The Role of Odour Perception in the Sensory Ecology of the Stable Fly, *Stomoxys calcitrans* L.

Thèse présentée à la Faculté des Sciences
Institut de Zoologie
Université de Neuchâtel

Pour l'obtention du grade de docteur ès Sciences

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Soutenue le 28 juin 2005

Université de Neuchâtel
2005

« […]Une mouche survient, et des chevaux s'approche,
Prétend les animer par son bourdonnement,
Pique l'un, pique l'autre, et pense à tout moment
Qu'elle fait aller la machine,
S'assied sur le timon, sur le nez du cocher.[…] »

Le Coche et la Mouche, Jean de la Fontaine

Contents

Remerciements

Je tiens tout particulièrement à remercier:

- Le Dr. Patrick Guerin, non seulement pour m'avoir offert l'opportunité d'effectuer une thèse en physiologie sensorielle, alors que cette branche de la biologie m'était inconnue; mais également pour m'avoir fait profiter de ses connaissances aussi bien sur le plan technique que scientifique; ainsi que pour sa disponibilité et pour m'avoir appris à travailler avec plus de rigueur et de précision.
- Les Prof. P.-A. Diehl, E. Städler, R. Tabacchi et le Dr. S. Schorderet-Weber pour avoir accepté de faire partie de mon jury de thèse et pour leurs commentaires avisés.
- Novartis (St.-Aubin) pour avoir fourni les mouches.
- Les personnes de l'administration (secrétaires et bibliothécaires) et de l'intendance (régisseur et concierges) pour leur disponibilité et leur sympathie.
- Mes collègues et amis du laboratoire de physiologie animale pour les discussions aussi bien scientifiques que politiques, pour les soirées thématiques, les pique-niques et les parties endiablées de ping-pong.
- Mes anciens collègues et amis du LEAE pour tous ces bons moments passés ensemble.
- Les nombreux étudiants qui ont suivi les travaux pratiques de physiologie sensorielle pour leur intérêt et surtout leur patience.
- Mes potes du volley pour ces inoubliables moments passés sur et en dehors des terrains.
- Mes anciens potes du Gymnase pour leur durable amitié et toutes ces aventures vécues ensemble.
- Mes vieux potes d'enfance pour être toujours là, ainsi que tous ceux qui n'entrent dans aucune de ces catégories, mais qui comptent pour moi.
- Ma famille et belle-famille pour leur soutien, leur confiance et leur intérêt.
- Sandrine pour son amour, sa joie de vivre, sa patience et sa présence dans les moments difficiles.

Résumé

Mots clés : *Stomoxys calcitrans*, mouche d'étable, olfaction, comportement, haleine, panse, substrats de ponte, électrophysiologie, chimie analytique, chambre de vol, bio essais

- Commune dans le monde entier, la mouche d'étable, *Stomoxys calcitrans* L., est un insecte nuisible du bétail. Males et femelles se nourrissent de sang et bien que la mouche d'étable s'attaque à un large éventail d'animaux, les grands herbivores comme les bovins et les équidés demeurent ses hôtes préférés. Pour la ponte, les femelles de la mouche d'étable utilisent toutes sortes de matériaux organiques en décomposition comme du foin fermenté, du compost et même des amas d'algues. Les déjections animales, et particulièrement lorsqu'elles sont mélangées à de la matière végétale en décomposition, sont également couramment exploitées par *Stomoxys* pour la ponte.

- L'haleine humaine et le CO_2 se sont avérés être attractifs pour *Stomoxys*. De plus, une réponse antennographique a été enregistrée suite à une stimulation avec de l'acétone, composé commun dans l'haleine des vertébrés.
- La mouche d'étable est activée par les composés volatils dégagés par la panse de vache et très probablement exhalés dans son haleine. La désorption thermique des constituants volatils de la panse en chromatographie gazeuse, couplée à des enregistrements électro-antennographiques, a permis la détection d'une trentaine de composés perçus par *S. calcitrans*. Nous avons déterminé ces composés qui appartiennent principalement aux classes chimiques suivantes : acides carboxyliques, alcools, cétones, aldéhydes, aromatiques, terpènes et sulfides. Certains composés du rumen, actifs en électro-antennographie, ont également été trouvés dans l'odeur dégagée par les poils de vache.
- Un seuil de détection très bas pour le dimethyl trisulfide, comparable à celui obtenu pour l'octénol, a été enregistré chez la mouche d'étable. De plus, le dimethyl trisulfide s'est avéré être attractif pour *Stomoxys* lors d'expériences en chambre de vol. L'acide butanoïque et le *p*-crésol ont également démontré un effet attractif pour la mouche

d'étable. Nous avons aussi observé un seuil de détection très bas pour certains terpènes comme le β-caryophyllene de même que pour certains aldéhydes comme le nonanal.

- Nous avons démontré que la mouche d'étable a besoin d'un apport continu en sang pour pondre de manière régulière et que, dans nos conditions de travail au laboratoire, un minimum de 3 jours avec un accès au sang est nécessaire pour obtenir les premiers œufs (Appendix 1).

- Nous avons démontré que *S. calcitrans* est capable de localiser un substrat potentiel de ponte, du crottin ou de la bouse dans notre cas, en se basant uniquement sur des indices olfactifs.
- Qu'elle ait un contact avec le substrat ou uniquement son odeur pour le localiser, la mouche d'étable démontre toujours une nette préférence de ponte pour le crottin de cheval par rapport à la bouse de vache. Nous avons aussi remarqué que les femelles pondent plus d'œufs en présence de l'odeur de crottin que lorsque aucune odeur n'est présente.
- L'analyse des composés volatils émis par le crottin et la bouse démontre que les composés perçus par *S. calcitrans* sont communément présents dans les deux substrats. Ces composés sont des acides carboxyliques, alcools, cétones, aldéhydes, aromatiques, terpènes et sulfides.
- Le CO_2 pourrait être en partie responsable de la préférence affichée par la mouche d'étable pour le crottin de cheval, ce dernier dégageant plus de CO_2 que la bouse de vache.
- Le contact avec un substrat est probablement important pour son acceptation comme lieu idéal de ponte.

- D'une manière générale, ce travail montre que *S. calcitrans* se base sur un éventail de composés volatils communs aux différentes ressources qu'elle exploite. Pour ne citer que les plus importants d'entre eux, le dimethyl trisulfide, l'acide butanoïque, le *p*-crésol, le β-caryophyllene et l'octénol, se retrouvent à la fois dans l'odeur des substrats de ponte et dans les effluves animales.

Summary

Key words : *Stomoxys calcitrans*, stable fly, olfaction, behaviour, breath, rumen, oviposition substrates, electrophysiology, analytical chemistry, wind tunnel, bio assays

- The stable fly, *Stomoxys calcitrans* L., is a worldwide pest on livestock. Both males and females are blood feeders, and although the stable fly is known to feed on a wide range of animals, large herbivores like bovids and equines remain the preferred hosts of *Stomoxys*. Female stable flies are known to oviposit in all kinds of decomposing organic matter like silage, rotting hay, garden compost and even in sea grass. Animal faeces especially when mixed with fermenting vegetal matter are also commonly exploited by *Stomoxys* females for oviposition.

- Human breath and CO_2 were shown here to attract stable flies, and acetone present in vertebrates breath elicited EAG responses from *S. calcitrans*.
- Stable flies were activated by rumen volatiles in a wind tunnel. These products are likely to be exhaled in bovine breath. Thermally desorbed rumen bolus headspace analysed by gas chromatography linked antennographic recordings yielded about thirty electrophysiologically active constituents for *Stomoxys* antennae. These volatile compounds were carboxylic acids, alcohols, ketones, aldehydes, aromatics, terpenoids and sulphides. Some of these EAG active compounds present in rumen were found to occur also in cow hair odour.
- A very low antennal threshold similar to that of oct-1-en-3-ol was recorded for dimethyl trisulphide and, moreover, this compound was shown to attract stable flies in the wind tunnel. Butanoic acid and the aromatic *p*-cresol were also shown to attract *Stomoxys* flies. Low antennal thresholds were also recorded to terpenoids like β-caryophyllene and aldehydes like nonanal.

- A continuous supply of blood was shown to be necessary for females to lay eggs regularly, and 3 days with access to blood was the minimum time required for oviposition under our laboratory conditions (Appendix 1).

- *Stomoxys* females were shown to rely on olfactory cues for finding an oviposition substrate. Dung odour by itself attracted flies for oviposition. Even without any contact with the substrate gravid females were able to localise either horse or cow dung.
- Horse dung was always preferentially selected over cow dung by gravid females whenever flies had contact with the substrate or just dung odour to rely on. We also observed a higher number of eggs laid when flies perceived horse dung odour than in the absence of an odour from a potential oviposition substrate.
- The analyses of volatile compounds emitted by horse and cow dung revealed that most of the chemostimuli perceived by stable flies occur in both substrates. These compounds are carboxylic acids, alcohols, ketones, aldehydes, aromatics, terpenoids and sulphides.
- Higher CO_2 levels were found over horse dung probably influencing stable fly choice for horse dung over cow dung for oviposition.
- Contact chemoreception and proprioreception are probably implicated in acceptance of a substrate for oviposition.

- Overall, this work shows that *S. calcitrans* rely on a set of volatile compounds that are common to the wide range of potential resources stable flies can exploit. To cite the most important of them, dimethyl trisulfide, butanoic acid, *p*-cresol, β-caryophyllene and oct-1-en-3-ol, occur in both oviposition substrates and in animal odours.

General Introduction

1. Biology of the stable fly *Stomoxys calcitrans* L.

Systematics and distribution

The genus *Stomoxys* belongs to the Stomoxyinae, a subfamily of the Muscidae (Diptera, Cyclorrhapha) representing a well-defined monophyletic group (Zumpt, 1973). The species *Stomoxys calcitrans*, the stable fly, is one among the 31 known species of this genus (Brues, 1913).

As a synanthropic fly, *S. calcitrans*, has gained worldwide distribution, rivalling the house fly in this respect. Much more common in temperate regions, it occurs very generally in the tropics, but in lesser numbers than in cooler climates. This may be due to the presence of parasites or predators (Muir, 1914). The question as to where it originated from has given rise to speculation. As 28 of 31 species belong to the Indo-Ethiopian region it is doubtful whether it was the temperate part of the Palearctic region that gave rise to the group as suggested by Brues (1913). More probably it was a tropical part of the Old World, and Zumpt (1973) suggests that the Oriental region has priority in this respect.

General morphology

In general appearance *S. calcitrans* resembles the common housefly (*Musca domestica* L.) and related spp. like *Fannia canicularis* L. and *Muscina stabulans* Fallén (Fig. 1). Its size is about the same as the housefly, from 4 to 7 mm. The distinguishing features of the stable fly are its mouthparts modified into a “piercing” organ (Fig. 2), a broader and shorter abdomen and a slightly different wing pattern in comparison to the house fly. The abdomen colour varies from grey to brown with a checkerboard of dark spots, and four dark lines run along the top of the thorax (Axtel, 1986). Males and females are very similar and both are blood feeders.

Stomoxys calcitrans

Musca domestica *Fannia canicularis* *Muscina stabulans*

Figure 1: General appearance of *S. calcitrans* and related spp. (modified from www.flycontrol.novartis.com).

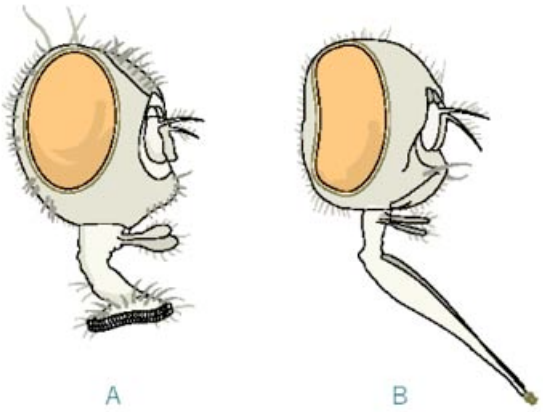

Figure 2: Schematic representation of the mouth parts from the house fly (A) and the stable fly (B) (modified from www.flycontrol.novartis.com).

Life cycle

Stable flies have the same life cycle as houseflies (Fig.3), but develop slightly slower. The eggs are white, elliptical, about 1 mm long by 0.3 mm wide (Fig. 3). They are laid in small groups (25-50) and a female will lay up to 800 eggs over a life-span of about 20 days. Oviposition takes place 4-8 days after copulation. Most of the eggs will hatch within 24 hours

of deposition (Axtel, 1986). The larvae are white and cylindrical (Fig. 3). Third-instar larvae are ready to pupate 6 to 8 days after hatching. Pupation occurs preferentially at 15°C (Axtel, 1986). The puparium of about 6 mm long gradually darkens to a rich, dark brown colour (Fig. 3). The pupal stage lasts 6-8 days. On completion of adult development, the adult pushes off the anterior end of the puparium with the ptilinum, an inflated sac that protrudes from the frontal region of the head (Axtel, 1986). The entire life cycle (egg to adult) requires 13-18 days at temperatures of 24°C to 30°C. Adult flies have a life-span of about 20 days.

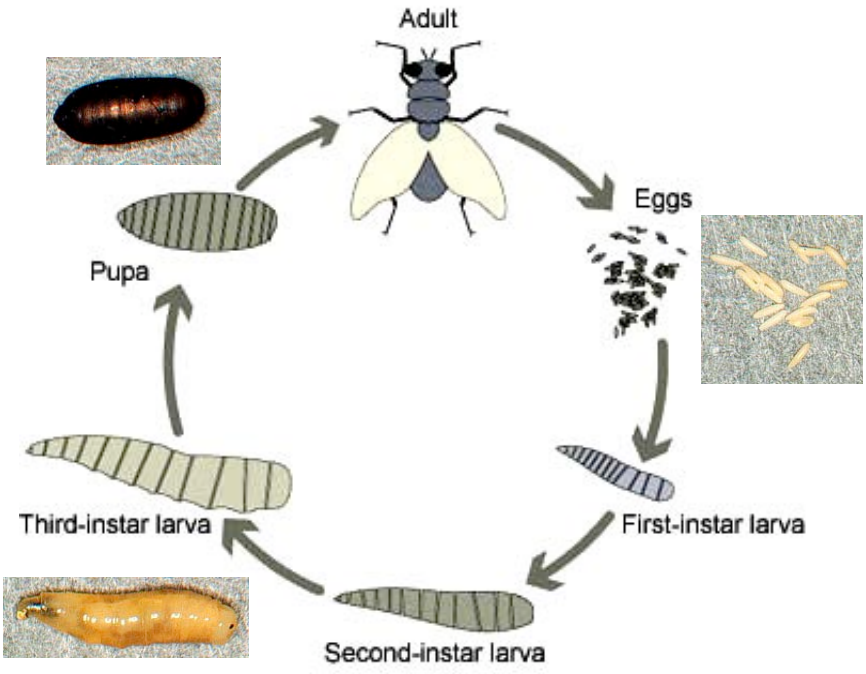

Figure 3: Life cycle of the stable fly *S. calcitrans* (modified from www.flycontrol.novartis.com, photos courtesy of Nebraska University).

Mating occurs for a few flies as early as 2 days after emergence, but most (89 %) have mated by day 5. Copulation lasts 4-6 min and first matings are only 60 % successful. Males inseminate more than one female (six on the average). On the other hand, females will mate only once (Harris *et al.*, 1966).

Feeding behaviour

Flies depend on blood for successful mating as well as survival, and repeated blood meals are needed for continued egg production (Appendix 1). Despite that they are known to suck liquids from decaying fruits and other parts of plants (Zumpt, 1973), nectar feeding is apparently common in stable flies. Sugar meals may provide the flies an immediate source of energy for flight activity but sperm transfer is very low or non-existent for males fed only sucrose solutions, and eggs will develop only until stage I in the females (Jones *et al.*, 1985; Jones *et al.*, 1992). Adult flies of both sexes, less than 2-3 h old, refuse to feed on blood, sugar solution or water. A sugar meal is taken when more than 3 h old and a blood meal is readily accepted when the fly is about one day or older. It takes a few minutes for a fly to feed to engorgement. This time probably varies depending on the physiological state of the fly, and the type of host and feeding site selected. Digestion of a blood meal will last 14-21 h depending on temperature. Furthermore, the rate of blood digestion can also be affected by the presence of a sugar meal, as those flies that take a sugar meal require a longer time to digest blood than those feeding on blood alone (Lee & Davies, 1979). Stable flies take two blood meals on warm days and one a day in cold weather. The biting activity tends to be light to moderate between 20 °C and 25 °C, it increases between 25 °C and 30 °C and becomes heavy at higher temperatures (Wang & Gill, 1970). Feeding flies are found on the lower parts of animals, especially on the lower half of the legs and they will attack humans as readily as livestock (Axtel, 1986).

Under lab conditions stable fly activity is almost completely diurnal with a peak about 4 h after lights-on. Starvation affects fly activity. Males and females show the same basic unimodal pattern of daytime activity (Schofield & Brady, 1996). Studies on the biting cycle of *S. calcitrans* in Egypt have revealed that in hot months, there are two peaks of biting activity, one in the morning and one in late afternoon, with a marked drop about noon but in the colder season only a single peak in activity occurs around 2 p.m. (Hafez & Gamal-Eddin, 1959). Gibson & Torr (1999) also reported a late afternoon peak in the arrival of *Stomoxys spp*. to baits in the field and a peak of host-searching behaviour occurring between 24 to 30 h after the previous blood meal.

Vision

S. calcitrans shows peaks of spectral sensitivity at 350-365 nm, 450-550 nm and 625-640 nm (Agee & Patterson, 1983; Lehane, 1991). Holloway & Phelps (1991) found that cotton cloth dyed phtalogen blue targets attracted three times more *Stomoxys* spp. than Alsynite fiberglass panels. As many stable flies were caught at an odour source (acetone, octenol and carbon dioxide) in the presence or absence of a visual target (Torr, 1989), it is concluded that stable flies rely probably less on vision than tsetse flies for host location. Nevertheless *S. calcitrans* shows a difference in the response to shapes decreasing in order of preference: horizontal rectangle, circle, square and vertical rectangle. The propensity to alight on a target is greater if it is large, dark and not striped (Gibson & Torr, 1999).

Economical importance

The economical importance of *S. calcitrans* results from two factors: disturbance by biting activity and transmission of pathogens. Important economical losses are encountered when stable flies are present in large numbers (Campbell *et al.*, 1977). Cattle are particularly sensitive to the annoyance caused by stable flies resulting in weight loss and dramatically reduced milk production (Cheng, 1958; Stork, 1979; Campbell *et al.*, 1987). For the year 1965 estimated losses in cattle production attributed to stable flies accounted for 142 millions US$ in the United States (Lehane, 1991). Due to its feeding behaviour, the stable fly acts as a mechanical vector and may transmit a large variety of pathogens. Indeed pathogens absorbed from infected hosts survive some time in the blood meal residues remaining on mouthparts (Foil *et al.*, 1987) and are readily transmitted to the next host the fly will alight on for feeding. Stable flies are important in the transmission of trypanosomes: *Trypanosoma evansi* causing Surra in horses, camels, dogs and cattle, and *T. equinum*, which causes “Mal de Caderas” in equines, cattle, sheep and goats (Lehane, 1991). *S. calcitrans* is also associated with the transmission of the nematode *Habronema majus*, a stomach worm of equines (Lehane, 1991). Morgan & Miller (1976) reported the successful mechanical transmission of hog cholera virus through *S. calcitrans* from infected pigs to susceptible ones. *S. calcitrans* was also found to act as an efficient mechanical vector of capripox virus and African swine fever. Moreover the viruses could survive at least two days in infected flies enhancing the risks of transmission (Mellor *et al.*, 1987). In summary, stable flies are potentially able to transmit a large variety of pathogens ranging from helminth, protozoans, bacteria, to viruses (Zumpt, 1973). Other

authors even suggested that *S. calcitrans* may have been implicated in a possible early transmission of HIV from apes to man (Eigen *et al.*, 2002).

Classical control measures for reducing fly infestations consist in removing or dispersing any substrate that can serve as a breeding site (rotting straw, manure), so that it dries out before the maggots complete their development; spraying cattle with repellents or applying contact insecticides to fly resting surfaces (Zumpt, 1973). When fly populations are low, biological control measures consisting in the release of parasitoids like *Spalangia nigroaenea* or *Muscidifurax zaraptor* (Hymenoptera: Pteromalidae) attacking stable fly pupae were shown to significantly reduce the production of adult stable flies in cattle feedlots (Weinzierl & Jones, 1998). British scientists (Birkett *et al.,* 2004) are developing a push-pull strategy that consists in modifying host natural odour with dispensers, so that it becomes less attractive, potentially enhancing the number of flies caught in traps deployed in the vicinity.

2. Olfactory system

2.1 Olfactory chemoreception in insects

The sense of smell is considered as the oldest of our senses and is present in all phyla (Mustaparta, 2002). Basically, olfaction is defined by the ability to perceive volatile substances. In insects, olfaction is a primary sense (Kaissling, 1971), even though other modalities like vision, contact chemoreception and proprioreception play also a crucial role. The first example of the importance of olfaction for insects was the discovery of species specific sex pheromones in moths produced by females and that attract males for reproduction (Schneider, 1992). Interest went further with investigations into the perception of the external environment by insects and the role of odours in the location of food, habitat and oviposition sites. The perception of odour is accomplished by receptors mainly located on the antennae and palps.

Morphology of insect olfactory organs

The antennae of insects bearing olfactory receptors vary quite a lot in shape and size between species (Fig. 4), but all are adapted for the perception of airborne volatile compounds. The featherlike antennae of male moths provide a striking example of the adaptation of this organ to sieve the air (Fig. 4).

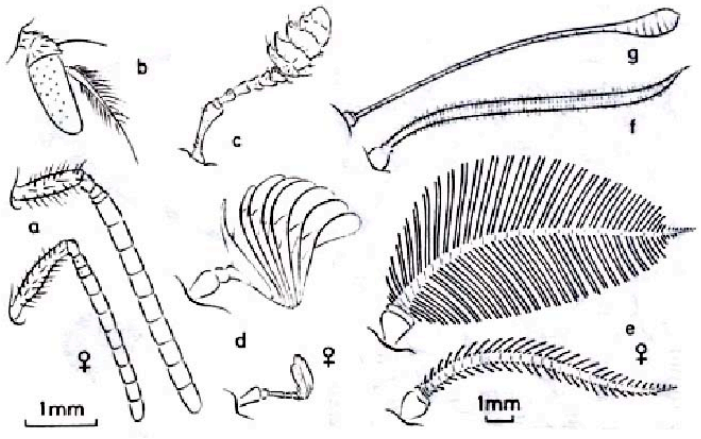

Figure 4: Variation in shapes of insect antennae. a honey bee; b flesh fly; c carrion beetle; d scarabid beetle; e saturniid moth; f hawk-moth; g butterfly (from Kaissling, 1987)

Antennae are covered with olfactory sensillae of different types: trichoid (long hairs), basiconic (short hairs), coeloconic (pit-peg), styloconic (subconical structure), clavate (club-like shape) and many other types and sub-types. They are comprised of cuticular and cellular components. The cuticular part is characterised by the presence of numerous pores that allow the chemicals to enter the sensillum. The pores vary in diameter from 10 to 25 nm and also in density, from 5 μm^{-2} on the trichoid sensillae of *Bombyx* to 125 μm^{-2} on the plate sensillae of *Apis* (Chapman, 1998). The cellular part is derived from the epidermis. The number of neurons in an olfactory sensillum varies among the different types of sensillae and insects, from two in the trichoid pheromone-specific sensillae of male moths to over twenty in the basiconic sensillae of grasshoppers. A typical olfactory trichoid sensillum is presented on figure 5. The dendrite extends into the hair in the receptor lymph which has a high K^+ concentration maintained by ion pumps (Kaissling & Thorson, 1980). The axon extends without connections from the neuron to the antennal lobe where it arborizes on an olfactory glomerulus (Hansson, 1995; Mustaparta, 1996). All the olfactory neurons responding to the same compound project their axons in the same glomerulus. Thus, a topographic map of receptor activation on the antennae is represented in the brain (Vosshall *et al.*, 2000).

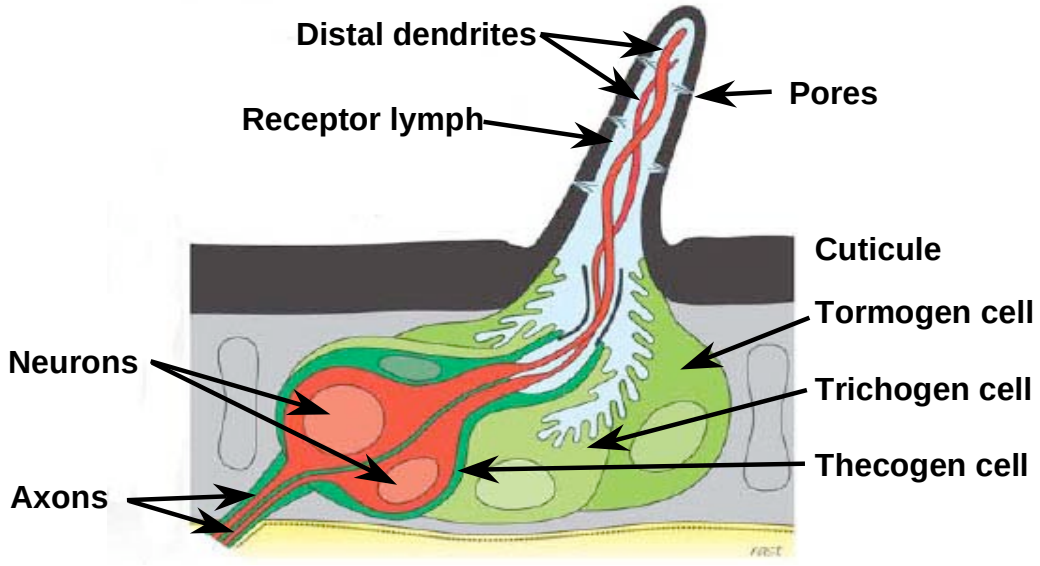

Figure 5: Diagrammatic section through an olfactory sensillum (modified from Kaissling, 2004)

Functioning of the receptor system

Two major processes comprising many events are involved in producing the neuronal response. The first one is the capture of the odour molecules and second one is the transduction, which converts the chemical signal into an electric one.

Perireceptor events correspond to the extracellular steps of the incoming odour. As most odorant compounds are lipophilic substances they will easily dissolve on the epicuticular lipids on the surface of the sensillum, but they will not dissolve in the insect's sensillum lymph. To reach the dendritic membrane, hydrophilic proteins (odour-binding proteins, OBPs) combine with the odour (Jacquin-Joly & Merlin, 2004). These proteins are not only carriers, but also play a role in the selection of olfactory information (Leal, 2003). When the complex odour-OBP reaches the dendrite surface, the odour molecule interacts with a receptor site. It is not yet clear if the complex binds to the receptor or if the odour detaches from the OBP to complex with the receptor (Hansson, 1995; Jacquin-Joly & Merlin, 2004). The binding initiates a cascade of events inside the dendrite leading to the nervous activity (Fig. 6). The formation of action potentials is preceded by a transduction mechanism activated by the binding of the stimulus molecule. Through G-protein and phospholipase activation, inositol triphosphate (IP3) is released in the cell and induces an increase of Ca^{2+} levels. The calcium activates a different secondary messenger system involving a protein kinase (PKc),

which phosphorylates the ion channel involved in the depolarisation of the chemosensory cell by opening cation channels (Hansson, 1995; Mustaparta, 2002; Jacquin-Joly & Merlin, 2004). This depolarisation is called the receptor potential. It will travel down the dendrite until it reaches the spike initiation site where it initiates action potentials that propagate along the axon to the brain. Some odours produce a hyperpolarisation of the membrane, acting through a different second messenger system with cAMP (Chapman, 1998).

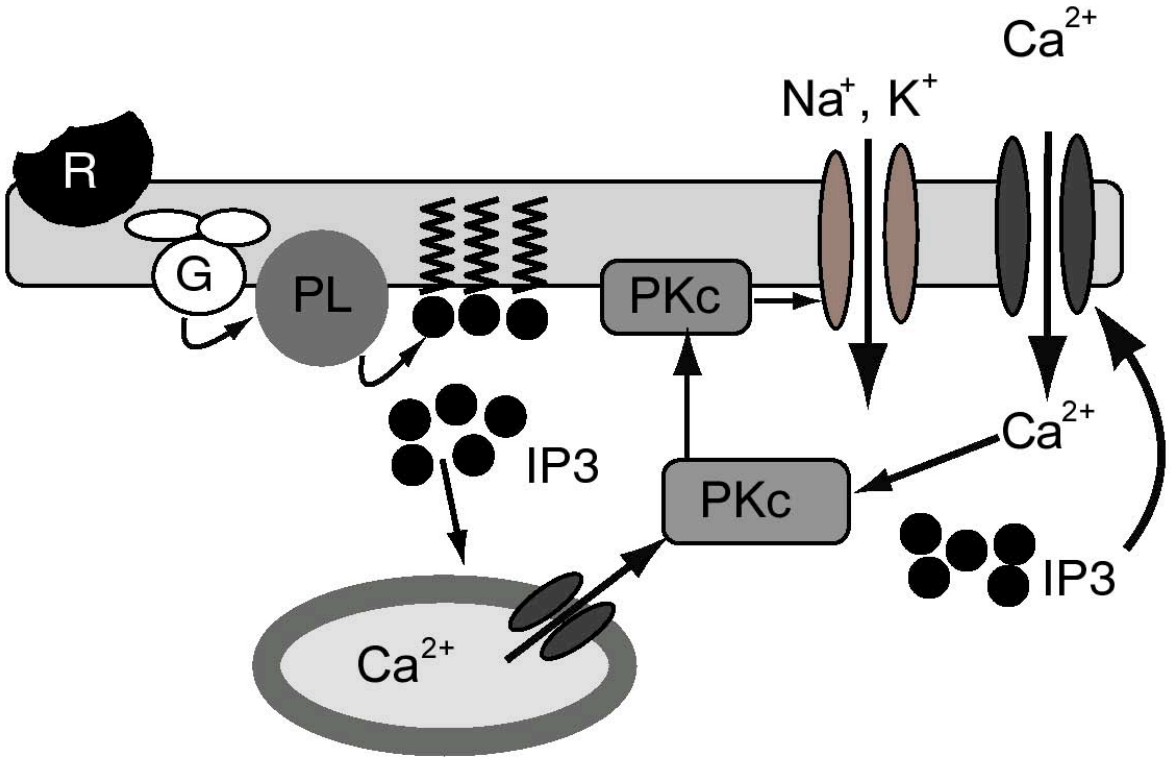

Figure 6: Schematic representation of the peripheral events in the olfactory receptor neuron. The receptor protein (R) binds with the odour molecule, thus activating a G-protein (G) and a phospholipase (PL), which mediates the release of Inositol triphosphate (IP3). The IP3 activates the release of calcium from internal calcium stores and entrance via ion channels into the cell. Ca^{2+} ions in turn stimulate a protein kinase (PKc), which phosphorylates the ion channels involved in the flux of cations into the cell (redrawn from Hansson, 1995).

The odour molecule must disassociate from the olfactory receptor to make it available for further stimulation. One possibility for degradation of the odour is through an enzymatic process. Two types of enzymes have been identified in Lepidoptera (*Antheraea*), an esterase and an aldehyde oxidase. However, since the kinetics of enzyme activity may not be adapted

to stimulus duration, it has been suggested that the OBP itself acts first in an oxidised form in the inactivation process followed by the enzymatic activity (Hansson, 1995; Chapman, 1998).

Specificity

Receptor neurons vary in their specificity. The most highly specific receptor cells are the ones for pheromone detection as they effectively respond to a single or few related compounds and show high selectivity even for specific isomers. Resource related odours are usually perceived by more generalist receptor cells that can bind with a range of compounds differing quite widely in their chemical structure. However, Anderson *et al.* (1995) found that in *Spodoptera littoralis* some receptors neurons were responding only to one or two types of compound indicating that some resource-associated receptor cells may also be quite specific.

The specificity of a neuron is assumed to correlate with the presence of the receptor in the dendritic membrane. Neurons responding to a single compound may contain only one type of receptor with specific binding properties (shape, conformation), on the contrary, neurons responding to different types of chemicals may have several types of receptor sites. Neurons responding to compounds of similar structure may have only a single receptor site that will bind with a more or less appropriate fit with odour molecules with a characteristic structure. The fitting of the molecule with the receptor may induce a distinct firing rate and thus elicit different behaviours (Chapman, 1998).

2.2 Sense organs of *S. calcitrans*

Lewis (1971) described in detail the superficial sense organs present on stable fly antennae. The antenna is constituted of three segments. The scape is the short basal segment carrying no sensilla. The second segment is the pedicel which bears only articulated bristles with a probable mechanical function and contains also the Johnston's organ acting as an air speed indicator. The third segment or funiculus is the biggest in size (0.55 mm long) and is covered by a high density of sensillae belonging to four major types: 700 trichoid, 4000 basiconic, 100 clavate and about 40 styloconic sensillae. All these sensillae vary in shape, dendritic organisation and are thought to be distributed on the funiculus according to their resistance to

mechanical damage with the less robust ones located on the ventral side partially protected when the antennae lie at rest. The structural organisation of the stable fly sensory system is extremely similar to that of the house fly described by Kelling (2001).

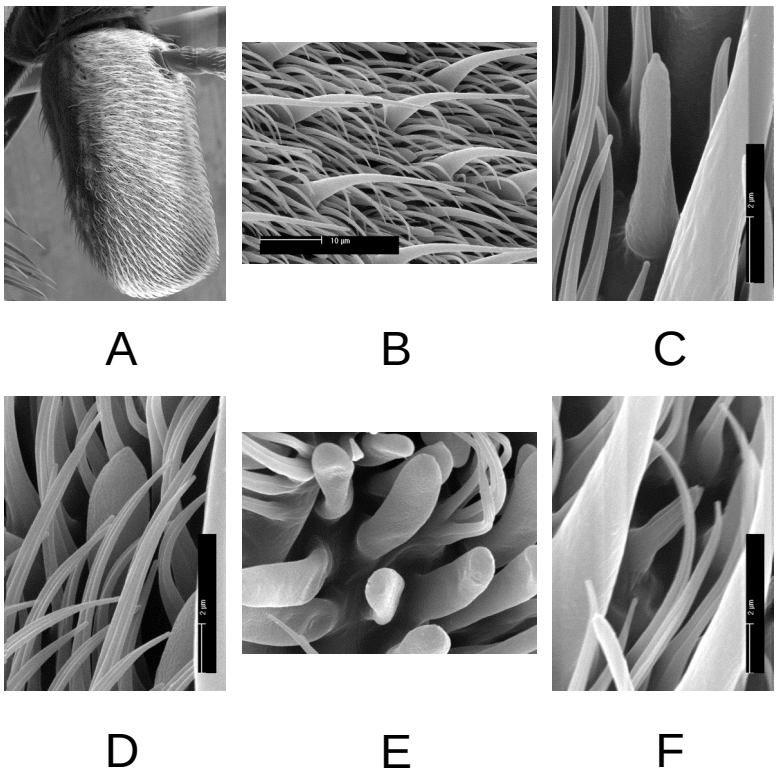

Figure 7: Scanning electron microscope images of the funiculus of a female *S. calcitrans* (A), trichoid sensillae (the biggest structures represented in B), a basiconic sensillum (C), clavate sensillae (D-E) and a styloconic sensillum or grooved peg (F) (images courtesy of M. Vlimant, University of Neuchâtel, CH)

2.3 Electrophysiological methods

Electroantennogram

The electroantennogram (EAG) technique is a simple approach to investigate stimulus-response characteristics of antennal receptor cells of insects. It represents summed fractions of receptor potentials of many olfactory sensillae activated by a particular stimulus (volatile

compound). Recordings can be made using the antenna either excised, or attached to an isolated head or to the whole insect. In our case, we used the technique described by Guerin & Visser (1980), where the reference (indifferent or grounded) electrode is inserted through the cut head of the insect to inside the pedicel and the recording electrode is brought into contact with the tip of the funiculus (Fig. 8). Clean and humidified air is blown continuously over the antenna at a constant rate and odours to be tested are injected into the air stream. The amplitude of the EAG response provides information on the selectivity and sensitivity of the receptor cells responding to an odour. The amplitude is also correlated to the dose tested, the biggest the dose inducing the biggest depolarisations until it reaches a plateau.

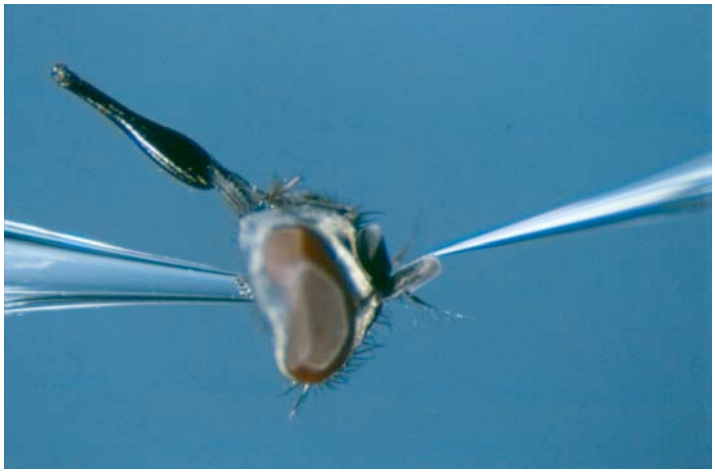

Figure 8: Image representing the cut head of a stable fly with the reference electrode (left) inserted in the head and the recording electrode (right) in contact with the funiculus (photograph by P. Jeanbourquin).

Electroantennogram linked to gas-chromatography (GC-EAD)

Gas-chromatography (GC) is a technique used in analytical chemistry. It allows the separation of all the constituents of a mixture of volatile products depending on their polarity and their molecular weight. EAG recordings combined with GC are employed to identify biologically active components of mixtures of volatiles from natural substrates. In this manner the selective response from the antenna can be related directly to constituents in a chemical separation, as illustrated in figure 9.

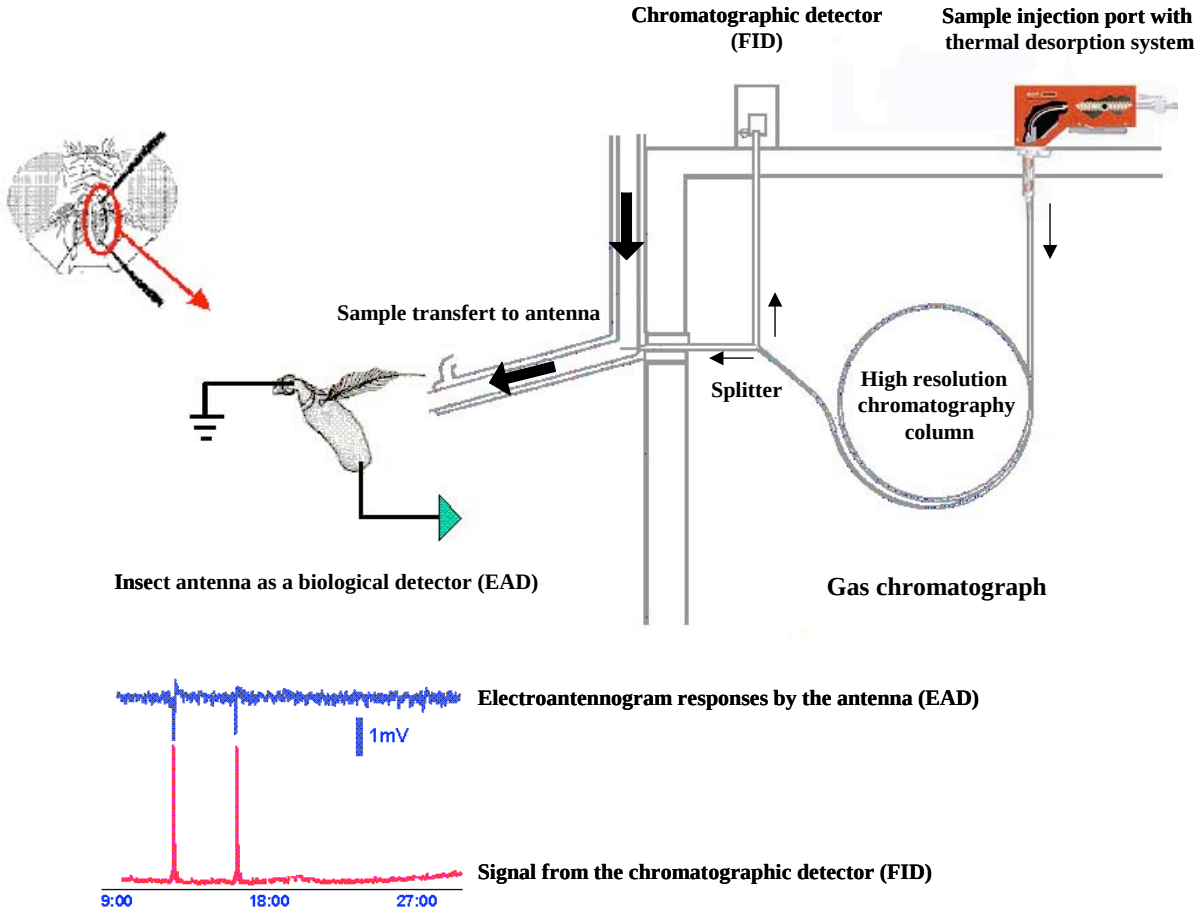

Figure 9: Schematic representation of the functioning of GC-EAD.

3. Haematophagous insects

3.1 Origin and evolution

The evolution of the blood-sucking habit, widely documented in Lehane (1991), is believed to have occurred along two main routes. In the first route it is suggested that haematophagous form of life may have developed in response to a close association with vertebrates. Non blood-sucking insects may have been attracted to the resting site of vertebrates (e.g. nests) because it provided them a warm and humid environment with an abundant supply in food. Initially feeding on organic debris with chewing-like mouthparts, insects living in close

contact with the host may have started occasional feeding on blood leaking from wounds. Once insects regularly encountered blood it is likely that its high nutritional value favoured the development of physiological, behavioural and morphological adaptations allowing them to regularly exploit blood as a resource. Insects with no clear adaptations for the blood-sucking way of life may also have developed prolonged associations with vertebrates away from the resting site. One of the most important factors for such a relationship was probably the use of the host's faeces as a larval habitat. Dung is a limited resource and one way for an insect to be the first colonizer is to form an increasingly close association with the vertebrate that provides it. Such a close association can be seen in the horn fly *Haematobia irritans*, which as an adult is permanently associated with large vertebrates, only leaving them to oviposit. Probably feeding initially on body secretions or open wounds, the ancestral female may have slowly incorporated blood in its diet. Selection is likely to have favoured the progressive development of piercing mouthparts leading to full haematophagy. Blood feeding among male insects could be explained by the advantage for the male to also become associated with the vertebrate enhancing its chances of finding a mate.

The second route for the evolution of the blood-sucking habit suggests that blood feeding developed in some insect lineages from ancestral insects, which were morphologically pre-adapted for piercing surfaces. Entomophagous insects for instance may have been attracted to vertebrates because of the other insects surrounding them, or they may have regularly encountered the vertebrates around wet areas, which are their breeding sites. These predatory insects like the ancestors of the blood feeding bugs and possibly some actual blood-feeding Diptera would have had physiological (efficient protein digesting enzymes) and morphological (piercing mouthparts) adaptations facilitating the switch to haematophagy. It has also been argued that haematophagy may have arisen in some insect groups from plant feeding ancestors. As many plant-feeding insects possess piercing and sucking mouthparts, they would have been also pre-adapted for haematophagy and, as stated above, attraction to free-living vertebrates may have occurred in order to feed on body secretions or to use dung as a larval medium.

3.2 Feeding preferences and relationship with hosts

Blood-sucking insects feed on a wide range of different host animals. For some insects, particularly some permanent ectoparasites like the human body louse, the host chosen may be very specific but for others, host choice is not so restricted. The commonest hosts are probably large herbivores like cattle and horses. As herbivores show a social behaviour and because they normally move slowly from one pasture site to another, they represent a large visible and abundant reliable food source for many temporary ectoparasites (Lehane, 1991). Large herbivores with a large accessible skin area may also encounter more difficulties in getting rid of biting flies that can distribute on the whole body surface to feed. By feeding on large animals, flies therefore diminish the risks of being killed by host defensive behaviour. The suitability of large herbivores as hosts for blood-sucking flies is demonstrated by the numerous species feeding on equines and bovids (Table 1).

Table 1: Species of biting flies commonly found on cattle and horses (modified from Williams *et al.*, 1985)

Blood-sucking flies	Species
Stable flies	*Stomoxys calcitrans*
Horn flies	*Haematobia irritans*
Horse flies	*Tabanus spp., Hybomitra spp.*
Deer flies	*Chrysops spp.*
Mosquitoes	*Aedes spp., Anopheles spp., Culex spp., Culiseta spp., Psorophora spp.*
Biting gnats	*Culicoides spp., Leptoconops spp.*
Black flies	*Simulium spp., Prosimulium spp., Cnephia spp.*

Studies conducted in Egypt by Hafez & Gamal-Eddin (1959) showed that the preferred host for *S. calcitrans* was the donkey, followed by the horse, buffalo, cow, camel, sheep and goat. Stable flies obviously prefer hosts with a large body size and an accessible skin with short and thin hairs. At our latitudes stable flies are considered as serious pest of cattle and horses (Axtel, 1986) and found in great numbers in livestock productions. Livestock facilities with many grazing animals in confined areas provide flies an easy accessible and inexhaustible

resource of food. Moreover, manure produced in large amount by herbivores provides *Stomoxys* females suitable breeding media in close proximity to the food source, diminishing the risks of predation when searching for oviposition substrates. Evolutionary processes leading to the association of *S. calcitrans* with large vertebrates like cattle and horses seem evident in that these herbivores provide all the necessary requirements for its development.

Opportunistic species are usually ecological and physiological generalists with a high intrinsic rate of increase and short generation time. They can rapidly increase in numbers when environmental factors are favourable and can shift their realized niches with morphological and physiological preadaptations to avoid competition and to make use of new adaptive opportunities (Kim, 1985). *S. calcitrans* can be considered as an opportunistic blood-sucking fly. Probably less competitive than other insects utilising the same resources, the stable fly has evolved a more generalist behaviour.

Stomoxys possess a relatively short development cycle (20 days) and produces eggs in large numbers with up to 800 eggs per female (Axtel, 1986). Producing large number of eggs may be a strategy for counteracting the effects of high mortality due to competition and predation. Dung is a limited resource and intense competition occurs to utilise it as a larval feeding site. Less competitive than other insects (e.g. *Haematobia irritans*) in colonizing this oviposition substrate, the stable fly may have widened the range of media used for oviposition. This is supported by the fact that *Stomoxys* flies can breed in sea grass deposits or even in accumulated dead bodies of mayflies (Zumpt, 1973).

Stable flies are able to feed on a wide variety of hosts (Hafez & Gamal-Eddin, 1959; Zumpt, 1973). Once more, we suspect *Stomoxys* to act as an opportunistic fly attacking the closest host and only showing preferences for a given host when several are immediately available. *S. calcitrans* tends to aggregate in sites where resources are abundant and localised. Humans by holding large mammals within a limited space for their own purposes have created a new niche quickly adopted by the stable fly that has become a worldwide pest on livestock.

Considering the range of substrates used for oviposition and the variety of hosts the stable fly can feed on, it is plausible to consider that evolution has favoured the development of an olfactory system tuned above all to the perception of the volatiles that are common to a wide range of host odours and oviposition substrates used by *S. calcitrans*.

4. Outline of the thesis

The aim of this thesis is to determine the role that olfaction plays for *S. calcitrans* in finding resources. Most of the studies dedicated to the olfaction in stable flies to date have dealt with chemostimuli arbitrarily chosen for their known activity on other haematophagous arthropods. In this study we deliberately focused on the volatile compounds present in natural substrates of importance to *S. calcitrans*. Thus we selected attractive media or sources of volatiles for *S. calcitrans* that are likely to be encountered and used by flies for resource location in nature.

In a first chapter we deal with volatile compounds implicated in host finding. Human breath and carbon dioxide were tested in wind tunnel assays, but we principally focused our research on rumen metabolites. Rumen was chosen for its rich volatile profile and the fact that many compounds found in rumen odour are likely to be exhaled in the breath of bovines, among the preferred host for *S. calcitrans*. We consequently tested the biological significance of rumen odour at an electrophysiological and behavioural level. After analysis of rumen volatile by GC-EAD we identified potential chemostimuli for host finding in stable flies and tested their efficiency in attracting *S. calcitrans* in the wind tunnel.

In a second chapter we assessed the role of olfaction in finding a suitable substrate for oviposition. Preferred sites for oviposition in stable flies are well known. Many efforts are made to render these sites unsuitable for larval development but little interest has been shown in understanding how flies locate them. For that purpose, we conducted oviposition assays and observed *S. calcitrans* behaviour in a wind tunnel on exposure to horse and cow dung odours. We then analysed the volatile composition of these odours and determined the compounds eliciting electroantennogram responses in stable flies. We finally identified some volatiles that could be critical for oviposition in stable flies.

In the final chapter we discuss the advantages and limitations of the different methods used in this research project. Finally, we conclude with the relevance of the results obtained with

regard to the sensory and behavioural strategies developed by *S. calcitrans* for finding resources suitable for its reproduction.

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Host odour components implicated in host location by *Stomoxys calcitrans* L.

Abstract :

Human breath was tested in the wind tunnel and its attractiveness for stable flies is discussed with regard to its carbon dioxide content and other constituents. In addition, the importance of rumen volatiles exhaled in bovine breath for the activation and attraction of host-seeking flies was investigated to show that rumen volatiles activate *Stomoxys calcitrans* in the wind tunnel but attractiveness was low. Thermally desorbed rumen headspace was analysed by gas chromatography linked antennographic recordings. Analysis of rumen odour extracts yielded about thirty electrophysiologically active constituents. Five chemostimulants, each one belonging to a different chemical class, were selected from them to test for behavioural responses. Dimethyl trisulfide, butanoic acid and *p*-cresol were found to attract *Stomoxys*, whereas oct-1-en-3-ol and skatole failed to attract flies in the wind tunnel. We also found dimethyl trisulfide and β-caryophyllene to be perceived at very low concentrations approaching the sensory threshold for oct-1-en-3-ol. The importance of products of different functionality detected as chemostimuli in rumen headspace and occurring also in animal emanations is discussed in relation to host location in stable flies.

Key words : stable fly, olfaction, breath, rumen, electrophysiological recordings, wind tunnel

Introduction :

Haematophagous arthropods depend on blood for reproduction and egg development, and rely on chemical and physical stimuli to find hosts (Cork, 1996; Gibson & Torr, 1999). Most of the chemostimuli generally originate from bacterial activity and are released from specific body regions of the host. Human skin emanations, for instance, are composed of numerous volatiles (Bernier *et al.*, 2000) and some of these compounds are produced by skin microflora (Braks *et al.*, 1999) are known to attract mosquitoes (Meijerink *et al.*, 2000). Vale (1980) also reported the attractiveness of ox body odours to some blood-sucking flies. Another important source of volatile emission is breath. Human breath has been shown to activate stable flies (Warnes & Finlayson, 1985), tsetse flies (Syed, 2002), mosquitoes (Healy & Copland, 1995) and ticks (McMahon & Guerin, 2002). Its attractiveness to blood-sucking insects is often associated to its high carbon dioxide content. CO_2 is considered a strong activator showing added effects when mixed to other volatiles compounds (Otalora-Luna *et al.*, 2004; Vale & Hall, 1985), and its efficiency as a bait for trapping various species of haematophagous insects is widely documented (Roberts, 1971; Vale, 1980). Breath is also composed of numerous volatile compounds (Wahl *et al.*, 1996; Phillips *et al.*, 1999), and ticks and tsetse flies have been shown to be attracted to rumen metabolites (Syed, 2002; Donzé *et al.*, 2004) that are expelled in bovine breath.

In this chapter we describe the attractiveness of human breath as tested in a wind tunnel to *Stomoxys* and the efficiency of breath as an attractant is compared to carbon dioxide. We also recorded the activation and attraction exhibited by stable flies on exposure to rumen odour in the wind tunnel. In order to determine which host odour constituents are critical for the stable fly we analysed the responses of antennal receptor cells to rumen volatiles by electroantennography linked gas chromatography. From these recordings we selected chemostimulants in different chemical classes showing the lowest sensory thresholds in *Stomoxys* and tested their efficiency in activating and attracting stable flies in a wind tunnel.

Material & Methods :

Insects. Pupae of *Stomoxys calcitrans* were supplied by Novartis, St-Aubin, Switzerland and kept in an environmental chamber until emergence under a LD 12:12 h photoperiod with 26-28°C, 65% RH in light and 22°C, 90% RH in dark with light increasing and decreasing for 2 h at dawn and dusk. Newly emerged flies were sexed daily and approximately 30 flies were dispatched in mesh cages (15 x 25 x15 cm). Flies had access to cotton imbibed daily with 5 ml 10% sucrose solution, changed daily. Experiments were run with 1-2 days old unmated flies.

Rumen volatile collection. 50 g of fresh rumen collected in a slaughter-house (La Chaux-de-Fonds, CH) was introduced into a 1-L gas-wash flask. The flask was flushed with N_2 in order to avoid any oxidization of the substrate and the formation of other volatiles, capped with a T-connector and left for 1 h for equilibration at room temperature. N_2 was blown into the bottle at 50 ml min^{-1} and the headspace collected at the other end of the T-glass connector with a commercial Tenax™ GR cartridge (6 mm OD, Gerstel®, Switzerland). Rumen volatiles collection lasted 20 min.

Electroantennograms (EAG). Electroantennograms from *S. calcitrans* antennae were recorded as described in Guerin & Visser (1980). The antenna was held in a humidified charcoal-filtered air stream (90–100% RH, 23 ± 2°C) delivered at 1 m sec^{-1} via a glass water-jacketed tube (7 mm ID) whose outlet was about 1 cm from the preparation. The EAG signal was recorded on 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). Antennal stimulation was made as described in Guerenstein & Guerin (2001) by passing 1 ml of charcoal-filtered air through a 5 ml polypropylene syringe (BD Plastipak™, Spain) containing the stimulus and this was injected in the air stream (above). Serial dilutions in methylene chloride (DCM, Merck, analytical grade) were prepared for each chemical tested, except for acetone that was diluted in nanopure water and for ammonium where an aqueous (125 mM) solution of NH_4OH was used, and 10 µl was deposited on a small filter paper strip

(0.8 cm x 3 cm) that was inserted into the stimulus syringe after solvent evaporation. Taking into account the amount delivered from the stimulus syringe, the dilution in the humidified air stream and the surface area of the antenna, the relative amount of synthetic reaching the antenna has been estimated and corresponds to approximately 1/10000 of the amount placed on the filter paper.

Gas chromatography (GC) linked electroantennographic detection (EAD). To use the antenna as a biological detector (Arn *et al.*, 1975), a *Stomoxys* antenna was mounted (Guerin & Visser, 1980) between two glass electrodes (2 mm OD) filled with a 0.1 M KCl solution. Electroantennographic responses were recorded in parallel (GC-EAD) with the flame ionisation detector of the GC (5300, Carlo Erba Instruments) on a computer through a 16-bit analogue/digital IDAC box (Syntech, The Netherlands). The GC was equipped with a thermal desorption unit (TDS 2, Gerstel®, Germany) and a cooled injection system (CIS 3, Gerstel®, Germany) installed on the head of a high-resolution capillary column. Two types of columns (polar and apolar) were used for the analyses of rumen volatiles by GC-EAD (FFAP, L 30 m, ID 0.25, df 0.25 and an SE-30, L 30 m, ID 0.25, df 0.15, both from BGB Analytik, Switzerland). Rumen volatiles initially trapped on a Tenax™ GR cartridge were thermally desorbed in splitless mode via the TDS 2 programmed to increase from 30°C to 220°C at a rate of 30°C min^{-1}. A heated (240°C) transfer line evacuated the volatiles to the CIS 3 cooled to -80°C with liquid nitrogen where volatiles were cryotrapped. At the end of the TDS 2 transfer the CIS was subjected to ballistic increase (12°C sec^{-1}) in temperature to reach 240°C to transfer, in splitless mode, the volatiles onto the high resolution capillary column. Carrier gas was H_2 at 50 cm sec^{-1} and the temperature programmes were set at 40°C for 5 min with a linear increase to 230°C at 5°C min^{-1} for the polar FFAP column, and 40°C for 5 min with a linear increase to 120°C at 3°C min^{-1}, then 5°C min^{-1} to 200°C and finally 8°C min^{-1} to 280°C for the apolar SE-30 column. The column effluent was split equally with an effluent splitter (Gerstel®, Germany) between the FID (set at 250°C) and a heated (250°C) transfer line that conveyed the column effluent to a glass water-jacketed tube (7 mm ID), where the charcoal-filtered air stream of humidified air (90–100% RH, above) at 1 m sec^{-1} carried the volatiles to the fly antenna (Steullet & Guerin, 1994). The relative amount of synthetic reaching the antenna has been estimated and corresponds to approximately 1/16000 of the amount injected on-column in the gas chromatograph taking into account the split ratio, the dilution in the humidified air stream and the surface area of the antenna.

An averaged GC-EAD trace was redrawn from the three most representative GC-EAD analyses of rumen odour extract on the SE-30 column. Only antennal responses to a given constituent of the extract recorded in at least two traces were taken into account for determination.

Gas chromatography coupled mass spectrometry (GC-MS). Biologically active constituents of rumen odour extracts that caused EAG responses from *S. calcitrans* antennae were identified by gas chromatography linked mass spectrometry (Steullet & Guerin, 1994). TDS 2, CIS 3 and the GC oven were run under the same conditions as for GC-EAD (above) on a HP 5890 Series II gas chromatograph linked to a mass selective detector (HP 5971 A) with He at 30 cm sec^{-1} as carrier gas. Chemical identity of biologically active compounds in rumen odour extracts was determined using mass spectrum library matches (Wiley and Nist98). The Kovat's retention indices (RI) of these tentatively identified compounds were compared with those of injected synthetic analogues when available. The biological activity of the synthetic analogues was established by EAG (Appendix 2) or confirmed from the literature.

Wind tunnel and stimulus delivery. The wind tunnel (170 cm long, 60 x 60 cm) constructed of non-reflecting glass was as described in Syed & Guerin (2004) with the following modifications depending on the stimuli tested.

Tests with human breath and carbon dioxide (CO_2). The downwind end (fly release point) of the nylon net flight cylinder (1.5 m long x 20 cm diam., 1 mm mesh) was closed by a metal grid (3 mm mesh size) with a centred 3 x 3 cm opening in order to stop released flies from escaping downwind. The wind tunnel was wrapped in a white paper cover along the sides, top and floor without any optomotor cues. Flies tested in the wind tunnel were transferred individually into plastic release cages (transparent PVC cylinders, 13 cm long, 4.5 cm diam.). Their downwind ends were covered with nylon netting (1 mm mesh) and their upwind ends were fitted with a flat sliding nylon mesh (1 mm) door. Cages with flies were kept in the environmental cabinet (28°C, 65% RH.) with the wind tunnel for at least 1 h before tests. The

release cage was placed horizontally at the downwind end of the tunnel its upwind opening aligned against that of the metal grid attached to the flight cylinder (see above).

Before breakfast human breath was collected in a Tedlar^{TM} bag (25 L). Breath was sucked from the bag using a membrane aquarium pump (WISA DBGM, Germany) and delivered at 1 L min^{-1} into the wind tunnel via an aluminium tube (4 mm ID) attached to a funnel (4 cm diam.) bent downwind at a height of 35 cm. The 4 cm-diam. funnel openings were perforated with 16 holes (0.4 mm diam.). Diluted CO_2 from a gas tank (2% CO_2 in N_2) was introduced into the wind tunnel via a stimulus delivery system (Syntech, NL). The flow rate was set in order to obtain an increase of 70-90 ppm (BINOS 1, ± 10 ppm resolution, Leybold-Heraeus, Germany) at the fly release point, corresponding to the increase in CO_2 obtained with breath delivered at 1 L min^{-1}. The odour plume structure was checked visually by generating a plume of ammonium acetate. The plume covered the flight cylinder uniformly from 15 cm downwind of the aluminium funnel to the tunnel exit.

After 2 min of acclimatisation in the wind tunnel, the nylon-mesh door was lifted slowly and flies were successively exposed for 2 min to odour-free air (control) and then for 2 min to the test product from two funnels placed side by side. Effects of test stimuli were expressed as the percentage of flies that were activated upon exposure to test stimuli and the percentage of flies attracted to the odour source. We considered that a fly was activated when it flew out of the release box and that it was attracted when it flew more than 50 cm towards the source corresponding to 1/3 of the distance between the release cage and the odour source. Flies that were activated (%) or attracted (%) during the control period were discarded. Differences in activation and attraction between treatments were calculated using Fisher's exact test. As no significant differences in either activation or attraction to breath and CO_2 between males and females were recorded, data from both sexes were pooled (Appendix 3).

Tests with odours from rumen bolus and synthetics. For these tests we removed the nylon flight cylinder and allowed flies to exhibit a free flying behaviour. As stable flies are known for flying preferentially near the ground, we lowered the delivery funnels to 8 cm above base of the tunnel and likewise the insect release point. We used bigger cylindrical release cages (10 cm diam., 15 cm long) made of metallic-netting (3 mm grid) and fitted with a metal mesh (1.3 mm) flap attached to the cage on a hinge to permit opening of the release cage with a

string on a pulley from outside the wind tunnel. In order to help flies to orient in the wind tunnel we provided them with visual cues as well. One pale blue cardboard strip (6 cm wide, 0.2 mm thickness) was attached in the centre of the wind tunnel running from odour-delivering funnels to the fly release cage and narrower ones (1 cm wide) were pasted along both sides of the wind tunnel and parallel to its length at a height of 6 cm from the floor. The fly release cage containing five flies was introduced into the wind tunnel and flies were given a 3-min acclimatisation period to odour-free air. The door was then gently opened and flies were exposed for 3 min to the test stimuli. We recorded the first flight behaviour exhibited by each fly on leaving the release cage using The Observer software (Noldus Technologies, The Netherlands). From these observations we calculated the percentage of activated flies as flies leaving the cage, and the percentage of attracted flies as flies casting in the odour plume and flying more than 50 cm upwind towards the odour source. Differences between test odours and control were assessed using the Chi-square test. We also recorded the total number of oriented flights in the odour plume during the whole test period and calculated the mean number of oriented flights per activated fly. Differences in the number of oriented flights between treatments were statistically assessed with Mann-Whitney tests. As no significant differences in either activation, attraction or the number of oriented flights between males and females were recorded, data from both sexes were pooled (Appendix 3).

Rumen. 50 g of rumen bolus (test) from a freshly slaughtered cow and 50 ml distilled water (control) were directly introduced into 1-L gas-wash flasks and caped with a T-glass connector. One end (injection) of the T-connection was linked to the stimulus delivery system (Syntech, NL) blowing charcoal filtered air at 450 ml min^{-1} through the bottle via Teflon tubing, the other end (exhaust) was linked to the aluminium tube bearing the funnel in the wind tunnel. The variation in CO_2 concentration on stimulation with rumen bolus was measured at the funnel exit and at the fly release point with a Li-820 gas analyser (Li-Cor®, ± 5 ppm resolution, DMP, Switzerland).

Synthetic chemicals. Dimethyl trisulfide (>98%, Sigma-Aldrich), oct-1-en-3-ol (>97%, Merck), butanoic acid (>99.5% puriss. p.a., Fluka), *p*-cresol (>99% puriss. p.a., Fluka) and skatole (>99% purum., Fluka) were diluted in methylene chloride (DCM) to obtain solutions

containing 100 ng μl^{-1}. A polyethylene dispenser (1.27 cm diam., 3 cm long, 0.8 mm thick walls, Kartell, Italy) was filled with 100 µl of a solution corresponding to 10 µg of the test substance. It was then inserted into a 1-L gas-wash bottle through which charcoal-filtered air passed at 400 ml min^{-1}. We used a dispenser with 100 µl DCM as control.

Results :

Electroantennograms (EAG). EAG responses from a female *Stomoxys* antenna were recorded to acetone at 100 µg in the stimulus cartridge and to ammonium (NH_3) at around 17 ppb on the antenna. Carbon dioxide (CO_2) at approximately 100 ppm on the antenna also elicited EAG responses from a male *Stomoxys* antenna (Fig. 1). The EAG response to oct-1-en-3-ol at 1 µg is provided as reference (Fig. 1).

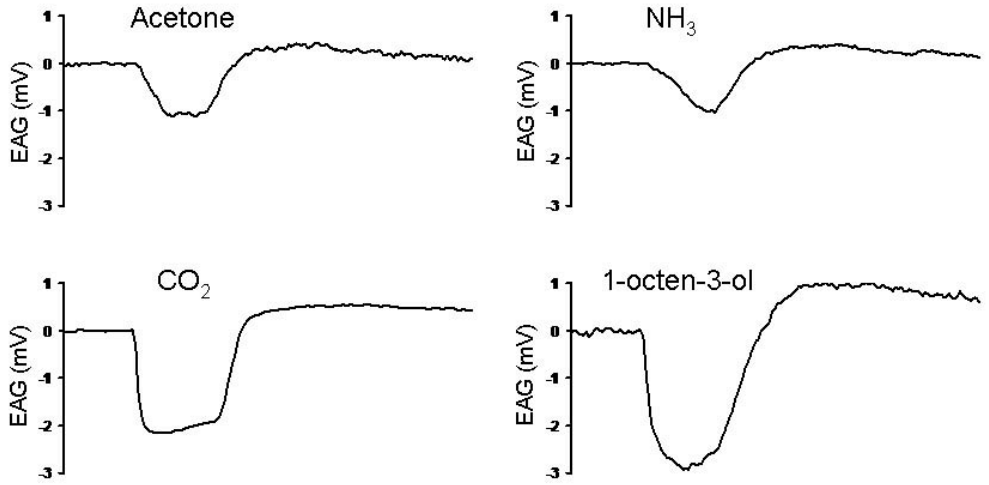

Figure 1: EAG responses of *S. calcitrans* male and female antennae (see above) to host chemostimuli. Acetone was at 100 µg in the stimulus syringe, NH_3 corresponded to 17 ppb and CO_2 to 100 ppm on the antenna, oct-1-en-3-ol as reference was at 1 µg in the syringe.

Dose response curves obtained to increasing doses of synthetic compounds found in rumen odour (Fig. 2) revealed oct-1-en-3-ol to be the compound to which *Stomoxys* showed the lowest threshold. EAG responses to this substance were already discernable at 1 ng in the stimulus syringe, meaning at most 100 femtograms on the antenna upon stimulation (see M&M). More interestingly, the stable fly antenna showed responses to dimethyl trisulfide with a threshold also around a hundreds of a femtogram and an increase in EAG response amplitude with increasing doses. The EAG responses recorded for butanoic acid, isovaleric acid and *p*-cresol shared a similar pattern, with discernable responses between 100 ng and 1 µg in the stimulus syringe and a sensory threshold situated around 10 pg. Finally, skatole appeared to be perceived when 10 to 100 pg reached the antenna, i.e. showing the higher threshold among the compounds tested.

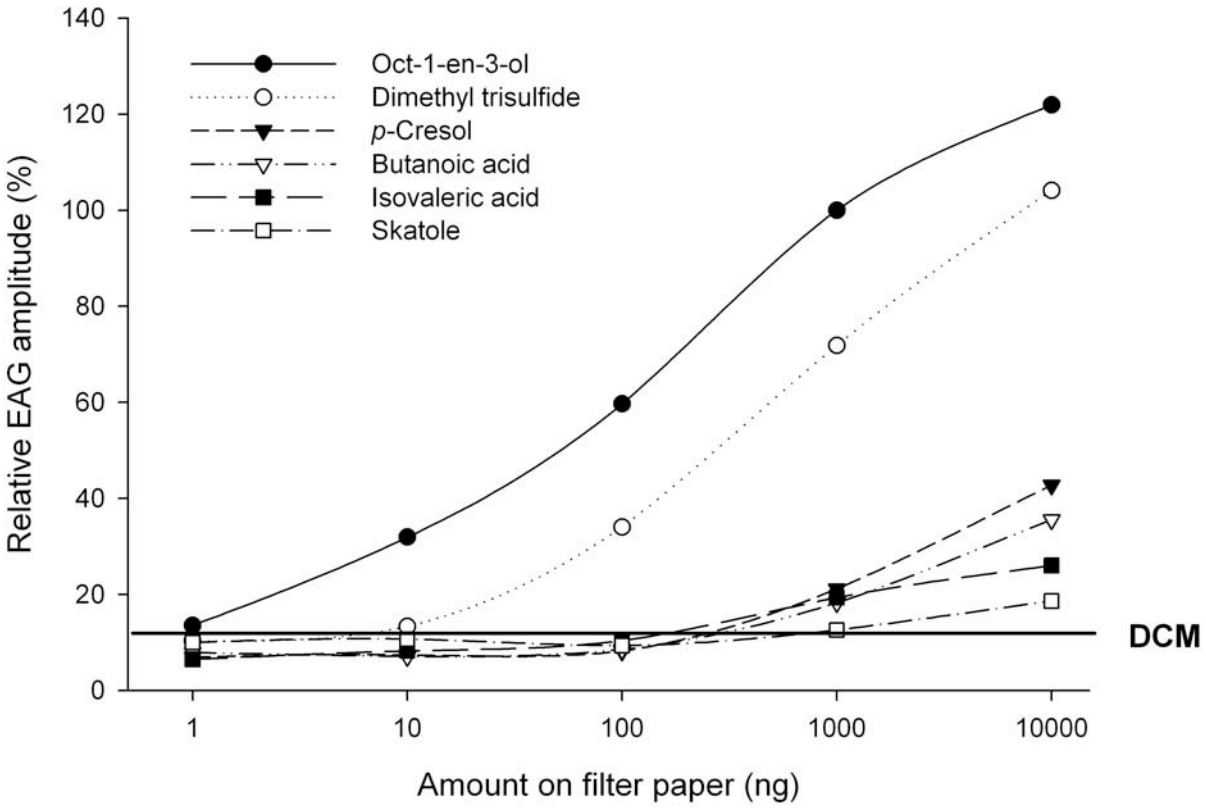

Figure 2: EAG responses of *S. calcitrans* antennae (N=3) to six chemostimuli found in rumen odour. Responses are normalised using 1 µg of oct-1-en-3-ol on filter paper as reference (100%) and the response to methylene chloride (DCM) provides an estimation of the response threshold for each compound.

Dose response curves were also recorded to increasing doses of synthetic compounds of plant origin (Fig. 3), among them β-caryophyllene, α-humulene and decanal that were detected in rumen odour. EAG responses were still recorded to β-caryophyllene, α-humulene and the two aldehydes (nonanal and decanal) at 10 ng in the stimulus syringe. So the antennal thresholds for these products were in the picogram range. Significant EAG responses to bornyl acetate were recorded at 10 µg on the filter paper. Eugenol and linalool elicited higher EAG responses than DCM at between 10 to 100 µg in the stimulus syringe.

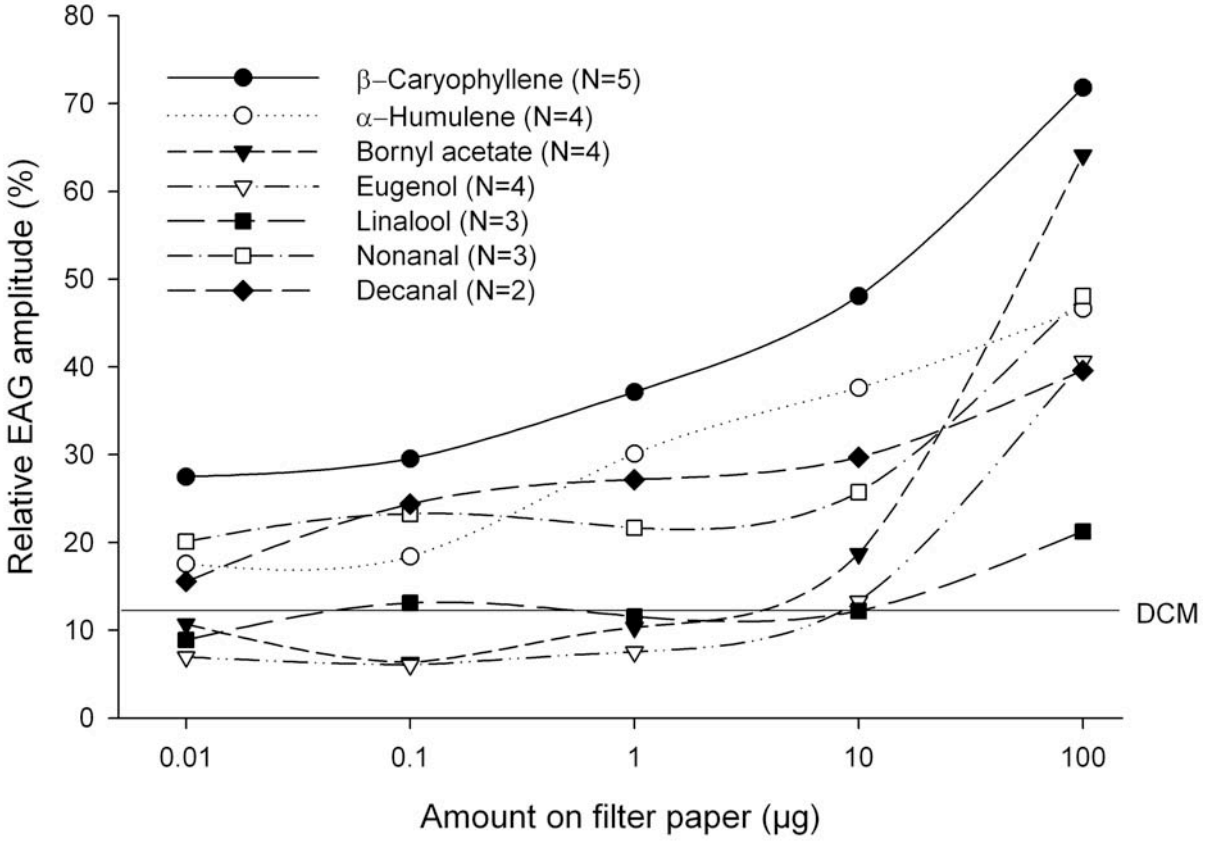

Figure 3: EAG responses of *S. calcitrans* antennae (N ≥ 2 and ≤ 5) to seven chemostimuli of plant origin. Responses are normalised using 1 µg of oct-1-en-3-ol on filter paper as reference (100%) and the response to methylene chloride (DCM) provides an estimation of the response threshold for each compound.

Gas chromatography linked electroantennographic detection (GC-EAD). The EAG responses of *Stomoxys* to increasing doses of six products injected at successively lower doses onto a high resolution gas chromatographic column (Fig. 4) showed that the responses to oct-1-en-3-ol and dimethyl trisulfide persisted at the lowest dose injected (200 pg) corresponding to at most a 1/10 of a picogram perceived by the antenna (see M&M). The sensory threshold for these two products was lower than 1 pg. At 20 ng injected, an EAG response was recorded to *p*-cresol, corresponding to a sensory threshold estimated to be in the range of the picogram. The thresholds recorded for the acids (butanoic and isovaleric) were found to be slightly higher than for *p*-cresol, at around 1-5 pg. No significant EAG response was recorded to skatole at the highest dose injected (100 ng) indicating that the threshold for this product was higher than 10 pg but probably lower than 100 pg (Fig. 2).

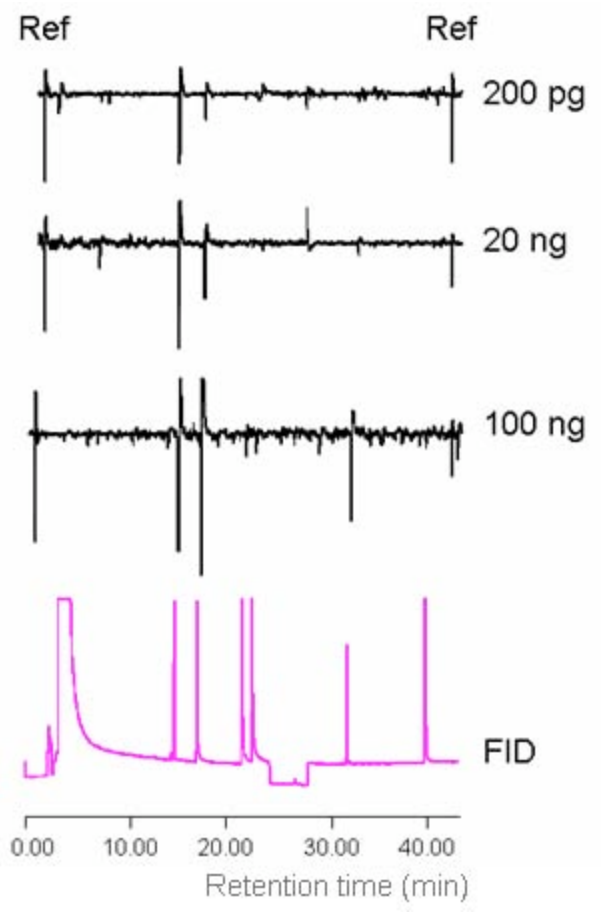

Figure 4 : EAG responses of *Stomoxys* females to increasing doses of 6 products injected at successively higher doses (amount on the right) onto a gas chromatographic column (FFAP). The lowest trace is the flame ionisation detector (FID) response to the following 6 products listed in the order of elution: dimethyl trisulfide, oct-1-en-3-ol, butanoic acid, isovaleric acid, *p*-cresol, skatole. Reference responses (Ref) were recorded at the start and end of the analysis using oct-1-en-3-ol at 1 µg in a stimulus syringe.

Rumen odour contained various classes of volatile compounds that elicited EAG responses from *S. calcitrans* in GC-EAD analyses (Table 1). Separation on the polar column (Fig. 5) revealed high amounts of fatty acids and EAG responses were recorded to acetic, propanoic, isobutyric, butanoic, isovaleric and hexanoic acid. Even when present in low quantities (in the nanogram range), alcohols also elicited EAG responses, and among them, 2-ethylhexan-1-ol, octan-3-ol, (*Z*)- hex-3-en-1-ol, oct-1-en-3-ol, heptan-1-ol and octan-1-ol. Ketones like octan-3-one, 6-methylhept-5-en-2-one and undecan-2-one also elicited EAG responses. Terpenoids eluting from the apolar phase like dihydrocarvone, trans-geranyl acetone and α-humulene evoked EAG responses (Fig. 5). Numerous rumen constituents elicited EAG responses on both the apolar and polar phases like acetophenone, dimethyl trisulfide, *p*-cresol, skatole and the terpenes citronellene, D-limonene, β-cyclocitral and β -caryophyllene. Approximately 86 % of the EAG active compounds were tentatively identified in GC-MS, but a few that elicited EAG responses remained undetermined because of their occurrence in low amounts in the extract. Moreover, EAG responses sometimes coincided with GC peaks composed of more than one product in which the antennal response was not attributed to one candidate but to all the determined compounds associated with the recorded EAG active fraction i.e. peaks 4, 8 on SE-30 and peaks 9, 12, 14 on the FFAP phase.

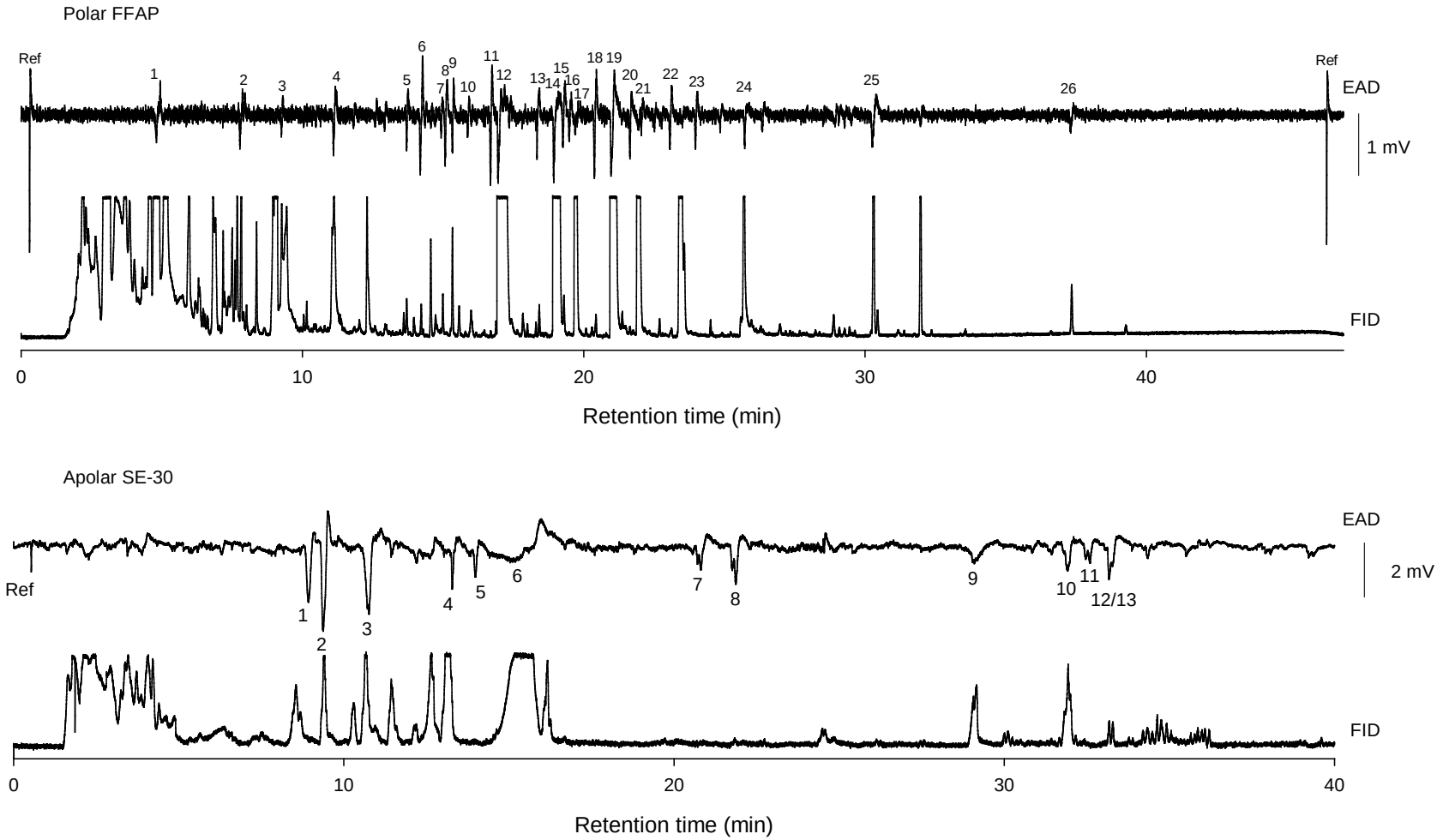

Figure 5: Analysis of fresh rumen volatiles by gas chromatography (upper chromatograph: FFAP phase, lower chromatograph: SE-30 phase) coupled to antennogram recording from *S. calcitrans*. The lower trace is the flame ionisation detector (FID) and the upper trace the electroantennogram responses recorded to the biologically active constituents of the rumen odour extract. The identity of numbered EAG active constituents was confirmed by GC-MS (see Table 1 for details). Reference responses (Ref) were recorded at the start and end of the analysis using oct-1-en-3-ol at 1 µg in a stimulus syringe.

Table 1: List of tentatively identified (by GC-MS) constituents of rumen headspace that evoked EAG responses from *Stomoxys* antennae during GC-EAD analysis (Fig. 5). Results are reported for an apolar column (SE-30) and for a polar column (FFAP). For EAG values and compounds respective proportions (%) the S.E represents the standard error calculated from three GC-EAD traces.

	SE-30					**FFAP**				
Compound name	% ± S.E.	EAG response N°	RI [(1)]	RI [(2)]	Mean EAG ± S.E. (mV)	%	EAG response N°	RI [(1)]	RI [(2)]	EAG (mV)
acetic acid						12.4	12	1461	1457	1.16
propanoic acid *						13.5	14	1541	1541	1.15
isobutyric acid *						8	17	1567	1568	0.32
butanoic acid *						13.6	19	1622	1625	1.04
isovaleric acid *						12.3	21	1667	1669	0.25
hexanoic acid *						6	24	1839	1841	0.57
2-ethylhexan-1-ol	4.1 ± 0.6	4	1018	1015	1.24 ± 0.70					
octan-3-ol						2	9	1390	1388	0.64
(*Z*)-hex-3-en-1-ol *						2	9	1393	1391	0.64
oct-1-en-3-ol *						0.1	11	1449	1448	1.19
heptan-1-ol *						12.4	12	1458	1457	1.16
octan-1-ol *						0.7	15	1556	1558	0.55
decanal *	0.4 ± 0.1	8	1184	1186	2.25 ± 0.30					
acetophenone *	0.4 ± 0.1	5	1034	1100	0.82 ± 0.33	0.2	20	1665	1660	0.75
octan-3-one *						5.6	4	1248	1244	0.69
6-methylhept-5-en-2-one *						0.7	5	1338	1347	0.61
undecan-2-one *						13.5	14	1538	1543	1.15

* = synthetic analogue known to elicit EAG responses from *S. calcitrans* (Appendix 2)

[(1)]: RI of natural product, [(2)]: RI of injected synthetic analogue in GC-MS analyses or RI from the literature (www.flavornet.org and Syed, 2002)

A percentage lower than 0.1 % is considered as a trace amount. Tr, trace.

Table 1: continued

Compound name	SE-30 % ± S.E.	EAG response N°	RI [(1)]	RI [(2)]	Mean EAG ± S.E. (mV)	FFAP %	EAG response N°	RI [(1)]	RI [(2)]	EAG (mV)
skatole *	7.5 ± 0.3	9	1341	1370	0.65 ± 0.22	1.4	26	2498	2495	0.31
p-cresol *	57.0 ± 1.8	6	1042	1213	0.39 ± 0.07	5.6	25	2085	2085	0.54
dimethyl trisulfide *	0.7 ± 0.4	1	942	949	2.28 ± 0.69	0.7	6	1361	1377	1.02
citronellene *	8.2 ± 0.1	2	951	-	3.02 ± 0.38	8.9	1	1028	1056	0.47
D-limonene *	4.1 ± 0.6	4	1018	1022	1.24 ± 0.70	3.6	3	1180	1193	0.37
dihydrocarvone	0.3 ± 0.0	7	1171	-	1.52 ± 0.22					
β-cyclocitral *	0.4 ± 0.1	8	1184	1233	2.25 ± 0.30	0.5	18	1607	1598	1.08
β -caryophyllene *	9.0 ± 0.6	10	1396	1438	1.13 ± 0.26	0.1	16	1564	1590	0.45
trans-geranyl acetone *	0.2 ± 0.0	12	1424	1431	1.34 ± 0.62					
α-humulene *	1.9 ± 0.3	13	1433	1472	1.04 ± 0.29					
3,7-dimethyl-(*Z*)-oct-2-ene	9.9 ± 0.2	3	972	-	2.86 ± 0.70					
1-methyl-3-(1-methylethyl)-cyclohexene						3.3	2	1143	-	0.58
1-propenylbenzene						0.1	7	1384	-	0.39
2,3,6-trimethylhepta-1,5-diene						Tr	8	1386	-	0.87
unidentified						0.1	10	1414		0.38
unidentified						0.3	13	1516		0.75
unidentified						0.2	22	1715		0.58
unidentified						0.1	23	1763		0.58
unidentified	0.2 ± 0.0	11	1414		1.27 ± 0.18					

* = synthetic analogue known to elicit EAG responses from *S. calcitrans* (Appendix 2)

[(1)]: RI of natural product, [(2)]: RI of injected synthetic analogue in GC-MS analyses or RI from the literature (www.flavornet.org and Syed, 2002)

A percentage lower than 0.1 % is considered as a trace amount. Tr, trace.

Wind tunnel experiments. On exposure to human breath 77.5% of the flies were activated and 70.0% were attracted towards the source. The activation level of stable flies stimulated with CO_2 reached 65%, and 52.5% of the flies flew more than 50 cm upwind (Table 2). Despite a higher percentage in activation and attraction recorded on stimulation with breath, no significant difference was found between human breath and carbon dioxide ($p > 0.05$, Chi-square test).

Table 2: Behavioural responses of *S. calcitrans* to human breath and to a 70-90 ppm CO_2 increase (at fly release point) in a wind tunnel; N= total number of flies tested

Treatments	**N**	**% activation**	**% attraction**
Human breath	40	77.5	70.0
CO_2	40	65	52.5

Rumen volatiles significantly activated 65.7% of stable flies as against 37.1% for the control (Table 3). No significant effect on attraction was recorded. Flies showed a low propensity (14.3%) to fly towards the source at first try. However, when only the actively flying flies were considered, these flies responded positively to rumen volatiles showing a higher rate of oriented flights than the controls (Table 3).

Table 3: Behavioural responses of *S. calcitrans* to rumen volatiles in a wind tunnel. The test odour was from 50 g rumen bolus and the control was from 50 ml distilled water. Oriented flights in plume represent the mean number per activated fly; *: P<0.05, **: P< 0.01, ***: P<0.001 compared to control, Chi-square test (activation + attraction) and Mann-Whitney (oriented flights) test; 70 flies were tested.

	Control	**Rumen**
% of flies activated	37.1	65.7^{***}
% of flies attracted	7.1	14.3
Oriented flights in plume	0.6	1.5^{**}

We measured an increase in carbon dioxide concentration of 20 ± 10 ppm at the funnel exit for rumen odour but due to the dilution the gas analyser did not detect any variation in CO_2 at insect release point.

Among the synthetic compounds tested in the wind tunnel, three of them were shown to activate and attract flies in a manner significantly different to the control (Table 4). Dimethyl trisulfide was the most active compound tested eliciting 72.5% activation and 41.3% attraction, resulting in an average of 2.2 oriented flights in the plume per activated fly. It was followed by butanoic acid accounting for 67.5% activation, 21.3% attraction and 1.7 oriented flights per fly in average. Similarly, *p*-cresol was responsible for activation in 75.0% of flies with 21.25% attraction and 1.3 oriented flights per activated fly. oct-1-en-3-ol and skatole were both found no different from the control in activating (57%) and attracting flies towards the source, 17.5% and 13.25%, respectively (Table 4). But a significant difference in oriented flights (1.3) was recorded during the exposure to oct-1-en-3-ol.

A mixture (Mix 1) of *Stomoxys* chemostimuli composed of dimethyl trisulfide and oct-1-en-3-ol each at 10 µg in a dispenser showed an intermediate efficiency in activation (70%) and attraction (21.25%) in comparison to that obtained when either synthetic was tested alone at 10 µg. The percentage of activation (70%), attraction (21.25%) and the number of oriented flights (1.8) recorded for this binary mixture remained, nevertheless, significantly different from that obtained to the control. A higher percentage of activation (83.8%) was recorded with a second mixture (Mix 2) of chemostimuli composed of dimethyl trisulfide, butanoic acid and *p*-cresol each at 10 µg in a dispenser. Attraction was high (28.8%) to mixture 2 but lower than to dimethyl trisulfide alone (41.3%). Correspondingly, fewer oriented flights (1.5) were recorded than with dimethyl trisulfide alone (2.2). However, attraction and the number of oriented flights to mixture 2 were higher than with either butanoic acid or *p*-cresol tested alone at the same individual concentration (Table 4).

Table 4: Behavioural responses of *S. calcitrans* to synthetic compounds in a wind tunnel. The test consisted of 10 µg of test substance in a dispenser and the control was methylene chloride (DCM). Oriented flights in the plume are the mean number per activated fly; *: P<0.05, **: P< 0.01, ***: P<0.001 compared to control, Chi-square test (activation + attraction) and Mann-Whitney (oriented flights) test; 80 flies were tested per treatment.

	% of flies activated	**% of flies attracted**	**Oriented flights in plume**
Control (DCM)	48.8	7.5	0.7
Dimethyl trisulfide	72.5**	41.3***	2.2***
Butanoic acid	67.5*	21.3*	1.5**
Oct-1-en-3-ol	57.5	17.5	1.3*
p-Cresol	75.0***	21.25*	1.3*
Skatole	57.5	13.75	0.9
Mix 1[a]	70**	21.25*	1.8***
Mix 2[b]	83.8***	28.8***	1.5**

a: dimethyl trisulfide and oct-1-en-3-ol, each one at 10 µg in the dispenser

b: dimethyl trisulfide, butanoic acid and *p*-cresol, each one at 10 µg in the dispenser

Discussion :

Breath and carbon dioxide. Human breath was shown to activate and attract a marginally higher percentage of stable flies in our wind tunnel assays in comparison to CO_2 presented at the same concentration. However, as no significant differences were recorded, the influence of other chemical constituents of breath on *Stomoxys* behaviour remains questionable. On the one hand, De Jong & Knols (1995) reported that breath-related chemicals do not enhance the attractiveness of CO_2 alone to *An. gambiae* s.s., whereas studies on the same species of mosquitoes (Healy & Copland, 1995), on tsetse flies (Warnes, 1990) and on ticks (McMahon & Guerin, 2002) characterised human breath acts as a stronger attractant than CO_2 alone. Mukabana *et al.* (2004) also suggested that breath is a key factor responsible for variability in human attractiveness to the anthropophilic mosquito *An. gambiae*. Moreover, we observed that among the numerous compounds found to occur in human breath (Wahl *et al.*, 1996; Phillips *et al.*, 1999), some volatiles are perceived by stable flies. Acetone for instance that is also found in cow breath (Spinhirne *et al.*, 2004), has been shown to elicit activation in stable

flies (Schofield *et al.*, 1997) and upwind orientation has been recorded by Warnes & Finlayson (1985) as well as by Alzogaray & Carlson (2000). Cilek (1999) also reported that Alsynite fibreglass cylinders used as traps and baited with acetone caught significantly more stable flies than no odour. We might also expect other heavier molecules like acetophenone, acetic acid and propanoic acid, nonanal, limonene, endobornyl acetate and phenol known to occur in human breath (Phillips *et al.*, 1999) and eliciting EAG responses from stable fly antennae to play a role in host seeking by stable flies. Despite some evidence that breath does contain potential attractants, the importance of carbon dioxide for stable flies, accounting for 4.5% of breath, should not be neglected. Indeed, laboratory experiments with CO_2 (5%) conducted by Gatehouse & Lewis (1973) in a large slow-speed wind tunnel, showed that CO_2 enhanced *S. calcitrans* activity. Similarly, Schofield & Brady (1997) recorded an increase in sinuosity and angular velocity whereas the linear flying velocity of stable flies was decreased in response to CO_2, with a response threshold around 60 ppm. Warnes & Finlayson (1986) recorded increasing EAG responses from *Stomoxys* antennae to increasing concentrations of CO_2 with a logarithmical increase in antennal responses up to approximately 2 %. They also found that flies responded to an increase in concentration (0.01 to 5 %) by an increase in flight activity measured as the number of take-offs, and that hungrier flies showed a better response to low levels (Warnes & Finlayson, 1985). Field studies conducted by Vale (1980) demonstrated that CO_2 was as attractive as ox odour to Stomoxyinae and Cilek (1999) confirmed that dry ice could increase stable fly captures in cylinder traps as much as 25-fold compared with blank controls. The general occurrence of CO_2 in a range of hosts and oviposition substrates (Chapter 3) and its efficiency in attracting stable flies by itself, suggests that CO_2 is of major importance for *S. calcitrans* to locate relatively close resources. As described by Knols & Meijerink (1997) for host finding in mosquitoes, CO_2 that is highly volatile and its diffusion characteristics may influence the resource seeking behaviour of stable flies from up to 20 meters, whereas attraction from longer distances occurs in response to less volatile molecules that are more susceptible to travel longer distances in discrete packets (Murlis & Jones, 1981) with a sufficiently high intrinsic concentration to initiate behaviour.

Rumen. We found that rumen volatiles can activate stable flies but we only recorded weak attraction. Although attractive for ticks (Donzé *et al.*, 2004) and tsetse flies (Syed, 2002),

rumen volatiles did not demonstrate such a clear effect on stable flies. The fact that we used quite high amount (approximately 50 ml) of rumen in comparison to the two other studies (0.4-1 ml) may partly explain such a result in a similar way as Knols *et al.* (1997) showed that an undiluted acidic extract of Limburger cheese was repellent to *An. gambiae* but highly attractive when diluted. Nevertheless, the number of oriented flights made in the plume was significantly higher than with the control. This suggests that this biological substrate might contain important compounds other than carbon dioxide that are regularly liberated in ruminant breath. Indeed, the CO_2 concentration measured at the fly release point in the wind tunnel was very low and it would probably be false to exclusively attribute *Stomoxys* activation to the CO_2 released from rumen bolus after its exposure to air. Moreover, analysis of the volatile organic compounds in bovine breath conducted by Spinhirne *et al.* (2004) confirmed the presence of acetic acid, propionic acid, isovaleric acid, hexanoic acid, decanal and acetophenone, compounds we found in our rumen odour collections too and that elicited EAG responses from stable flies.

Dimethyl trisulfide was identified in rumen volatiles and despite the low amounts at which it was present, strong and consistent EAG responses were recorded. The low response threshold was furthermore confirmed with the dose response curve showing perception in the hundreds of femtograms range by the antenna. Dimethyl trisulfide like dimethyl disulfide arises from the oxidation of methanethiol, whose precursor is the essential amino acid methionine (Weimer *et al.*, 1999). It has been detected in a large range of materials under bacterial degradation and is likely to occur in livestock wastes (Mackie *et al.*, 1998; Louhelainen *et al.*, 2001; Le *et al.*, 2004) contributing to the unpleasant smell. It is also commonly encountered in cheese flavour thanks to the activity of lactic acid bacteria (van Kranenburg *et al.*, 2002). Bernier *et al.* (2000) reported the presence of dimethyl trisulfide in their analysis of human skin emanations and methanethiol, its precursor, is known to be produced by *Brevibacterium epidermidis*, a bacteria occurring on human skin (Braks *et al.*, 1999). Furthermore, the evaluation of volatile sulphur compounds in human breath conducted by Kaji *et al.* (1978) and Wahl *et al.* (1996) also revealed the occurrence of methanethiol and dimethyl disulfide suggesting a possible presence of dimethyl trisulfide in trace amount. Nilssen *et al.* (1996) demonstrated that dimethyl trisulfide from reindeer interdigital glands is a strong attractant for some calliphorids and the muscid fly *Hydrotaea anxia* in the field. Dimethyl trisulfide was also found in pig manure volatiles and attracted house flies in wind tunnel assays when mixed with butanoic acid and skatole (Cossé & Baker, 1996). Our wind tunnel observations

demonstrated a strong response from stable flies to dimethyl trisulfide and since the discovery of the importance of this product for *S. calcitrans* attractancy has been shown for other haematophagous insects like the sandfly *Lutzomiya longipalpis* (Bourquin, unpublished data) and the triatomine bug *Rhodnius prolixus* (Otalora-Luna, unpublished data) in the animal physiology laboratory at Neuchâtel.

Donzé *et al.* (2004) found volatile carboxylic acids to predominate as constituents in rumen odour but our first analysis on an apolar column (SE-30) did not yield such results. This is not really surprising as polar compounds and particularly acids are poorly resolved on apolar phases. Therefore we conducted a separation on a polar column especially designed for the separation of fatty acids (FFAP). The results obtained confirmed the presence of large amounts of acetic, propanoic, isobutyric, butanoic, isovaleric, pentanoic and hexanoic acids as constituents in rumen odour. Information about potential effects of carboxylic acids on stable fly host-seeking behaviour is scarce. Mihok *et al.* (1995) reported that D/L-lactic acid failed to increase stable fly catches with Vavoua traps and no attraction of *Stomoxys* was observed by Alzogaray & Carlson (2000) to compounds related to L-lactic acid. Still Vale (1980) recorded a repellent effect of acetic acid for Stomoxyinae and non-biting Muscidae. In our wind tunnel assays butanoic acid was shown to activate and attract *S. calcitrans*, and the importance of straight and branched carboxylic acids for host location in haematophagous arthropods has been widely demonstrated (Table 5).

Table 5: Haematophagous arthropods responding in behavioural and/or in electrophysiological assays to carboxylic acids.

Arthropods	**References**
Ticks	McMahon (1999); Donzé *et al.* (2004); Carty (2004)
Mosquitoes	Knols *et al.* (1997); Clements (1999); Bosch *et al.* (2000); Costantini *et al.* (2001)
Triatomine bugs	Guerenstein & Guerin (2001); Otalora-Luna (unpublished data)
Tsetse flies	Vale (1980); Syed (2002)

Previous studies demonstrated the occurrence of phenolic compounds in rumen (Syed, 2002; Donzé *et al.*, 2004) and Mohammed *et al.* (2003) attributed the presence of *p*-cresol, indole and skatole to tryptophan degradation by rumen bacteria. Our results confirmed their presence

as rumen constituents and consistent EAG responses were recorded to *p*-cresol and skatole. In the wind tunnel, only *p*-cresol elicited significant activation and attraction at 10 μg in the dispenser. Studies on tsetse flies showed that the attractiveness of cattle urine was entirely attributable to *p*-cresol and 3-n-propyl phenol (Bursell *et al.*, 1998) and Torr *et al.* (1995) partly attributed attraction towards ox odour to phenols. This is consistent with the occurrence of *p*-cresol in cow hairs odour (Appendix 4). Actually, the most widely used attractant for tsetse flies is a mixture that contains *p*-cresol, 3-n-propyl phenol, oct-1-en-3-ol and acetone. *S. calcitrans* was shown to respond to phenol, 3-methyl phenol, 4-methyl phenol and 2-methoxy phenol (Schofield *et al.*, 1995; Birkett *et al.*, 2004; Appendix 2) and Cilek (1999) reported that a modified blend without acetone significantly increased stable fly collection in cylinder traps, nearly 6-fold compared with no odour. We suggest though that *p*-cresol is used by stable flies for host location.

Rumen was also found to contain various alcohols not detected previously as constituents with the apolar column and, among them, oct-1-en-3-ol was surprisingly detected only in trace amounts. As oct-1-en-3-ol is produced by enzymatic oxidation of linoleic acid, we were surprised to find it in the rumen, which is highly anaerobic. One may suspect aerobic processes to have set in during rumen collection and transportation to the laboratory. The first reference to this compound as an insect attractant was made by Hall *et al.* (1984), who discovered it as a tsetse fly attractant in cattle odour. Since then oct-1-en-3-ol has given rise to varying results with some attesting its attractiveness to tsetse flies, tabanids or mosquitoes (Hall *et al.*, 1984; French & Kline, 1989; Kline *et al.*, 1990) and others claiming its lack of effect on tsetse flies (Vale & Hall, 1985). Concerning *S. calcitrans* Mihok *et al.* (1995) recorded an increase of up to 3.7-fold in the number of flies caught with Vavoua traps baited with oct-1-en-3-ol. Similarly Holloway & Phelps (1991) described a significant effect of oct-1-en-3-ol on the efficiency of F3 traps for capturing stable flies. In contrast to these results, field studies (Mullens *et al.*, 1995; Cilek, 1999) showed that oct-1-en-3-ol failed to attract flies in cylinder traps. Moreover, Schofield & Brady (1997) reported very low activity from *Stomoxys* in response to oct-1-en-3-ol in wind tunnel experiments, and neither attraction nor orientation has been recorded in response to oct-1-en-3-ol in a triple cage olfactometer experiment with *S. calcitrans* (Alzogaray & Carlson, 2000). Our observations in the wind tunnel tend to corroborate the latter results in that no activation or attraction was recorded upon exposure to oct-1-en-3-ol. But, we still observed a significantly higher number of oriented flights in the plume compared to the control, but no additional effects were observed

even when oct-1-en-3-ol was mixed with the best *Stomoxys* attractant dimethyl trisulfide. Such findings are surprising in view of the low threshold recorded in electrophysiology. Why does *S. calcitrans* possess receptors highly tuned for oct-1-en-3-ol if this compound does not elicit any behavioural response? In our case, the most likely explanation could be that we did not test the appropriate dose for eliciting activation or attraction, or a restricted contribution of oct-1-en-3-ol to mixtures could also be another alternative.

Our rumen headspace analysis yielded also the following ketones as chemostimulants for *Stomoxys*: acetophenone, 6-methylhept-5-en-2-one, octan-3-one and undecan-2-one. Ketones arise from different biosynthetic pathways. Acetophenone derives from the amino acid L-phenylalanine, octan-3-one and undecan-2-one come from fatty acids degradation and 6-methylhept-5-en-2-one is an isoprenoid derivative. Interestingly, acetophenone and 6-methylhept-5-en-2-one, also detected as chemostimulants for *Stomoxys* in cow hairs (Appendix 4), are commonly encountered in various sources of volatiles known to attract haematophagous arthropods like human or bovine breath (Wahl *et al.*, 1996; Phillips *et al.*, 1999; Spinhirne *et al.*, 2004), human skin emanations (Bernier *et al.*, 2000) and human sweat (Meijerink *et al.*, 2000). However, Vale (1980) reported a repellent effect of acetophenone for tsetse flies, and adding slow-release dispensers baited with 6-methylhept-5-en-2-one on the back of susceptible heifers significantly reduced fly loads of *S. calcitrans, Musca autumnalis, Haematobia irritans, Hydrotaea irritans* and *Wohlfahrtia magnifica* (Birkett *et al.*, 2004). Furthermore, undecan-2-one found in odours of Bermuda grass infusions evoked EAG responses from *Culex* spp. but failed to attract them for oviposition (Du & Millar, 1999). All these observations provide some evidence for a repellent effect of these ketones. Nevertheless, a repellent activity can be a question of dose and we suggest that these products at low doses might at least contribute to the preference shown by some blood-sucking insects for hosts and other substrates.

Terpenoids were also detected in rumen headspace and citronellene, β-cyclocitral, D-limonene, dihydrocarvone, trans-geranyl acetone, β-caryophyllene and α-humulene were found to elicit EAG responses from *S. calcitrans*. Moreover, stable flies showed a very low perception threshold for β-caryophyllene and we found that many terpenoids evoke EAG responses from *Stomoxys* antennae (Appendix 2). From a general point of view, terpenoids have been found to stimulate many blood-sucking arthropods (Table 6). Although, it is not surprising that stable flies and mosquitoes (Bowen, 1992) that are known to feed on plants in

nature should respond to these typical plant products, the fact that even obligate haematophagous arthropods like tsetse flies (Syed & Guerin, 2004) and ticks (Guerin, unpublished data) do respond to terpenoids suggests that these chemostimuli may also be used for host location. Moreover, many of the terpenoids identified in rumen odour are also known from a range of vertebrate odours such as human breath (Phillips *et al.*, 1999), steer hair (Steullet, 1993), chicken feathers and sheep wool (Guerenstein, 1999). It is also worth mentioning their occurrence in the oviposition substrates exploited by gravid *Stomoxys* females (Chapter 3).

Table 6: Haematophagous arthropods responding to terpenoids.

Arthropods	**References**
Ticks	Carty (2004); Guerin (unpublished data)
Mosquitoes	Bowen (1992)
Triatomine bugs	Guerenstein (1999)
Sand flies	Dougherty *et al.* (1995)
Tsetse flies	Syed (2002); Syed & Guerin (2004)

In summary, we confirmed human breath and CO_2 to be strong attractants for stable flies. However, we suggest that key compounds other than CO_2 present in human breath contribute to its attractiveness. Furthermore, analysis of rumen volatiles revealed the occurrence of numerous EAG active compounds exhaled in bovine breath that belong to different chemical classes. Various carboxylic acids, alcohols, ketones, phenols, indoles, sulfides and terpenoids were detected as *S. calcitrans* chemostimulants. Moreover, we found stable flies to possess a very low perception threshold for some volatiles like dimethyl trisulfide and β-caryophyllene. So the heightened behavioural activity manifested by *S. calcitrans* on exposure to rumen headspace might be due to some of these volatiles. The percentages of activation and attraction obtained in the wind tunnel with dimethyl trisulfide, butanoic acid and *p*-cresol either presented individually or in a mixture confirmed the importance of rumen volatiles exhaled in bovine breath in host location by the stable fly.

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Olfactory stimuli involved in the selection of oviposition substrates by the stable fly

Abstract :

Horse and cow dung were tested for their potential to attract gravid stable flies *Stomoxys calcitrans* to a suitable site for oviposition. Dung odour by itself attracted flies for oviposition even without any contact with the substrate and gravid females were able to localise either horse or cow dung for oviposition. This was confirmed with wind tunnel experiments where horse and cow dung were shown to attract stable flies. However, when flies were proposed a choice between these two oviposition substrates, flies always chose horse dung over cow dung for oviposition, either when they had contact with the substrate or just dung odour to rely on. Furthermore, we observed that horse dung volatiles stimulated gravid females to lay more eggs than in the absence of odour. The analyses of volatile compounds emitted by horse and cow dung revealed different gas-chromatographic (GC) profiles but flies showed similar patterns of antennal responses to constituents of both substrates in GC-linked antennographic analyses. The volatile compounds that consistently elicited antennal responses in stable flies like carboxylic acids, alcohols, aldehydes, ketones, phenols, indoles, terpenoids and sulphides were found to occur in both dung odours. Apart from these products, horse dung was shown to release higher levels of CO_2 than cow dung. As CO_2 serves as an attractant for most of the blood-sucking insects, it may be partly responsible for the preference exhibited by *S. calcitrans* for horse dung.

Key words : stable fly, oviposition, olfaction, electrophysiological recordings, bioassay

Introduction :

Numerous observations in the field have demonstrated the low selectivity of stable flies in the choice of substrates on which to lay their eggs. It seems indeed that all kinds of decomposing organic matter of plant origin like silage, rotting hay, piles of grass clippings, garden compost piles and even sea grass deposits may form appropriate breeding media (Zumpt, 1973; Axtel, 1986; Broce & Haas, 1999). Cooked rice straw soaked with urine and cooked plant leaves were found to be highly attractive to *Stomoxys* gravid females yielding large numbers of flies and animal faeces were much less attractive when not mixed with fermenting plant material (Hafez & Gamal-Eddin, 1959). These authors also reported *S. calcitrans* preferences for equine (donkey and horse) manure especially when mixed with straw and wetted with urine, followed by cattle dung that attracted the ovipositing females only to a slight degree and finally sheep and goat droppings that were the least favoured oviposition substrates. Despite the apparent variety of potential substrates that can be selected by stable flies for egg deposition, it can be supposed that flies rely on a particular set of chemostimuli shared by all these substrates. As decomposing organic matter releases CO_2 and volatile compounds that arise from the degradation of carbohydrates, lipids and proteins through microbial activity, one can expect in all these potential oviposition substrates the production of carboxylic acids, sulphur containing compounds and aromatic compounds like indoles. Indoles could attract *Stomoxys* females in the same manner as fermented grass infusions, that also release indoles, attract female mosquitoes for oviposition (Beehler *et al.*, 1994; Millar *et al.*, 1994; Allan & Kline, 1996). Terpenoids should also be well represented in all the substrates containing vegetal matter and CO_2 that attracts numerous blood-feeding insects (Chapter 2) may also be implicated in the oviposition behaviour of *S. calcitrans.*

We hypothesise that flies primarily locate a suitable oviposition site at a distance using olfactory cues and then use information from contact chemoreceptors for the final acceptation or rejection of the substrate. We conducted experiments with horse and cow dung, potential oviposition substrates commonly encountered at temperate latitudes, and attempted to determine the key volatile compounds employed for oviposition by *S. calcitrans.*

Material & Methods :

Insects and oviposition substrates. Newly emerged flies were sexed daily and 15 males and 25 females were held in cotton mesh (1 mm) cages (15 x 25 x 15 cm) placed in an environmental chamber (conditions as described in Chapter 2). Flies were fed heparinized bovine blood (Veterinary School, University of Bern, Switzerland), and had access to cotton imbibed daily with 5 ml fresh blood. Four days after emergence 10 gravid females were placed in a bigger cage (30 x 30 x 30 cm) made of white nylon netting (2 mm) and used for the oviposition experiments with access to a 10% sucrose solution on cotton wool. The 15 remaining females were separated from males and used in wind tunnel experiments or GC-EAD recordings (see below). Fresh horse and cow dung were collected from animals pasturing in a farm located in Fleurier (Neuchâtel, Switzerland) and stored at 4°C until needed.

Volatile collection and fractions. 50 g of either cow or horse dung was introduced into a 1-L gas-wash flask. The flask was left 1 h for equilibration at room temperature. Charcoal-filtered air entered the bottle at 50 ml min^{-1}. Headspace was collected at the other end of the T-glass connector with a commercial Tenax™ GR cartridge (6 mm OD., Gerstel®, Switzerland). The collection lasted for 20 min. Fractions were obtained by manipulating the pH of aqueous samples of either cow or horse dung as in (Donzé *et al.*, 2004).

Electroantennograms (EAG). Electroantennograms from 2 days-old *S. calcitrans* females' antennae were recorded as described in Chapter 2. Approximately 2 g of either mule faeces, sheep bedding or rotten hay were introduced in syringes. The response to 100 ng of oct-1-en-3-ol (>97%, Merck) disposed on a small filter paper strip (0.8 cm x 3 cm) inserted into the stimulus syringe, was used as reference.

Gas chromatography (GC) linked electroantennographic detection (EAD). Analyses of dung volatiles were run on a polar high-resolution capillary column (FFAP, L 30 m, ID 0.25, df 0.25, BGB Analytik, Switzerland) as described in Chapter 2.

An averaged GC-EAD trace for each oviposition substrate was redrawn from the three more representative GC-EAD analyses of an extract from either cow or horse dung. Only antennal responses to a given constituent of the extract recorded in at least two traces were taken into account for identification.

Gas chromatography coupled mass spectrometry (GC-MS). Biologically active constituents of either cow or horse dung that caused EAG responses from *S. calcitrans* antennae were identified by gas chromatography linked mass spectrometry as described in Chapter 2.

Oviposition assays. The cage containing 10 gravid females was placed on a black cardboard paper (0.2 mm thickness) pierced with two 8.5 cm diam. holes that were covered with a black nylon netting (0.25 mm). A plastic dish (10 cm diam. x 5 cm) containing either 25 g of cow dung or horse dung was placed under one hole of the black piece of paper, and under the opposite hole a dish with water-soaked cotton was used as control. Eggs were counted after 24 h. Data were corrected for the area of the cage and holes in order to obtain the number of eggs laid per cm^2 over dung, control and elsewhere on the floor of the cage, and their respective numbers were statistically compared using Kruskal-Wallis tests. Four replicates were made for each treatment. The same procedure was used for choice experiments where the attractiveness of cow and horse dung was compared with 25 g cow dung in one dish and 25 g horse dung in the other. The cage was carefully washed and the position of holes changed for each replicate to avoid any bias. Moreover, a third experiment with empty plastic dishes was made as a control to check for any chemical contamination of the black netting covering holes. The concentration in carbon dioxide above both substrates was also recorded for 24 h with a Li-820 gas analyser (Li-Cor®, DMP, Switzerland) linked to a portable computer.

The same procedure was also used for oviposition experiments with a reconstituted blend of synthetics (> 98% pure) composed of acetic, propanoic, isobutyric, butanoic, isovaleric, pentanoic, 4-methyl pentanoic, hexanoic, benzoic acids, phenol, 3-ethyl phenol, 4-ethyl phenol, *m*-cresol, *p*-cresol, indole and skatole in methylene chloride (DCM, Merck, analytical

grade). A sealed polyethylene dispenser (1.32 cm diam., 3 cm long, 1.6 mm thick walls, Kartell, Italy) filled with 500 µg of each synthetic compound was placed in the plastic dish (10 cm diam. x 5 cm) situated under the test hole. As a control we used a dispenser filled with 800 µl methylene chloride (DCM).

For oviposition experiments where flies had complete access to dung we used a slightly different procedure, in that experiments were conducted in cotton netting (1 mm) cages (15 x 25 x 15 cm) and the plastic dish (9 cm diam. x 1.5 cm) containing either 20 g of either cow or horse dung was placed directly in the cage. Eggs laid in the oviposition substrate and elsewhere in the cage were counted and their respective proportions were compared for each substrate tested using the Mann-Whitney test.

Wind tunnel and stimulus delivery. The wind tunnel set-up was as described for the tests with human breath and carbon dioxide in Chapter 2.

For tests with natural substrates, 50 g of dung (test) and 50 ml distilled water (control) were directly introduced in 1-L glass bottles. For tests with acidic or phenolic fractions, 400 µl of the fraction (test) and 400 µl of methylene chloride (DCM control) were applied to a (125 mm diam.) filter paper (Schleicher & Schuell, Germany), which was immediately introduced in the 1-L glass bottles equipped with a T-glass connector. One end of the T-connection was linked to a stimulus delivery device blowing charcoal-filtered air at 400 ml min^{-1} through Teflon® tubing into the bottle. The other end was linked via Teflon® tubing to an aluminium tube (4 mm ID) releasing the gas-wash bottle headspace through a perforated (0.4 mm diam. holes) aluminium funnel (4 cm diam.) facing downwind at a height of 35 cm. The odour plume structure was checked as described in Chapter 2.

The response of gravid females was tested to odours emanating from cow and horse dung and to acidic and phenolic fractions obtained from dung. After 2 min of acclimatisation in the wind tunnel, the nylon-mesh door was lifted-up slowly and flies were successively exposed for 2 min to odour-free air (control) and then for 2 min to the test odour from two funnels placed side by side. Effects of test stimuli were estimated as the percentage of flies that were activated upon exposure to dung volatiles and the percentage of flies attracted to the odour

source. As flies were naturally active, we considered that a fly was activated when it flew more than 3 times in the flight cylinder and that it was attracted when it flew more than 50 cm from the release cage towards the source. Flies that were activated or attracted during the control period were discarded. The Observer software from Noldus Technologies (The Netherlands) was used for recording fly behaviour during observations. Chi-square and Fisher's exact tests were used for statistical comparison of the activation and attraction percentages between test odours. The variation in CO_2 concentration during stimulation with cow and horse dung was measured at the funnel exit and at the fly release point with the Li-820 gas analyser (Li-Cor®, DMP, Switzerland).

Results :

Electroantennograms. EAG responses from a female stable fly antenna were recorded to odours from potential oviposition media (Fig. 1). Mule faeces, sheep bedding and rotten hay evoked EAG amplitudes of 1.25, 1.31 and 2.61 mV, respectively. The EAG response of 1.74 mV elicited by oct-1-en-3-ol at 100 ng in the stimulus syringe is represented for comparison.

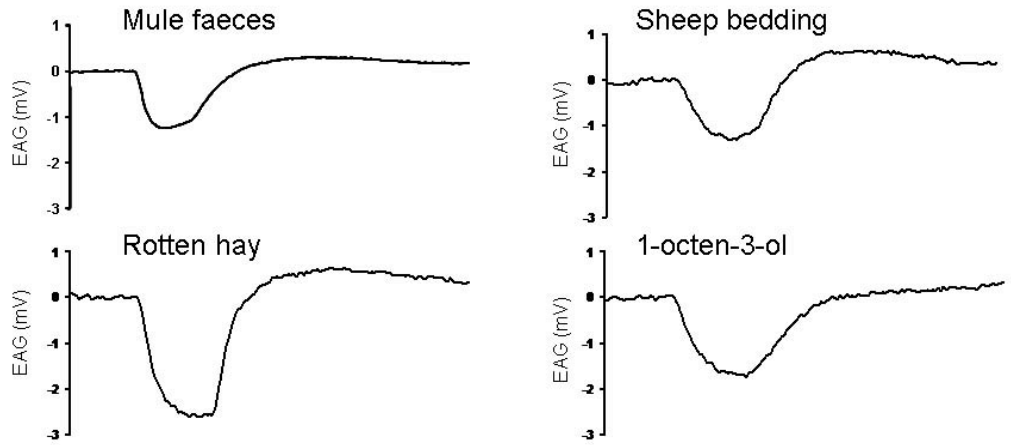

Figure 1: EAG responses of *S. calcitrans* female to various odours from potential oviposition substrates and to oct-1-en-3-ol at 100 ng in the stimulus syringe for comparison.

Gas chromatography linked electroantennographic detection. The EAG responses of a *Stomoxys* female to five carboxylic acids injected at 100 ng each onto a high resolution gas chromatographic column (Fig. 2) shows that the stable fly respond to C_3 to C_5 straight and branched carboxylic acids with a more marked response for C_4 acids (butanoic and isovaleric acids).

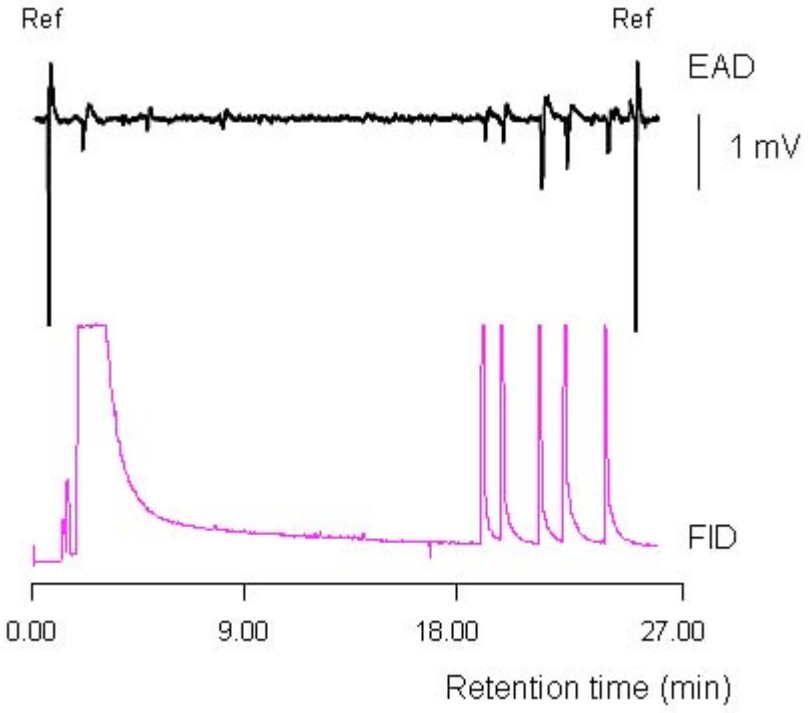

Figure 2 : Antennogram responses of a *Stomoxys* female antenna to a series of carboxylic acids each injected at 100 ng onto a polar gas chromatographic column (FFAP). The upper trace is the antenno-graphic detector response (EAD), the lower the response of the flame ionisation detector (FID) to the following carboxylic acids listed in order of elution: propanoic acid, isobutyric acid, butanoic acid, isovaleric acid, pentanoic acid. Reference responses (Ref) were recorded at the start and end of the analysis using oct-1-en-3-ol at 1 µg in a stimulus syringe.

Despite the different gas-chromatographic profiles recorded for cow and horse dung volatile collections on Tenax™ GR, flies showed similar patterns of antennal responses (Fig. 3). Of the 27 volatile compounds eliciting electroantennographic response (Table 1), stable flies consistently responded to 25 compounds occurring in both substrates. Responses n°15 to n°19

clearly visible in the averaged GC-EAD trace for horse dung volatiles, were also detected in the GC-EAD trace of cow dung volatiles but as these responses were less obvious they are not represented in figure 3 for purposes of clarity. Antennal responses to heptan-1-ol (response n°10) and β-caryophyllene (response n°13) were observed in only the odour of one substrate, respectively, cow and horse dung. However, GC-MS analyses revealed that these two compounds may occasionally occur also in the opposite substrate. Volatile compounds eliciting electrophysiological responses belong to different chemical classes including acids (acetic, propanoic, butanoic, isovaleric, hexanoic), alcohols (heptan-1-ol, octan-1-ol, octan-2-ol, octan-3-ol, oct-1-en-3-ol), aldehydes (decanal), ketones (octan-3-one, acetophenone), indoles (indole, skatole), phenols (phenol, *p*-cresol), sulfides (dimethyl trisulfide), terpenes (citronellene, D-limonene, β-cyclocitral, β-caryophyllene, α-humulene, borneol) and hydrocarbons (1-methylethylidene-cyclohexane). EAG responses n°11 and n°12 are corresponding to GC peaks composed of more than one constituent that might have contributed together to the amplitude of the electrophysiological response.

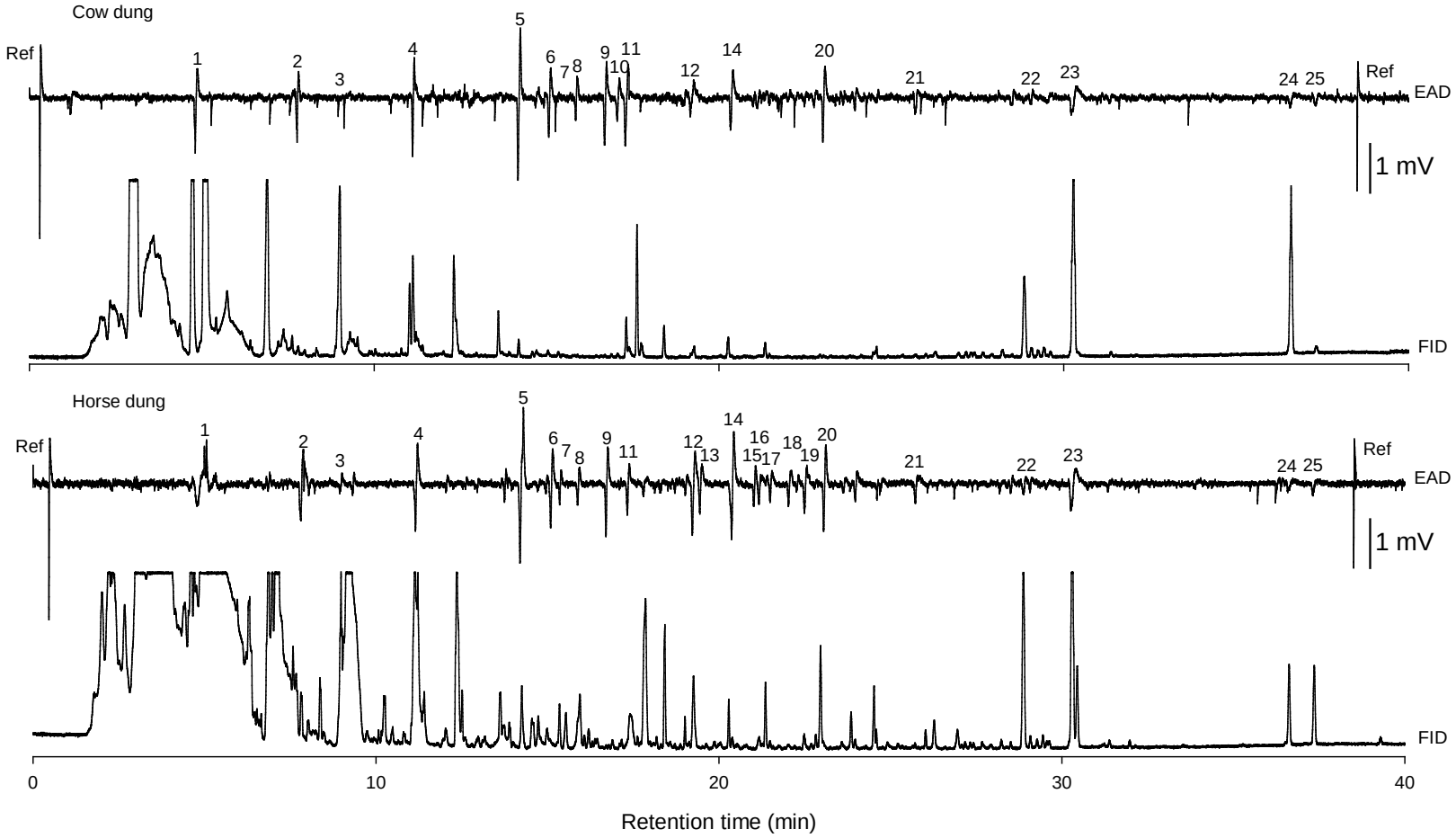

Figure 3: Antennal responses of *S. calcitrans* to cow and horse dung volatiles thermally desorbed onto a high resolution gas chromatographic column from a Tenax™ GR cartridge. The lower traces are the signal from the flame ionisation detector (FID), the upper traces the EAG responses of 3 female stable flies (averaged trace). The identity of the volatile compounds, corresponding to EAG responses represented here by numbers, is provided in Table 1. Reference responses (Ref) were recorded at the start and end of the analysis using oct-1-en-3-ol at 1 µg in a stimulus syringe.

Table 1: Determination of volatile compounds from horse and cow dung eliciting EAG responses from female stable flies on a FFAP high resolution gas chromatographic column (see Fig. 3). For EAG values and compounds respective proportions (%) the S.E. represents the standard error calculated from three GC-EAD traces.

Response N°	Compound name	% ± SE Cow dung	% ± SE Horse dung	RI [(1)]	RI [(2)]	EAG (mV ± SE) Cow dung	EAG (mV ± SE) Horse dung
1	citronellene *	5.5 ± 2.4	30.5 ± 9.1	1043	1056	0.80 ± 0.19	0.43 ± 0.18
2	1-methylethylidene-cyclohexane	0.7 ± 0.2	2.9 ± 0.2	1137		0.74 ± 0.18	0.72 ± 0.08
3	D-limonene *	17.3 ± 7.3	11.5 ± 0.6	1182	1193	0.68 ± 0.05	0.20 ± 0.06
4	octan-3-one *	7.5 ± 0.3	10.8 ± 0.8	1251	1244	0.98 ± 0.21	0.71 ± 0.22
5	dimethyl trisulfide *	2.8 ± 1.2	3.3 ± 0.8	1361	1384	1.24 ± 0.22	1.09 ± 0.28
6	octan-2-ol	0.6 ± 0.1	0.5 ± 0.1	1380	1352	0.73 ± 0.14	0.70 ± 0.19
7	octan-3-ol	2.3 ± 1.9	1.5 ± 0.6	1391	1391	0.64 ± 0.53	0.26 ± 0.15
8	unidentified	0.4 ± 0.0	3.4 ± 1.1	1411		0.39 ± 0.09	0.26 ± 0.14
9	oct-1-en-3-ol *	0.5 ± 0.2	0.2 ± 0.0	1449	1456	0.93 ± 0.24	0.71 ± 0.25
10	heptan-1-ol *	0.5 ± 0.0		1459	1457	0.55 ± 0.04	
11	acetic acid / decanal *	3.8 ± 0.6	1.8 ± 1.0	1475/1480	1457/1504	1.01 ± 0.06	0.53 ± 0.09
12	propanoic acid */ octan-1-ol *	1.8 ± 0.5	5.3 ± 0.6	1552/1555	1541/1558	0.53 ± 0.06	0.77 ± 0.18
13	β-caryophyllene *		0.2 ± 0.0	1558	1590		0.55 ± 0.10
14	β-cyclocitral *	0.2 ± 0.0	1.0 ± 0.4	1605	1598	0.71 ± 0.11	0.70 ± 0.22
15	butanoic acid *	Tr	0.2 ± 0.1	1634	1625	0.21 ± 0.02	0.24 ± 0.02
16	α-humulene *	Tr	0.8 ± 0.0	1640	1650	0.24 ± 0.03	0.25 ± 0.04
17	acetophenone *	Tr	0.3 ± 0.1	1653	1660	0.19 ± 0.01	0.29 ± 0.02
18	isovaleric acid *	Tr	0.4 ± 0.0	1676	1669	0.11 ± 0.02	0.28 ± 0.09
19	borneol	Tr	0.8 ± 0.2	1699		0.16 ± 0.02	0.36 ± 0.09
20	unidentified	0.5 ± 0.1	0.4 ± 0.1	1713		0.65 ± 0.12	0.52 ± 0.25
21	hexanoic acid *	0.4 ± 0.2	0.4 ± 0.1	1840	1841	0.43 ± 0.06	0.68 ± 0.36
22	phenol *	1.1 ± 0.1	0.9 ± 0.2	2006	2001	0.22 ± 0.01	0.16 ± 0.05
23	*p*-cresol *	31.1 ± 0.9	14.8 ± 3.6	2089	2085	0.44 ± 0.09	0.55± 0.20
24	indole *	22.1 ± 0.4	3.9 ± 2.5	2455	2455	0.18 ± 0.02	0.13 ± 0.01
25	skatole *	1.0 ± 0.1	4.1 ± 1.9	2499	2495	0.23 ± 0.03	0.19 ± 0.06

* = synthetic analogue known to elicit EAG responses from *S. calcitrans* (Appendix 2)

[(1)]: RI of natural product, [(2)]: RI of injected synthetic analogue or RI from the literature (www.flavornet.org and Syed, 2002)

A percentage lower than 0.1 % is considered as a trace amount. Tr, trace.

Oviposition assays. When horse or cow dung were compared to water as control, flies laid significantly more eggs over dung than over the control or elsewhere in the cage (Fig. 4). A mean of 3.45 eggs cm^{-2} and 1.96 eggs cm^{-2} were laid, respectively, on horse and cow dung, whereas the number of eggs deposited per cm^2 did not exceed a mean of 0.08 on the control and 0.39 elsewhere in the cage. However when given the choice between horse and cow dung, flies laid significantly more eggs over horse dung (Fig. 5), with a mean of 5.82 eggs cm^{-2} recorded over horse dung whilst no more than 0.04 eggs cm^{-2} were laid over cow dung. When no substrate was proposed, flies randomly deposited their eggs showing no clear choice for a preferred site inside the cage (data not shown).

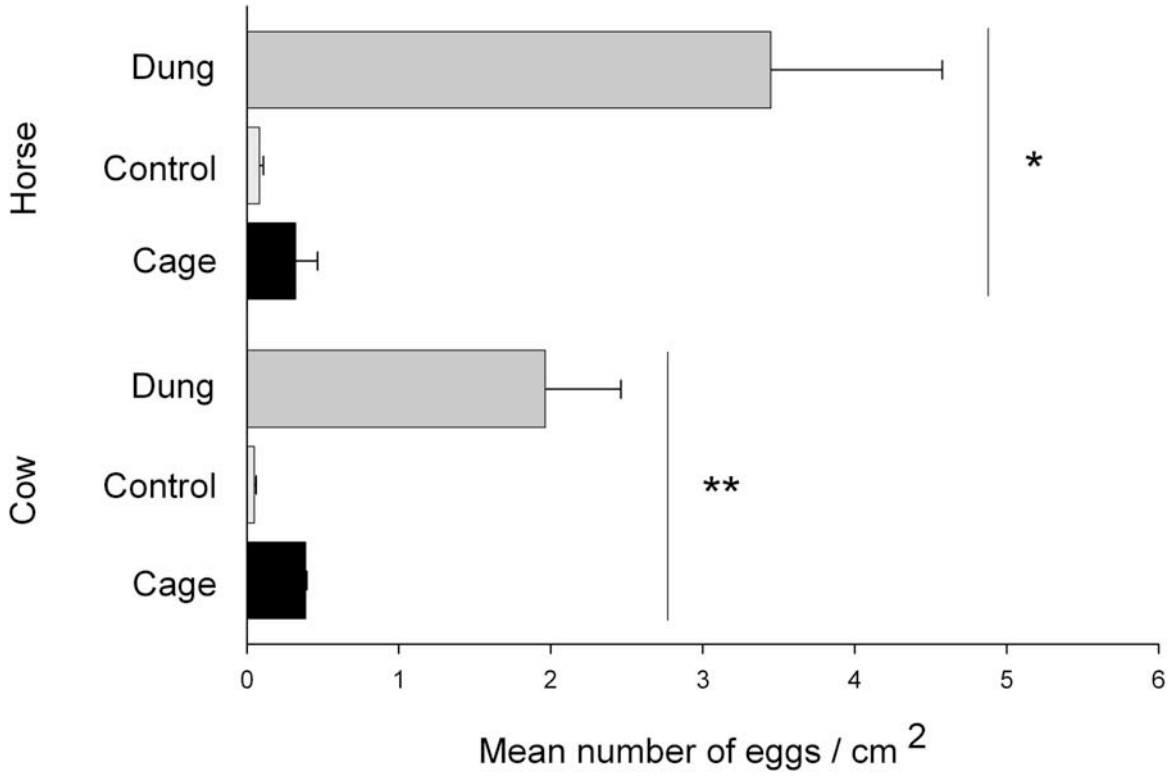

Figure 4: Mean number of eggs cm^{-2} laid by 10 gravid *Stomoxys* females over 24 h in response to either horse or cow dung volatiles; * p-value < 0.05, ** p <0.01, Kruskal-Wallis test; error bars represent the standard error (4 replicates for each treatment).

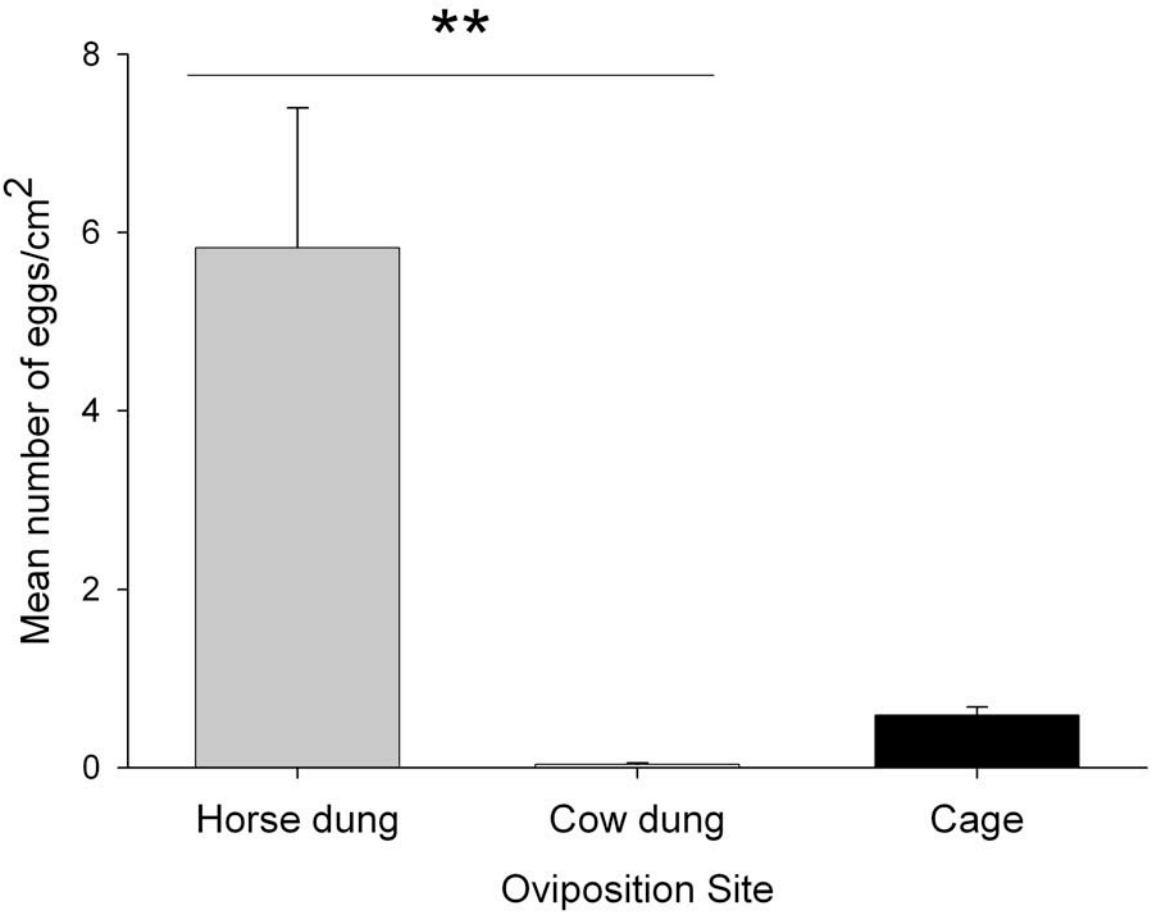

Figure 5: Mean number of eggs cm^{-2} laid by 10 gravid *Stomoxys* females over 24 h in choice-tests between horse and cow dung volatiles; ** $p < 0.01$, Kruskal-Wallis test; error bars represent the standard error for 4 replicates.

Stomoxys females did not show any significant preference for the synthetic blend composed of nine carboxylic acids (acetic, propanoic, isobutyric, butanoic, isovaleric, pentanoic, 4-methyl pentanoic, hexanoic and benzoic) and 7 aromatic products (phenol, 3-ethyl phenol, 4-ethyl phenol, *m*-cresol, *p*-cresol, indole and skatole) as released from a strongly-smelling polyethylene dispenser and laid their eggs randomly with an average of 0.08, 0.59, 0.36 eggs cm^{-2} over, respectively, the blend, the DCM control and elsewhere in the cage (Fig. 6).

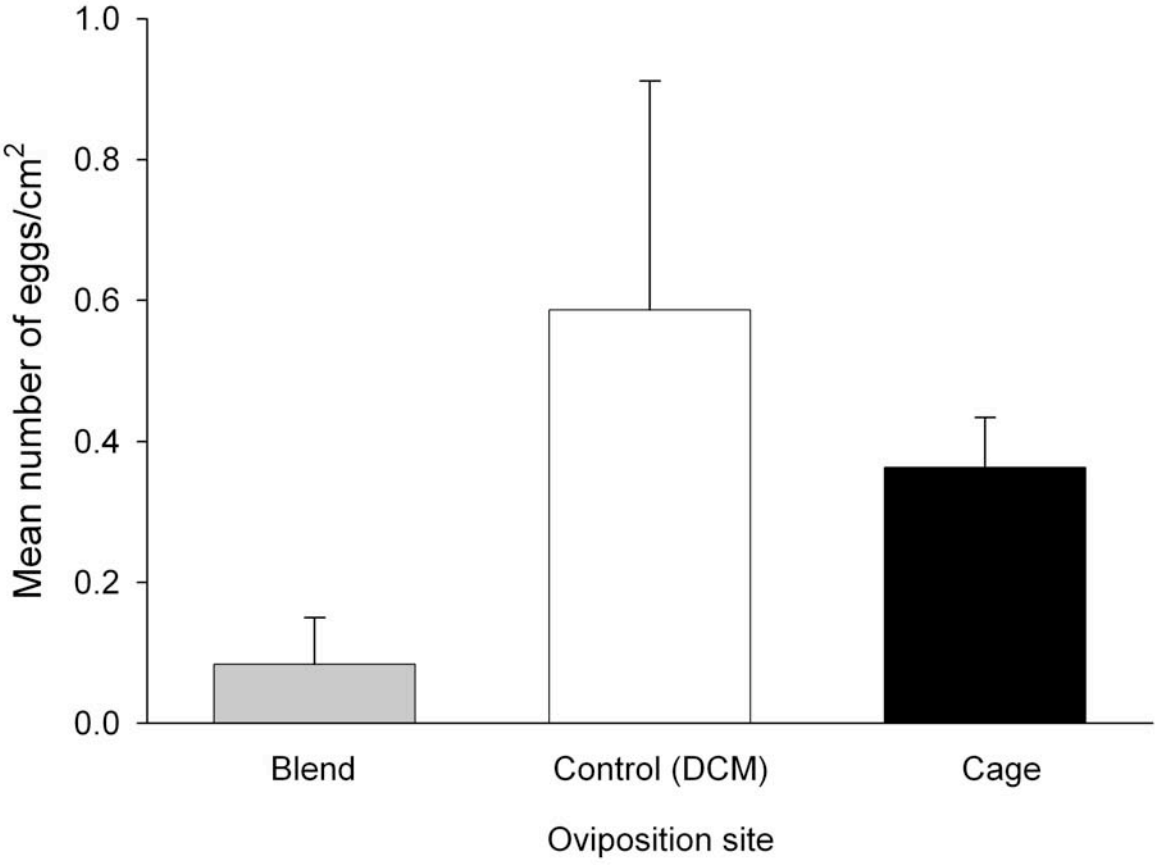

Figure 6: Mean number of eggs cm^{-2} laid by 10 gravid *Stomoxys* females over 24 h in response to a blend of synthetic chemicals (9 fatty acids and 7 aromatic compounds); no significant difference was recorded between oviposition sites (Kruskal-Wallis test), error bars represent the standard error for 4 replicates.

Oviposition experiments where flies had complete access to dung (Fig. 7) showed that when they were proposed horse dung as an oviposition substrate, a significantly higher number of eggs were deposited inside the dung with 547 eggs on average, than elsewhere in the cage with only 38 eggs on average. On the contrary, *Stomoxys* flies avoided cow dung for egg laying with only 5 eggs on average found in the dung and oviposited significantly more in its close vicinity with an averaged number of 506 eggs recorded.

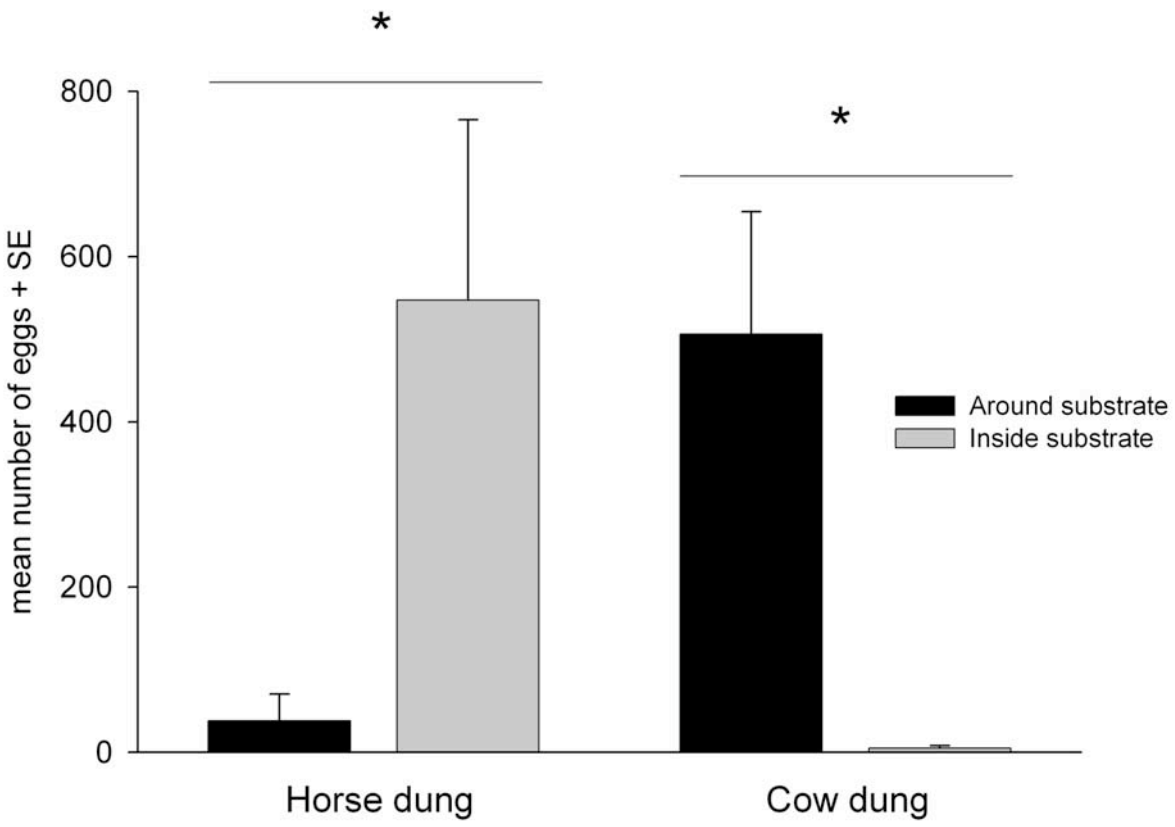

Figure 7: Mean number of eggs laid by 10 gravid *Stomoxys* females over 24 h in response to full access to either horse or cow dung; * $p < 0.05$, Mann-Whitney test; error bars represent the standard error for 3 replicates.

When volatiles from horse and cow dung were presented together *S. calcitrans* females laid in total significantly more eggs (about 796) than with any other odour (Table 2). A significant difference in the number of eggs laid was also recorded between horse dung assays with 453 eggs and oviposition trials in the absence of odour (261 eggs). Although not significantly different, cow dung odour with 419 eggs and the synthetic blend composed of acids and aromatics (324 eggs) yielded more eggs than the trials in absence of odour.

Table 2: Comparison of the total number of eggs laid by 10 gravid *Stomoxys* females over 24 h in the different oviposition assays; Anova (+ Fisher's PLSD post-hoc), $f_{4,15} = 11{,}054$, $p = 0{,}0002$; SE is the standard error for 4 replicates.

Oviposition assay type	Origin of odour	Total number of eggs ± SE
Odours presented individually (Fig. 4)	Horse dung	453 ± 98 [b]
	Cow dung	419 ± 26 [b,c]
Choice-test experiments (Fig. 5)	Horse + Cow dung	796 ± 41 [a]
Synthetic blend (Fig. 6)	Acids + Aromatics	324 ± 44 [b,c]
No odour presented	-	261 ± 74 [c]

The concentration of CO_2, a stable fly attractant, varied differently over horse and cow dung during the 24 h of continuous recording (Fig. 8). During the first 2 h an initial increase of about 10 to 20 ppm was recorded over both substrates, followed by a constant decrease to a value of 400 ppm. From 7 to 8 h after the beginning of the recordings the CO_2 concentration over both horse and cow dung was equivalent at around 400 ppm, approaching the ambient concentration (around 350 ppm). From this point, however, a different CO_2 level over the two substrates was recorded. While the concentration over cow dung remained approximately constant in the range of 390 to 400 ppm over time, the level of CO_2 over horse dung increased to reach a plateau around 420-430 ppm. From 12 h after starting the recordings an absolute difference of about 20 to 40 ppm was found between horse and cow dung. Differences in CO_2 concentration between horse and cow dung at the beginning of the recordings were due to build up of CO_2 in the environmental cabinet in which the recordings took place. This CO_2 concentration was influenced by the presence of the operator setting-up the experiment, but evidently not to the same extent on each occasion (Fig. 8). However, we were principally interested in the variations of CO_2 level above the two oviposition substrates over time.

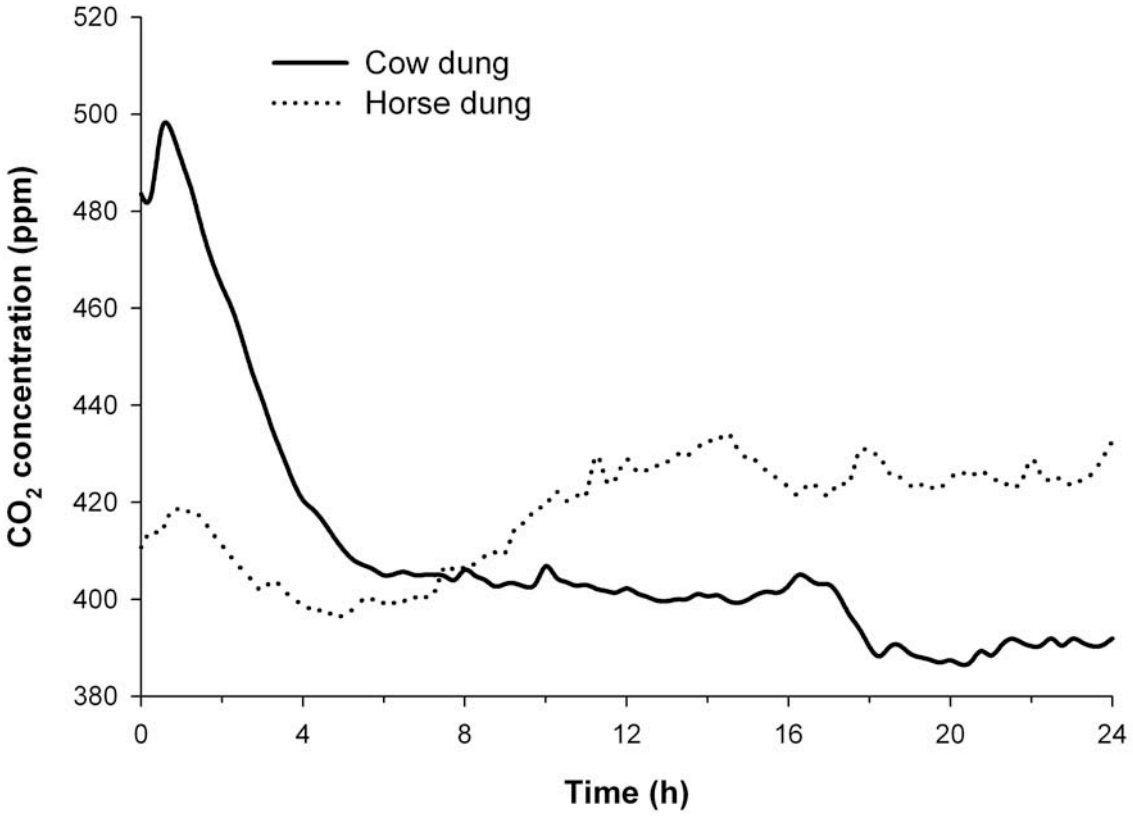

Figure 8: CO_2 levels (ppm) above 25 g of cow and horse dung over a 24-h period as recorded with a Li-Cor® gas analyser.

Wind tunnel experiments. Flies were similarly activated and attracted to the source upon exposure to volatile compounds emanating from either horse or cow dung (Table 3). Although not significantly different, a higher percentage of flies was activated and attracted when stimulated with horse dung volatiles: activated flies accounted for 77.3% with horse dung against 68.2% with cow dung and 68.2% and 63.6% were, respectively, attracted to the source. We also recorded some activation and attraction to dung acidic and phenolic fractions. Apart from activation by the cow dung phenolic fraction (30%), which was significantly lower than the activation recorded with horse dung volatiles (77.3%), no other significant differences in either activation or attraction were recorded when comparing complete substrates to fractions. The analysis of the cow dung acidic and phenolic fractions by gas

chromatography linked mass spectrometry revealed various acids, phenols and indoles (Table 3, footnote); horse dung acidic and phenolic fractions were not analysed.

Table 3: Behavioural responses of *S. calcitrans* gravid females following exposure to various stimuli in a wind tunnel. The % of flies that were activated and the % of flies attracted to the odour source were recorded. A fly was considered activated when it flew more than 3 times in the flight cylinder and attracted when it flew more than 50 cm from the release cage towards the source. Flies that were activated or attracted during control period were discarded. Chi-square and Fisher's exact tests were used for statistical comparison of the % activation and attraction between test odours. Letters represent differences among the stimuli tested ($p<0.05$).

Treatments	**N**	**N***	**% activation**	**% attraction**
Cow dung (CD)	32	22	68.2 [a,b]	63.6 [a]
Horse dung (HD)	32	22	77.3 [a]	68.2 [a]
CD acidic fraction[1]	16	12	50.0 [a,b]	50.0 [a]
HD acidic fraction	16	10	50.0 [a,b]	50.0 [a]
CD phenolic fraction[2]	16	10	30.0 [b]	30.0 [a]
HD phenolic fraction	16	10	60.0 [a,b]	60.0 [a]

N = number of flies tested

N* = nb. of flies that responded only during stimulus delivery

1 = acetic, propanoic, 2-methyl propanoic, butanoic, 2-methyl butanoic, 3-methyl butanoic, pentanoic, 4-methyl pentanoic, hexanoic, cyclohexane carboxylic, benzoic, benzene acetic and benzene propanoic acids

2 = phenol, 3-ethyl, 4-ethyl, 3-methyl and 4-methyl phenols, indole, skatole

We also noticed a high spontaneous activity in *Stomoxys*, resulting in activation in about 30 % of the flies during the 2-min control periods. This activation probably corresponds to escape behaviour, as flies quickly left the small plastic release cage but once on the nylon flight cylinder most of them generally remained still until the end of the control period.

Increases in CO_2 concentration of 43 ±15 ppm and 162 ±49 ppm were recorded at the odour delivery exit for horse and cow dung, respectively. These variations in CO_2 levels at one end of the wind tunnel yielded small fluctuations at the fly release point of 1 ±0.5 ppm for horse and 2 ±1 ppm for cow dung.

Discussion :

The data obtained from the oviposition assays suggest that olfaction plays a crucial role for flies when seeking suitable substrates to lay eggs. Effectively, in no choice experiments, flies which had only olfactory cues to rely on, always deposited significantly more eggs over the organic substrate, whatever its origin. We suggest that some volatile compounds common to both substrates are exploited by flies to find an oviposition substrate. Similar EAG responses recorded upon exposure to horse and cow dung headspace constituents as evidenced by GC-EAD reinforce this. Although gravid female stable flies did not show any significant difference in activation and attraction when exposed to volatiles from horse and cow dung in the wind tunnel, they demonstrated a clear preference for ovipositing over horse dung when proposed both substrates together. Furthermore, we found that odours emanating from horse dung have an influence on the number of eggs deposited, and that in the absence of olfactory cues indicating the availability of a potential breeding medium, gravid *Stomoxys* females laid fewer eggs.

In this study we isolated numerous compounds from horse and cow dung eliciting consistent EAG responses from *S. calcitrans* antennae using GC-EAD. These volatiles were acetic acid, propanoic acid, butanoic acid, isovaleric acid, hexanoic acid, heptan-1-ol, octan-1-ol, octan-2-ol, octan-3-ol, oct-1-en-3-ol, decanal, octan-3-one, acetophenone, indole, skatole, phenol, *p*-cresol, dimethyl trisulfide, citronellene, D-limonene, β-cyclocitral, β-caryophyllene, α-humulene, borneol and 1-methylethylidene-cyclohexane. The method used for the collection of dung volatiles is selective, and may not be appropriate for collections of all volatiles perceived by stable flies. Indeed the collection of headspace volatiles from cow and horse

dung by sampling on a Tenax™ GR sorbent, chosen for its low affinity with water, yielded amounts of volatiles that varied between replicates making any quantification difficult. Although thermal desorption of the sorbent is a useful tool to permit the detection of highly volatile and trace compounds that could be masked or diluted by the solvent (Pfannkoch & Whitecavage, 2000), its principal drawback is in the variation between successive collections producing different chromatographs. We overcame this problem by reconstituting an averaged GC-EAD trace from three replicates for each substrate. As we selected only EAG responses present in at least two traces, responses to compounds not consistently recovered from Tenax™ GR may have been omitted. We did observe the occasional occurrence of compounds known to elicit EAG responses in the stable fly like 6-methylhept-5-en-2-one, nonanal, benzoic acid and geranyl acetone (Appendix 2 and 4).

Now the question is which key compounds play a role in attraction of gravid females seeking an oviposition site. From a general point of view, the oviposition substrates selected by many blood-feeding dipteran females (stable flies, horn flies, mosquitoes) are mainly composed of decomposing vegetal matter in which bacterial degradation of carbohydrates and amino acids results in the production of volatile compounds that may attract flies. Many studies have highlighted the general occurrence of phenols and indoles from such substrates that have been shown to attract different species of mosquitoes for oviposition (Clements, 1999). Du & Millar (1999) identified compounds mediating oviposition in *Culex* mosquitoes from fermented infusions of Bermuda grass. They reported antennal responses to phenol, *p*-cresol, 4-ethyl phenol, indole, skatole and other non-aromatic compounds, and found that a reconstituted blend of 10 compounds was attractive to gravid *Cx. tarsalis* and *Cx. quiquefasciatus* and enhanced oviposition. Mboera *et al.* (2000) showed in field experiments that more *Cx. quinquefasciatus* egg rafts were deposited in water treated with low concentrations of skatole than in tap water. Phenol, 3-methyl phenol, 4-methyl phenol and 2-methoxy phenol were found to elicit EAG responses from *S. calcitrans* antennae (Schofield *et al.*, 1995; Birkett *et al.*, 2004; Appendix 2) and Cilek (1999) found that a mixture of *p*-cresol, 3-n-propyl phenol and oct-1-en-3-ol increased stable fly numbers in cylinder traps. Here we report the occurrence of phenol, 4-methyl phenol, indole and skatole as dung volatiles that elicited EAG responses from stable flies and the attraction of gravid females in wind tunnel experiments to dung phenolic fractions.

Other commonly occurring volatiles that could have an influence on gravid female *Stomoxys* are carboxylic acids. Broce & Haas (1999) who conducted field experiments on the colonization of cattle manure by stable and houseflies observed a clear preference by gravid stable flies for aged manure. They found no correlation between stable fly visitation and the various physiochemical parameters (pH, osmolality, CO_2 production, temperature, moisture content, ammonia) that were monitored. In fact, the attractiveness of aged manure to flies may be partly due to carboxylic acids as supported by studies on the anaerobic production of fermentation products in slurries of fresh and aged cattle manure conducted by Miller & Varel (2001). These authors found that whereas the concentration of aromatic compounds (phenols, indoles and benzoates) remained unchanged over time, aged manure produced twice the concentration of volatile carboxylic acids than fresh dung. Carboxylic acids have been shown to play a role for host location in many blood-feeding insects (Chapter 2) but very little is known about their potential influence on the oviposition behaviour of these insects. However, we observed activation and attraction of gravid females when tested with dung acidic fractions in wind tunnel experiments. So we suggest that some volatile carboxylic acids may act synergistically with other volatile compounds like phenols and indoles in attracting stable flies to oviposition sites. Although the oviposition assay with a mixture of carboxylic acids, phenols and indoles present in equal amount did not induce a clear egg laying behaviour by *Stomoxys* females, it would be premature to draw conclusions from this result knowing the importance of having compounds represented in relevant proportions to induce behavioural responses with mixtures of volatiles (Donzé *et al.*, 2004).

Apart from carboxylic acids and aromatic compounds, the *Stomoxys* chemostimulant dimethyl trisulfide originating from methionine degradation is worth mentioning because of its overall occurrence in potential oviposition substrates (Mackie *et al.*, 1998). This product is also found in fermented infusions of Bermuda grass and it stimulates oviposition in *Culex* mosquitoes (Du & Millar, 1999). It was also detected in pig manure and was shown to attract houseflies when combined with butanoic acid and skatole (Cossé & Baker, 1996). In our study GC-EAD recordings from *Stomoxys* antennae confirmed the presence of dimethyl trisulfide in horse and cow dung as an electrophysiologically active compound for the stable fly. Moreover, as reported in Chapter 2, the perception threshold of *Stomoxys* flies for this compound is in the 1/10 of a picogram range.

Among the various alcohols found in dung volatiles, oct-1-en-3-ol was detected in trace amount. Whereas the electrophysiological and behavioural activity of oct-1-en-3-ol on various

host-seeking haematophagous insects is widely documented (Gibson & Torr, 1999; Chapter 2), it seems that no potential influence on oviposition behaviour has been observed as yet. All studies of alcohols with a carbon chain length comprised between C_5 and C_{10}, were shown to elicit EAG responses from Stomoxys antennae (Schofield *et al.*, 1995; Birkett *et al.*, 2004; Appendix 2).

Aldehydes and terpenoids were also identified as volatile constituents of dung and were eliciting EAG responses from stable fly antennae. *S. calcitrans* is known to take nectar and pollen from plants as an energy source for flight (Zumpt, 1973; Jones *et al.*, 1985; Jones *et al.*, 1992) and, although we suspect these compounds to be used primarily for locating flowers, the low antennal thresholds recorded for β-caryophyllene, α-humulene, nonanal and decanal (Chapter 2) and the preference exhibited by *S. calcitrans* for oviposition substrates containing plant material (Hafez & Gamal-Eddin, 1959; Zumpt, 1973) suggest that we cannot exclude their importance in the location of a suitable oviposition substrate.

Interestingly, some plants mainly belonging to the Araceae family are known to mimic dung odour to attract pollinators and when Skubatz *et al.* (1996) analysed the composition in odoriferous compounds produced by the *Sauromatum guttatum* appendix he found a complex blend constituted of terpenoids, carboxylic acids, alcohols, ketones, aldehydes, phenols, indoles and sulphur containing compounds. Among more than 100 compounds listed, some of them like citronellene, limonene, β-caryophyllene, α-humulene, octan-3-one, oct-1-en-3-ol, octan-3-ol, octan-1-ol, *p*-cresol, indole, dimethyl trisulfide, decanal and propanoic acid, drew our attention because of their presence in our volatile analysis of cow and horse dung as chemostimulants for stable flies. Moreover, many species of Diptera associated with the recycling of dung like the false stable fly *Muscina stabulans* Fallen were captured with traps baited with *S. guttatum* appendices. Kite (1995) also compared the floral odour of *Arum maculatum* with the volatiles produced by cow dung, the normal breeding site of its main pollinator, *Psychoda phalaenoides* L. Apart from some terpenes and aldehydes (limonene, citronellene, β-caryophyllene, α-humulene, nonanal, decanal), indole and *p*-cresol that elicit EAG responses from *Stomoxys* antennae were found to be common to both odours.

Apart from less volatile organic compounds, the production of CO_2, a universal attractant for blood-sucking insects (Cork, 1996; Clements, 1999; Gibson & Torr, 1999; Chapter 2), is also

intimately associated with bacterial activity in decomposing organic matter. As we observed a different pattern of CO_2 release over time from the two substrates with an increase in CO_2 concentration over horse dung from 8 h after the beginning of the recordings, we suggest that due to the more fibrous texture of horse dung consistent with the less efficient digestive system of horses compared to that of ruminants, the microbial fauna had started the decomposition process accounting for the increase of CO_2. The formation of a crust over cow dung may also slow down the process of decomposition, explaining a constant low level of CO_2. As flies are thought to start laying eggs between 14 to 24 h (light phase) into the oviposition trial (personal observations) and considering the close similarity of antennal response patterns to volatiles from both substrates, we suggest that the clear preference for horse dung demonstrated by flies in the choice-tests may result from the higher levels of CO_2 being released. This phenomenon has been observed in fruit flies where a CO_2 stimulus simulating a lesion in a fruit and without the presence of other sensory inputs attracted gravid females for oviposition (Stange, 1999). Many studies have shown the potential of carbon dioxide in attracting stable flies (Vale, 1980; Cilek, 1999) and a behavioural response threshold around 60 ppm was reported by Schofield & Brady (1997). This observation supports our hypothesis that CO_2 may have influenced the oviposition behaviour of *Stomoxys* females in favour of horse dung. It also suggests that molecules other than carbon dioxide activated and attracted flies when stimulated with dung volatiles in the wind tunnel as the level of recorded CO_2 at fly release point (1-3 ppm increase) was at most a factor of 20 below the threshold of 60 ppm established by these authors.

Oviposition experiments in which flies had free access to dung, showed that they hid eggs only in horse dung and completely avoided cow dung preferring to oviposit in its vicinity through the net of the cage. As stated before, we observed the formation of a crust over cow dung as its superficial layer dries out. We suggest that flies unable to hide their eggs in cow dung oviposit around it, providing larvae with a close by food resource. On the contrary, horse dung, with its more fibrous structure, allows flies to provide their eggs with good protection from desiccation or predation. This hypothesis is in accordance with Hafez & Gamal-Eddin (1959) who reported that the oviposition substrates chosen by gravid *Stomoxys* females must be loose and porous. Furthermore, the avoidance of cow dung by stable flies cannot be attributed to physiochemical reasons that could affect larval development, as we obtained well-developed adult flies from eggs manually dispersed on cow dung (data not

shown). This just highlights the importance of contact chemoreception and proprioreception in the final acceptation of a substrate for oviposition (Städler *et al.*, 1995).

In summary, we have shown that stable flies rely on olfactory cues to locate from a distance a suitable substrate on which to lay their eggs and that odour from horse dung stimulates oviposition. We found carboxylic acids, alcohols, phenols, indoles sulfides and terpenoids in dung to elicit EAG responses from *Stomoxys* antennae. These products are commonly encountered in larval breeding media used by several blood-sucking insects. We also demonstrated that horse dung was the preferred substrate for oviposition and that its higher CO_2 content compared to cow dung may be responsible for such a preference. Finally, the importance of contact chemoreception and proprioreception for *S. calcitrans* females in acceptance of an oviposition substrate was suggested.

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General discussion and conclusions

Methods

The methods employed in this thesis permit some interpretation of the physiological and behavioural adaptations implicated in the sensory ecology of stable flies. From a general point of view the methods we used provide rather more qualitative than quantitative information. Electrophysiological recordings from antennal receptor cells linked to gas chromatography is suitable for detecting which compounds present in a complex odour may be biologically relevant to the stable fly. An electroantennogram (EAG) provides a measure of the summed receptor potentials of the olfactory cells activated in response to a given chemical thereby providing information about the presence on the antenna of receptor cells tuned to the chemical with an estimation of their number. This information cannot be directly linked with the biological function of a chemical and its role in attraction or repellence. Between the collection of volatiles emanating from a biological substrate to their delivery to the antenna from the gas chromatograph several steps are required in the course of which the possibilities of isolating and identifying chemostimuli may diminish (Agelopoulos & Pickett, 1998). When using an adsorbent for trapping volatiles we are confronted with its selectivity for compounds belonging to a certain set of classes (Sunesson *et al.*, 1995). The duration as well as sampling rate are other factors influencing the collection of volatiles. For example, with increased collection duration high boiling point compounds or those occurring in low quantities will be better represented but low boiling point products might break through the adsorbent. Direct thermal desorption from an adsorbent is a useful tool permitting the detection of highly volatile products in a sample and those at trace levels that could be masked by the solvent peak or diluted in conventional solvent extraction (Phillips & Greenberg, 1991; Wahl *et al.*, 1996; Pfannkoch & Whitecavage, 2000). The major fault with thermal desorption is in the variation between successive collections producing different chromatograms. Finally, it is worth mentioning the importance of the choice of the gas chromatographic phase (see Chapter 2) and the chromatographic settings (temperature and flow rate) in order to obtain highest resolution and the best separation of compounds with close retention times. This last point is primordial when a biological preparation is linked to the GC to permit sufficient time for the antenna to recover between the elution of two biologically active compounds. Having well separated peaks helps also in the identification of the compounds when the column is linked

to a mass spectrometer. When these steps have been optimized, the volatile profile obtained will be an approximate image of the natural representation of the volatiles in a given odour. The challenge then is to elaborate a blend of synthetic chemostimuli mimicking the natural odour.

Using the flight cylinder (Syed, 2002; Syed & Guerin, 2004) in the wind tunnel assays provides the advantage that the fly is always in the plume and potential visual disturbance is reduced. However, it restricts lateral displacement during flight resulting in interrupted flights and forcing the fly to reorient after each landing. The second set-up we used (Chapter 2) for testing rumen odour and synthetics allows the fly to exhibit a casting behaviour similar to that exhibited by moths (Kennedy, 1983; Kuenen & Cardé, 1994). It nevertheless yields one major disadvantage. Despite the presence of visual cues (blue lines) flies sometimes quickly lose the plume and land on some place in the wind tunnel to where odour is not likely to be blown. Apart from activation and attraction, the initial behaviours exhibited by flies, we also decided to estimate the average number of oriented flights per activated fly recorded during the 3 min of stimulation. It gives a more complete information regarding the attractiveness of an odour. Indeed flies that left the plume of chemostimuli early contacted it later when exploring the wind tunnel and initiated a casting upwind flight towards the source. Still we have to be cautious in interpreting these results because the same fly may exhibit an oriented flight several times.

Host-seeking behaviour (Chapter 2)

Human breath has been shown to attract *S. calcitrans* (Alzogaray & Carlson, 2000), and our wind tunnel experiments confirmed its attractiveness for stable flies. CO_2 on its own was found not only to activate *Stomoxys* (Gatehouse & Lewis, 1973; Warnes & Finlayson, 1985) but to be a strong attractant as well. Even if no significant differences in either activation or attraction was recorded here, stable flies were shown to be marginally more activated and attracted with human breath than with CO_2 alone. So acetone that elicited EAG responses from *Stomoxys* antennae and other chemostimuli present in breath might be responsible for such a difference. Many volatile chemostimuli released in bovine breath (Spinhirne *et al.*, 2004) also occur in human breath (Wahl *et al.*, 1996; Phillips *et al.*, 1999) including

carboxylic acids, alcohols, ketones, aromatics, sulfides and terpenoids that were found to elicit EAG responses from stable flies antennae. Interestingly, EAG responses recorded from tsetse flies to rumen odour were found to be extremely similar to those from the stable fly in that tsetse also responded to heptanol, octanol, acetic acid, propanoic acid, isobutyric acid, butanoic acid, isovaleric acid, hexanoic acid, *p*-cresol, indole, skatole, D-limonene and β-caryophyllene (Syed, 2002). Like tsetse, *S. calcitrans* also showed a very low perception threshold for β-caryophyllene and similar EAG responses to butanoic acid and *p*-cresol. Rumen odour strongly activated *Stomoxys* in the wind tunnel but in contrast to results obtained with tsetse flies (Syed, 2002) and ticks (Donzé *et al*., 2004), only a low percentage of flies were attracted towards the source. However, we report here for the first time the strong electroantennographic and behavioural responses from *S. calcitrans* for dimethyl trisulfide as a rumen constituent. Moreover, dimethyl trisulfide, butanoic acid, *p*-cresol each presented alone and their mixture were shown to be attractive for stable flies in the wind tunnel. Surprisingly, oct-1-en-3-ol one of the most widely documented chemostimuli for blood-sucking insects and skatole did not elicit either activation or attraction of *S. calcitrans* in our wind tunnel experiments.

Oviposition behaviour (Chapter 3)

Although several studies confirm the importance of olfaction in some haematophagous arthropods such as mosquitoes for oviposition (Bentley & Day, 1989; Clements, 1999), this is the first time that the role of olfaction for oviposition has been investigated for *S. calcitrans*. Despite the tendency exhibited by stable flies to oviposit everywhere in laboratory experiments, we succeeded in developing an oviposition assay that permitted us to demonstrate the importance of olfactory cues for stable flies for finding a suitable site for oviposition. In no-choice experiments *Stomoxys* was shown to be able to locate either horse or cow dung relying only on odour, and this was latter confirmed with observations in the wind tunnel where flies responded in a similar manner in terms of both activation and attraction to both dung odours. However, when given a choice *S. calcitrans* clearly shows a preference for horse over cow dung in oviposition bioassays, which confirms earlier field observations. Furthermore, the presence of horse dung odour induced female flies to lay more eggs than in absence of such an odour from an oviposition substrate. In experiments where flies could

contact the substrates *S. calcitrans* demonstrated a clear preference for horse dung. Apart from olfaction, this result highlights the importance of both contact chemoreception and proprioreception in the final acceptance of the oviposition substrate, although these senses were not studied in the present thesis.

In order to explain the preference mediated by olfaction, the EAG responses from *Stomoxys* to the odours of horse and cow dung were investigated. The results obtained by GC-EAD did not yield big differences in the patterns of EAG responses for the two substrates and most volatile chemostimuli consistently occurred among the odours of the two oviposition substrates. Carboxylic acids along with aromatic compounds like phenols and indoles that have been shown to influence other blood-sucking insects oviposition behaviour (Table 1) were more closely investigated in wind tunnel experiments where some activation and attraction by *Stomoxys* gravid females for the acidic and phenolic fractions of either horse or cow dung were recorded. Other compounds like dimethyl trisulfide and oct-1-en-3-ol were also detected as constituents of dung odours, and considering the low thresholds recorded for these products by EAG they could also play a role for oviposition by *Stomoxys*. It is also worth mentioning terpenoids, as the reported preference of stable flies for oviposition substrates containing plant material and the low sensory threshold recorded for β-caryophyllene suggest that these chemostimuli might also be useful indicators of the suitability of a site for oviposition. Apart from the above volatile compounds, the production of CO_2 by horse and cow dung over time was also investigated. Higher levels of CO_2 were found to occur over horse dung at the time when flies started to oviposit. CO_2 has since long time been described as a strong attractant for numerous host-seeking haematophagous insects (Gibson & Torr, 1999) and is probably also implicated in the location of oviposition substrates. CO_2 is likely to be released from grass infusions shown to attract mosquitoes for oviposition (Millar *et al.*, 1992; Isoe & Millar, 1995; Allan & Kline, 1996) but its precise influence on gravid mosquito females is not reported. Gravid fruit fly females were also shown to rely on CO_2 for oviposition (Stange, 1999). We suggest that the preference for horse dung exhibited by *S. calcitrans* for oviposition could be partly attributed to its CO_2 levels.

Concluding remarks

Two major points come out of this study. Firstly, it appears that *S. calcitrans* shares a number of chemosensory features with other haematophagous arthropods. The most obvious similarity lies in the almost universal importance of CO_2. Among the other compounds perceived by many blood-feeding arthropods it is probably appropriate to attribute major role to volatile compounds originating from bacterial degradation of amino acids. Indeed some amino acids are termed essential as they are not synthesised by animals and have to be absorbed from plants. As for humans, several amino acids are essential for insects (Chapman, 1998). Apart from CO_2 and ammonium that elicited EAG responses from *Stomoxys* antennae**,** bacterial breakdown of these otherwise rather non volatile amino acids yields volatile end products that may inform haematophagous arthropods of their presence in a resource (Table 1).

Table 1: Behavioural and electrophysiological responses exhibited by various haematophagous arthropods to chemostimuli originating from the degradation of essential amino acids *

Degradation product	Essential amino acid (precursor)	Haematophagous arthropod	References
Carboxylic acids (C_1-C_6)	lysine, threonine, histidine, methionine, valine, leucine, isoleucine, L-phenylalanine	*Stomoxys calcitrans*	Schofield *et al.* (1995); this thesis
		Glossina spp.	Vale (1980); Syed (2002)
		Mosquitoes	Cork & Park (1996); Knols *et al.* (1997); Bosch *et al.* (2000)
		Lutzomyia longipalpis	Dougherty *et al.* (1999)
		Amblyoma variegatum	Steullet (1993); Steullet & Guerin (1994); Donzé *et al.* (2004)
Phenols (*m*-cresol, *p*-cresol)	L-phenylalanine, L-tryptophan	*Stomoxys calcitrans*	Schofield *et al.* (1995); Cilek (1999); this thesis
		Glossina spp.	Bursell *et al.* (1988); Den Otter (1991); Syed (2002)
		Mosquitoes	Millar *et al.* (1992); Beehler *et al.* (1994); Allan & Kline (1996); Cork & Park (1996); Collins & Blackwell (1998)
		Tabanid flies	Gibson & Torr (1999)
		Amblyoma variegatum	Steullet (1993); Steullet & Guerin (1994); Donzé *et al.* (2004)
Indoles (Indole, skatole)	L-tryptophan	*Stomoxys calcitrans*	This thesis
		Mosquitoes	Millar *et al.* (1992); Beehler *et al.* (1994); Allan & Kline (1996); Collins & Blackwell (1998)
		Amblyoma variegatum	Donzé *et al.* (2004)
Sulfides (dimethyl trisulfide)	methionine	*Stomoxys calcitrans*	This thesis
		Aedes aegypti	Harraca (unpublished data)
		Lutzomyia longipalpis	Bourquin & Kroeber (unpublished data)
		Glossina spp.	Harraca (unpublished data)
		Rhodnius prolixus	Otalora-Luna (unpublished data)

* Degradation scheme of essential amino acids from Weimer *et al.* (1999)

Secondly, our results show that *Stomoxys* may rely on similar compounds for both oviposition and host finding. Apart from CO_2, we report that various carboxylic acids, alcohols, ketones, phenols, indoles, sulfides and terpenoids eliciting EAG responses occur consistently in bovine breath, cow odour and oviposition substrates like horse and cow dung. This is consistent with the opportunistic behaviour exhibited by stable flies. As discussed in Chapter 1, *S. calcitrans* is a generalist species feeding on a wide range of hosts and ovipositing in all kinds of decomposing organic matter. Evolution may have favoured an olfactory system permitting the perception of products that are common and consistently associated with all these resources, and we suggest that certain volatiles when presented together at relevant proportions might attract *S. calcitrans* whenever it is seeking a resource giving flies invaluable information with regard to the location of proteins sources for larval development as well as for their own survival and development.

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Appendix 1

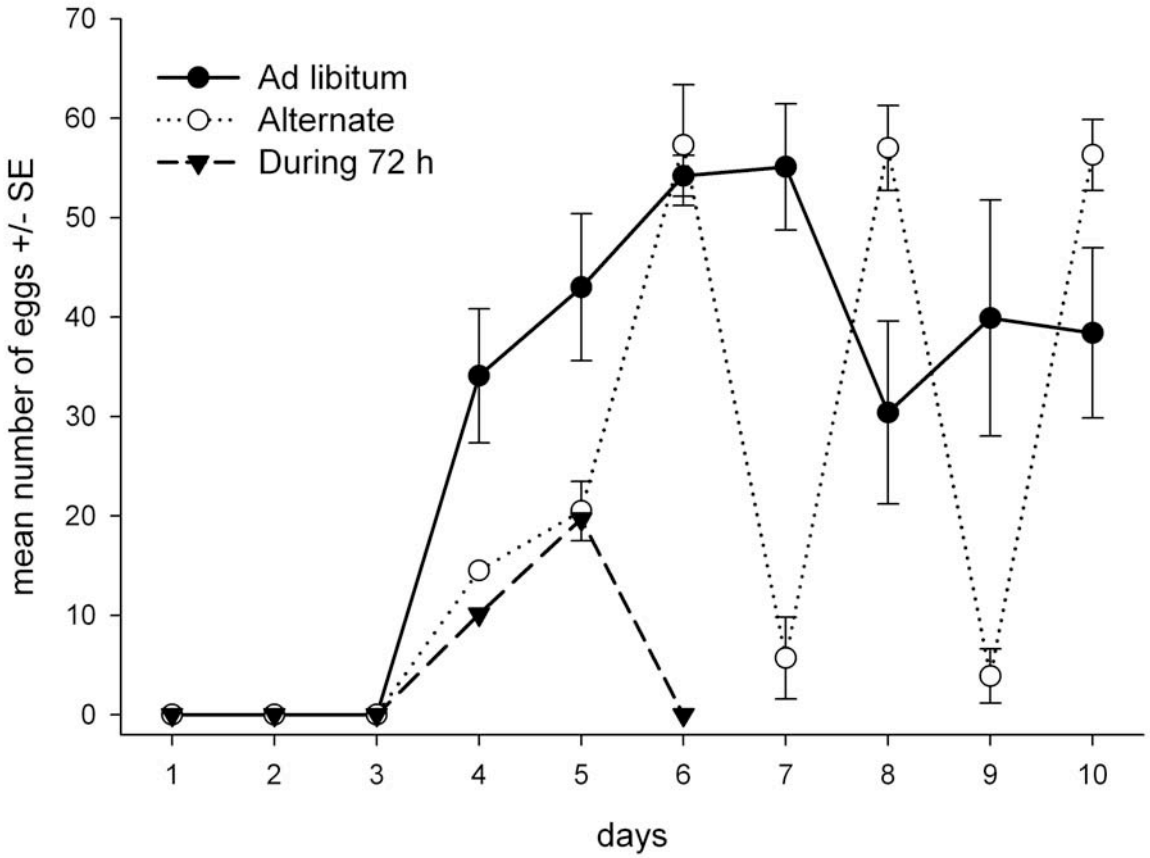

Figure 1: Effect of blood feeding on the dynamics of egg deposition by *S. calcitrans*. Females had full access to blood (ad libitum, plain circles), access to blood for one day (first blood meal on day 1) and 10 % sucrose solution the following day (alternate, open circles), or were fed with blood for 72 h and then only with sucrose solution (during 72 h, triangles). When females had access ad libitum to blood, they laid eggs regularly. On the other hand, when the access to blood occurred only every second day, the females laid eggs only the day after the blood meal. The minimum time with full access to blood required for oviposition in *Stomoxys* females is 3 days (72 h).

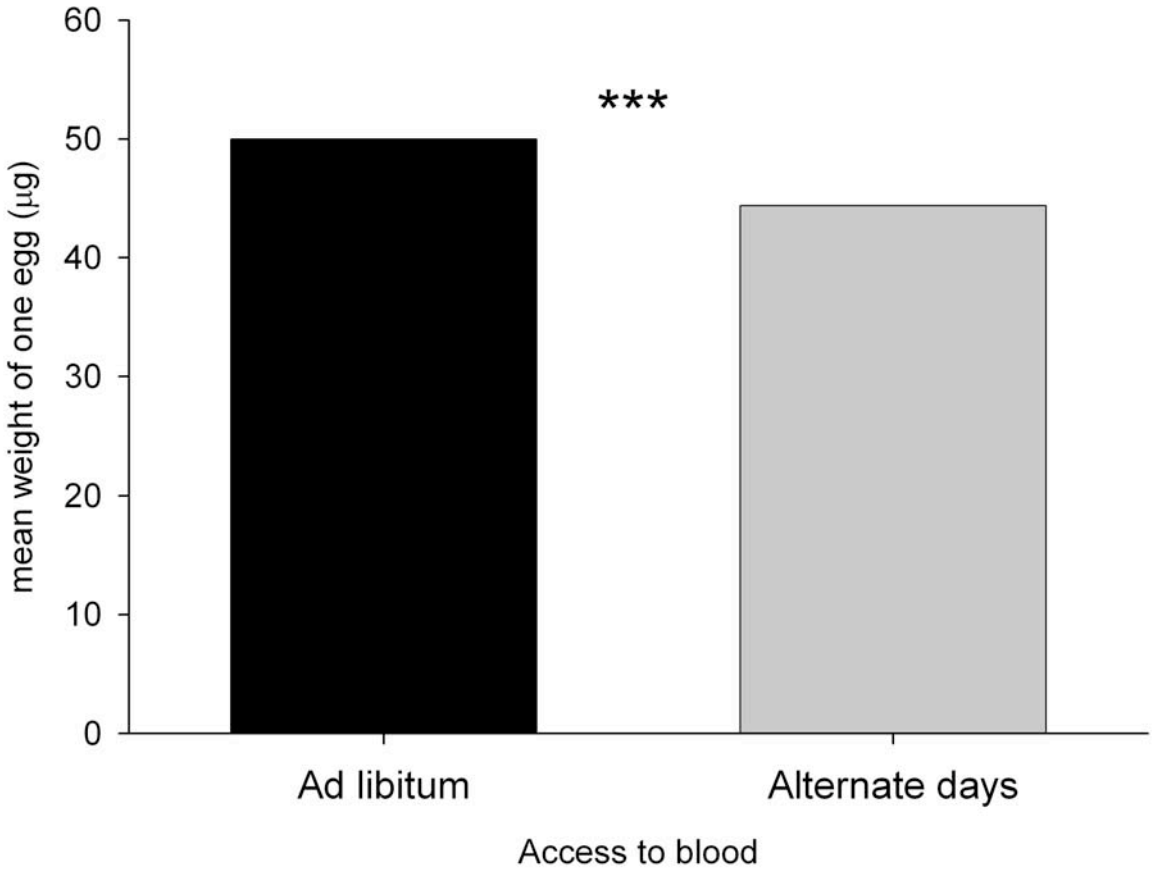

Figure 2: Effect of the feeding rate on the weight of eggs deposited by *S. calcitrans*. Asterisks above bars indicate a significant effect of the access to blood on the weight of eggs (T-test, t=6.262, P<0.001). The reduced access to blood in the treatment where females had access to blood during one day and to a 10 % sucrose solution the following day, negatively affected the weight of the eggs laid by females.

Appendix 2

Table 1: List of synthetic compounds eliciting EAG responses from *S. calcitrans*.

Type	**Chemical class**	**Compound**	**Relative EAG response (%)** [a]	**Dose tested (ng)** [b]
Fatty acid derivative	Acids	C_3-C_5 straight	13-35	100
		C_3-C_4 branched	10-25	100
		butanoic	15	10
		3-methyl-propanoic	22	10
		pentanoic	9	10
		hexanoic	33	**10^5**
	Alcohols	C_6-C_{10}	14-77	50
		(*Z*)-hex-3-en-1-ol	154	**10^5**
		oct-1-en-3-ol	32.4	0.2
	Aldehydes	C_5-C_{10}	18-39	50
	Ketones	acetone	43	**10^5**
		2-butanone	71.6	**10^4**
		octan-3-one	85	**10^5**
		undecan-2-one	273	**10^5**
Amino acid derivative	Phenols	phenol	24	100
		p-cresol	28	10
		m-cresol	25	100
		2-methoxy-phenol	21	100
		2-nitro-phenol	153	**10^5**
	Indoles	indole	55	**10^5**
		skatole	9	200
	Sulfides	dimethyl trisulfide	81	0.2
	Others	methyl benzoate	202	**10^5**
		methyl salicylate	106	**10^5**
		acetophenone	47	50
		eugenol	31	50
		p-tolyl ester	23	100

Table 1: continued

Type	Chemical class	Compound	Relative EAG response (%) [a]	Dose tested (ng) [b]
Isoprenoid or derivative	Monoterpenes	citronellene	139	200
		(*Z*)-citral	19	50
		(*E*)-citral	23	50
		β-cyclocitral	86	**10^5**
		citronellal	70	50
		(+)-camphor	107	50
		bornyl acetate	63	50
		geranyl acetate	46	20
		trans-geraniol	65	50
		trans-geranyl acetone	60	**10^5**
		menthyl acetate	10	200
		(+)-terpinen-4-ol	14	50
		(+/-)-limonene	11	50
		γ-terpinene	33	50
		β-myrcene	28	50
		carvone	22	50
		trans-dihydrocarvone	9	**10^5**
	Sesquiterpenes	β-caryophyllene	59	20
		α-humulene	55	20
	Others	linalool	61	100
		6-methylhept-5-en-2-one	153	**10^5**

[a] Relative EAG response estimated on the basis of the EAG response to a reference stimulus (oct-1-en-3-ol at 1 µg in the stimulus syringe)

[b] Amount of synthetic product injected for GC-EAD recordings; "dose" in bold letters is the amount of synthetic product placed on filter paper in the stimulus syringe for EAG recordings. (**!** Dose tested = lowest dose tested in our experiments ≠ lowest dose eliciting an EAG response)

Table 2: List of synthetic compounds that did not elicit EAG responses from *S. calcitrans*.

Type	Chemical class	Compound	Highest dose tested (ng)*
Fatty acid derivative	Acids	acetic	100
	Alcohols	C_3-C_5	50
		2-ethylhexan-1-ol	100
Amino acid derivative	Phenols	Phenol, 2-ethyl	100
		Phenol, 3-ethyl	100
		Phenol, 4-ethyl	100
		o-cresol	100
		Phenol, 2-propyl	100
		Phenol, 3-propyl	100
		Phenol, 3-propyl	100
		Phenol, 3-methoxy	100
		Phenol, 4-methoxy	100
	Sulfur-compounds	dimethyl sulfide	**10^5**
		dimethyl disulphide	200
		dipropyl sulphide	**10^5**
		dipropyl disulfide	**10^5**
		dimethyl sulfoxide	**10^5**
	Other	*p*-cymene	200
		benzaldehyde	200
		styrene	**10^5**
Isoprenoid or derivative	Monoterpenes	α-pinene	50
		β-pinene	50
		β-phellandrene	200
		δ-3-carene	200
	Sesquiterpenes	α-farnesene	200

* Amount of synthetic product injected for GC-EAD recordings; “dose” in bold letters is the amount of synthetic product placed on filter paper in the stimulus syringe for EAG recordings

Appendix 3

Statistical analysis of the responses of males and females *S. calcitrans* to odour stimuli in the wind tunnel

1. Human breath: observed frequencies

	Females	Males	Totals
Activation	15	16	31
No activation	5	4	9
Totals	20	20	40

	Females	Males	Totals
Attraction	13	15	28
No attraction	7	5	12
Totals	20	20	40

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	.143
Chi Square P-Value	.7050
G-Squared	.144
G-Squared P-Value	.7047
Contingency Coef.	.060
Phi	.060
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.476
Chi Square P-Value	.4902
G-Squared	.478
G-Squared P-Value	.4894
Contingency Coef.	.108
Phi	.109
Cty. Cor. Chi Square	.119
Cty. Cor. P-Value	.7301
Fisher's Exact P-Value	.7311

2. CO_2: observed frequencies

	Females	Males	Totals
Activation	15	11	26
No activation	5	9	14
Totals	20	20	40

	Females	Males	Totals
Attraction	12	9	21
No attraction	8	11	19
Totals	20	20	40

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	1.758
Chi Square P-Value	.1848
G-Squared	1.777
G-Squared P-Value	.1825
Contingency Coef.	.205
Phi	.210
Cty. Cor. Chi Square	.989
Cty. Cor. P-Value	.3200
Fisher's Exact P-Value	.3203

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.902
Chi Square P-Value	.3422
G-Squared	.906
G-Squared P-Value	.3413
Contingency Coef.	.149
Phi	.150
Cty. Cor. Chi Square	.401
Cty. Cor. P-Value	.5266
Fisher's Exact P-Value	.5273

3. Rumen: observed frequencies

	Females	Males	Totals
Activation	21	25	46
No activation	14	10	24
Totals	35	35	70

	Females	Males	Totals
Attraction	6	4	10
No attraction	29	31	60
Totals	35	35	70

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	1.014
Chi Square P-Value	.3138
G-Squared	1.018
G-Squared P-Value	.3130
Contingency Coef.	.120
Phi	.120
Cty. Cor. Chi Square	.571
Cty. Cor. P-Value	.4500
Fisher's Exact P-Value	.4504

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.467
Chi Square P-Value	.4945
G-Squared	.469
G-Squared P-Value	.4933
Contingency Coef.	.081
Phi	.082
Cty. Cor. Chi Square	.117
Cty. Cor. P-Value	.7327
Fisher's Exact P-Value	.7343

4. Dimethyl trisulfide: observed frequencies

	Females	Males	Totals
Activation	29	29	58
No activation	11	11	22
Totals	40	40	80

	Females	Males	Totals
Attraction	15	18	33
No attraction	25	22	47
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	8.705E-31
Chi Square P-Value	>.9999
G-Squared	-1.288E-14
G-Squared P-Value	•
Contingency Coef.	1.043E-16
Phi	1.043E-16
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.464
Chi Square P-Value	.4957
G-Squared	.465
G-Squared P-Value	.4954
Contingency Coef.	.076
Phi	.076
Cty. Cor. Chi Square	.206
Cty. Cor. P-Value	.6497
Fisher's Exact P-Value	.6500

5. Butanoic acid: observed frequencies

	Females	Males	Totals
Activation	27	27	54
No activation	13	13	26
Totals	40	40	80

	Females	Males	Totals
Attraction	7	10	17
No attraction	33	30	63
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	0.000
Chi Square P-Value	•
G-Squared	0.000
G-Squared P-Value	•
Contingency Coef.	0.000
Phi	0.000
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.672
Chi Square P-Value	.4123
G-Squared	.675
G-Squared P-Value	.4113
Contingency Coef.	.091
Phi	.092
Cty. Cor. Chi Square	.299
Cty. Cor. P-Value	.5846
Fisher's Exact P-Value	.5856

6. Oct-1-en-3-ol: observed frequencies

	Females	Males	Totals
Activation	24	22	46
No activation	16	18	34
Totals	40	40	80

	Females	Males	Totals
Attraction	6	8	14
No attraction	34	32	66
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	.205
Chi Square P-Value	.6510
G-Squared	.205
G-Squared P-Value	.6510
Contingency Coef.	.051
Phi	.051
Cty. Cor. Chi Square	.051
Cty. Cor. P-Value	.8211
Fisher's Exact P-Value	.8213

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.346
Chi Square P-Value	.5562
G-Squared	.347
G-Squared P-Value	.5556
Contingency Coef.	.066
Phi	.066
Cty. Cor. Chi Square	.087
Cty. Cor. P-Value	.7686
Fisher's Exact P-Value	.7695

7. *p*-Cresol: observed frequencies

	Females	Males	Totals
Activation	30	30	60
No activation	10	10	20
Totals	40	40	80

	Females	Males	Totals
Attraction	12	5	17
No attraction	28	35	63
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	0.000
Chi Square P-Value	•
G-Squared	0.000
G-Squared P-Value	•
Contingency Coef.	0.000
Phi	0.000
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	3.660
Chi Square P-Value	.0557
G-Squared	3.749
G-Squared P-Value	.0528
Contingency Coef.	.209
Phi	.214
Cty. Cor. Chi Square	2.689
Cty. Cor. P-Value	.1010
Fisher's Exact P-Value	.0993

8. Skatole: observed frequencies

	Females	Males	Totals
Activation	22	24	46
No activation	18	16	34
Totals	40	40	80

	Females	Males	Totals
Attraction	7	4	11
No attraction	33	36	69
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	.205
Chi Square P-Value	.6510
G-Squared	.205
G-Squared P-Value	.6510
Contingency Coef.	.051
Phi	.051
Cty. Cor. Chi Square	.051
Cty. Cor. P-Value	.8211
Fisher's Exact P-Value	.8213

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.949
Chi Square P-Value	.3301
G-Squared	.959
G-Squared P-Value	.3274
Contingency Coef.	.108
Phi	.109
Cty. Cor. Chi Square	.422
Cty. Cor. P-Value	.5161
Fisher's Exact P-Value	.5179

9. Mix 1: observed frequencies

	Females	Males	Totals
Activation	31	25	56
No activation	9	15	24
Totals	40	40	80

	Females	Males	Totals
Attraction	12	5	17
No attraction	28	35	63
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	2.143
Chi Square P-Value	.1432
G-Squared	2.160
G-Squared P-Value	.1416
Contingency Coef.	.162
Phi	.164
Cty. Cor. Chi Square	1.488
Cty. Cor. P-Value	.2225
Fisher's Exact P-Value	.2222

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	3.660
Chi Square P-Value	.0557
G-Squared	3.749
G-Squared P-Value	.0528
Contingency Coef.	.209
Phi	.214
Cty. Cor. Chi Square	2.689
Cty. Cor. P-Value	.1010
Fisher's Exact P-Value	.0993

10. Mix 2: observed frequencies

	Females	Males	Totals
Activation	34	33	67
No activation	6	7	13
Totals	40	40	80

	Females	Males	Totals
Attraction	11	12	23
No attraction	29	28	57
Totals	40	40	80

Summary table for Activation

Num. Missing	0
DF	1
Chi Square	.092
Chi Square P-Value	.7618
G-Squared	.092
G-Squared P-Value	.7617
Contingency Coef.	.034
Phi	.034
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Summary table for Attraction

Num. Missing	0
DF	1
Chi Square	.061
Chi Square P-Value	.8049
G-Squared	.061
G-Squared P-Value	.8049
Contingency Coef.	.028
Phi	.028
Cty. Cor. Chi Square	0.000
Cty. Cor. P-Value	>.9999
Fisher's Exact P-Value	>.9999

Appendix 4

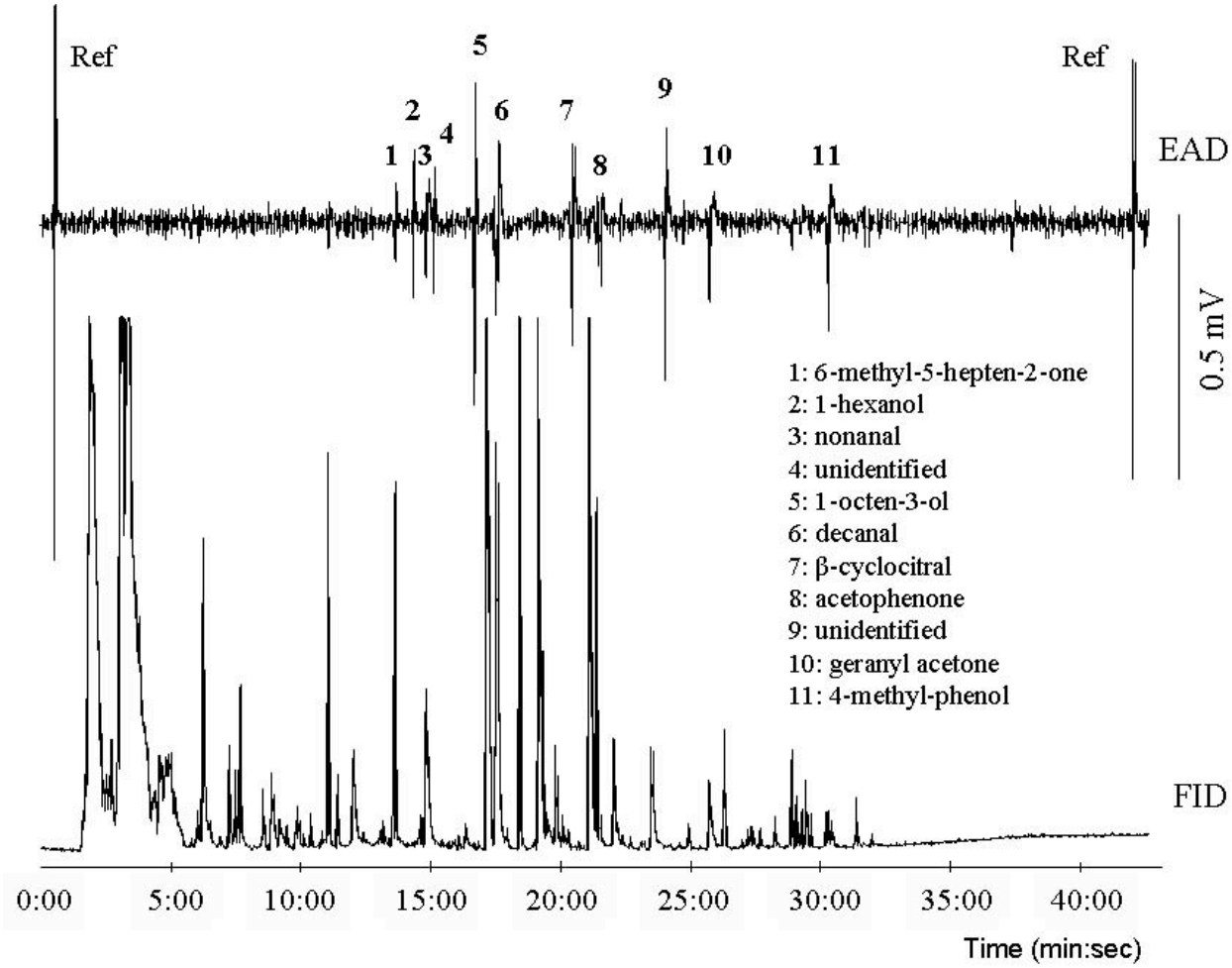

Figure 1: Antennogram responses of *S. calcitrans* to cow hairs volatiles thermally desorbed onto a high resolution chromatographic column (FFAP) from a Tenax™ GR cartridge. The lower trace is the signal from the flame ionisation detector (FID), the upper trace the antennographic responses (EAD) of a female stable fly. Reference responses (Ref) were recorded at the start and end of the analysis using oct-1-en-3-ol at 100 ng in the stimulus syringe.

Appendix 5

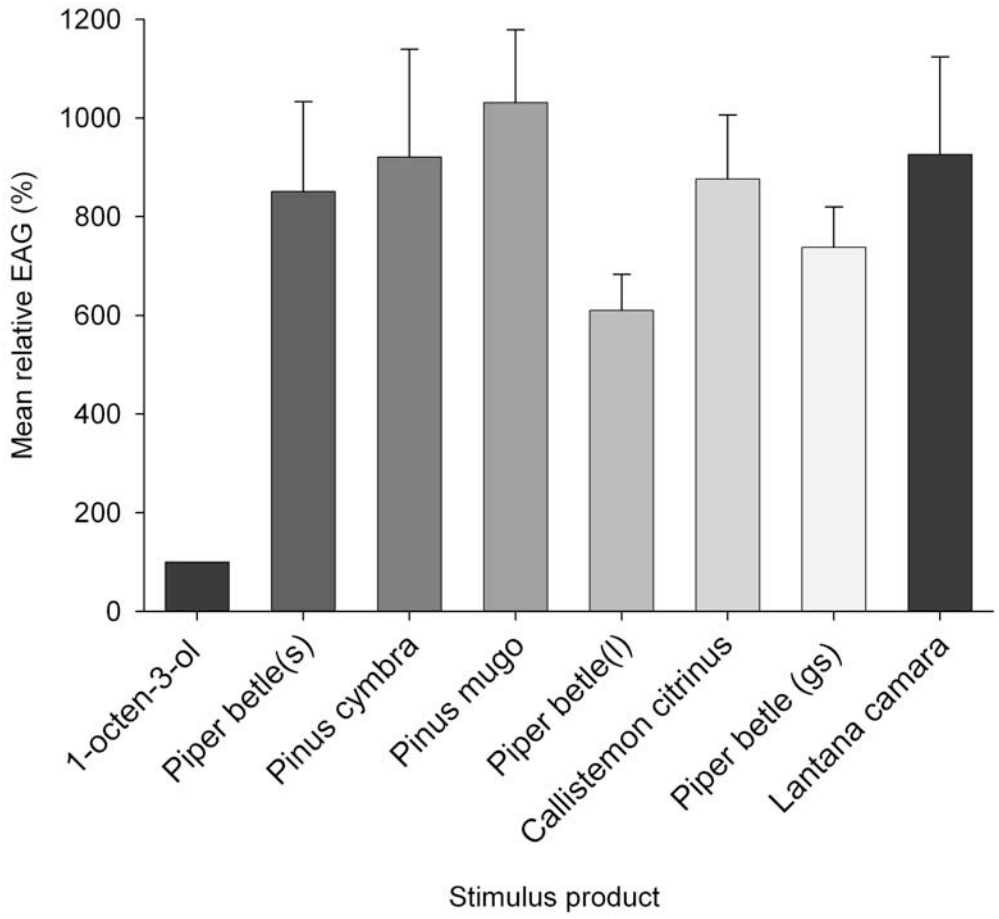

Figure 1: Relative EAG responses recorded from *S. calcitrans* males (N=2) to various essential oils of plants, each at 10 µl (neat) in the stimulus syringe. Oct-1-en-3-ol at 100 ng in the stimulus syringe was used as reference. These EAG responses indicate that the antennae of *S. calcitrans* carry receptor cells responding to plant related compounds like terpenes. Similar EAG responses were recorded to these oils from *Aedes aegypti* and *Glossina pallidipes* antennae by Mohottalage (2002). This author made also a very complete list of the constituents present in these essential oils that consist mostly of terpenes. For Piper betle oil, s: stems, l: leaves, gs: ground stems.

Mohottalage M.S.B. (2002) Chemistry, insecticidal and insect neurophysiological activity of some essential oils from Sri Lanka with emphasis on *Piper betle* L. (Piperaceae) leaf oil. PhD thesis, pp. 186, Institute of Chemistry, University of Neuchâtel, Switzerland.