Tsetse fly responses to volatile plant compounds

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Le Doyen, Prof. P. Kropf

"Es ist mir in den Wissenschaften gegangen
wie einem, der früh aufsteht,
in der Dämmerung die Morgerröte, sodann aber
die Sonne ungeduldig erwartet
und doch, wie sie hervortritt,
geblendet wird."

– Johann Wolfgang von Goethe (1749-1831)

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Summary

Tsetse flies (Diptera, Glossinidae) constitute 31 species and subspecies divided into three subgenera. They are currently confined in sub-Saharan Africa and occupy three preferred habitats: savannah, riverine zones and forest. Both sexes are strictly haematophagous and can transmit during their blood meal trypanosomiasis which causes sleeping sickness in humans and Nagana in animals. It is estimated that about 60 million people and 50 million cattle are continuously at risk in 20 African countries resulting in enormous economic losses.

Tsetse flies locate hosts using visual cues including contrast, shape and colour at short range, and olfactory cues at a distance. Tsetse fly population dynamic is characterised by a slow intrinsic growth rate as they are ovoviviparous, so visual baited traps and targets can deplete a population in only a few years. The major olfactory stimulants used in field traps to control tsetse flies are 1-octen-3-ol, 3-*n*-propylphenol, *p*-cresol and acetone.

Plants not only provide the habitat and shelter for tsetse flies but also provide females with sunlight-protected places for larviposition. Little attention has been paid to the flies' propensity for finding suitable cover under vegetation. Keeping in mind the role of peridomestic resting sites, it is important to consider volatile chemicals derived from plants for the development of new odour-baited traps to attract tsetse from all 3 subgenera. It is possible that the tsetse flies make parsimonious use of chemostimuli (e.g. 1-octen-3-ol present in both host odours and plant volatiles) for host finding and shelter under plants.

Sensory responses of tsetse flies from all three African habitats to volatile products commonly found in plants as well as host odours were recorded using the electroan-

tennogram techniques. Products included monoterpenes, sesquiterpenes and green-leaf volatiles. We have shown, that tsetse species from all three African habitats perceive volatile plant compounds in a similar manner, several at detection thresholds similar to 1-octen-3-ol. Of main interest for behavioural studies is L-turpentine, as it is a simple terpene mixture with a low detection threshold, with limonene and α-pinene as main components , along with the monoterpene ocimene and aromatic *p*-cymene, and it is relatively easily obtained, inexpensive and can be produced directly in Africa.
Tsetse flies are fast flyers, so we filmed the responses of *G. pallidipes*, *G. brevipalpis* and *G. swynnertoni* in the wind tunnel using a 3D recording system. By combining the sensitising effect of CO_2 with the test compounds we were able to increase the number of activated flies by tenfold and were therefore able to detect differences in the behavioural responses of tsetse flies to treatments. Our results show that tsetse flies respond to a series of single plant compounds and their mixtures. The single plant compounds tested with the best activation potential were S-(-)-limonene and *p*-cymene. The best results were achieved with the monoterpene mixture comprising L-turpentine showing a very good activating effect on *G. pallidipes*, *G. swynnertoni* and *G. brevipalpis* and also inducing many flies to perform a local search at the upwind end of the wind tunnel. At the optimal dose tested (10μg/min) L-turpentine was comparable to human breath in terms of activating and inducing local search behaviour by *G. pallidipes* and *G. swynnertoni*.
In our study we found no differences in behavioural responses of male and female *G. pallidipes* to human breath, CO_2 alone and 1-octen-3-ol, products usually associated with hosts, but we could detect differences in behavioural responses of males and females to volatile plant compounds (limonene, *p*-cymene and L-turpentine). As pregnant *G. brevipalpis* responded only to L-turpentine but not human breath one could speculate that volatile plant compounds not only carry information about hosts, but also indicate larviposition or resting sites, both of which are of greater importance for females due to the higher energy demands of flying than for males.
To assess whether volatile plant compounds could be of use in the flied, i.e. increasing trap and target encounters, we tested if plant compounds attract tsetse flies to a target and therefore introduced a phthalogen blue sphere into the wind tunnel to test com-

bined visual and olfactory stimuli. We showed that L-turpentine in the presence of CO_2 increases the time and distance spent flying around a target and also affects the relative hight of flight to the target and of the local search at the target. With L-turpentine presented, flight to the target was more directed than with CO_2 alone and the local search around the sphere was lower in elevation.

The results presented in this thesis show that tsetse flies not only perceive volatile plant compounds, but that these vectors of disease, especially females, are attracted by terpenes and simple terpene mixtures presented with and without a visual target at amounts 1000 to 10'000 times lower than background CO_2 levels. Overall this work suggests that volatile plant compounds hold promise for integration into semiochemical-based control techniques for tsetse flies.

Key words : Sensory ecology, tsetse fly, *Glossina*, *G. pallidipes*, *G. swynnertoni*, *G. brevipalpis*, *G. fuscipes fuscipes*, *G. palpalis gambiensis*, electrophysiology, behaviour, wind tunnel, 3D video tracking

Résumé

Les glossines (Diptera, Glossinidae) constituent 31 espèces et sous-espèces réparties en trois sous-genres. Elles sont actuellement limitées à l'Afrique sub-saharienne et préfèrent trois habitats: la savane, les zones riveraines et la forêt. Les deux sexes sont strictement hématophages et peuvent transmettre la trypanosomiase au cours de leur repas de sang qui provoque la maladie du sommeil chez les humains et le nagana chez des animaux. Environ 60 millions de personnes et 50 millions de bovins dans 20 pays africains risquent continuellement l'infection, ce qui provoque des pertes économiques énormes.

Les mouches tsé-tsé détectent les hôtes à proximité à l'aide de repères visuels, dont le contraste, la forme et la couleur, et de signaux olfactifs à distance. Comme elles sont ovovivipares, la dynamique de population des mouches tsé-tsé est caractérisée par un taux de croissance faible. Des pièges et cibles appâtés peuvent ainsi épuiser une population en quelques années seulement. Les stimulants olfactifs principalement utilisés dans les pièges sur le terrain contre les mouches tsé-tsé sont l'1-octène-3-ol, le 3-*n*-propylephénol, le *p*-crésol et de l'acétone.

Les plantes fournissent aux mouches tsé-tsé non seulement habitat et refuge, mais en plus elles offrent des endroits protégés du soleil pour la larviposition aux femelles. La faculté naturelle des mouches pour trouver une couverture adaptée sous la végétation n'a attiré que peu d'attention. Tenant compte du rôle des sites de repos péridomestiques, il est important de considérer les produits chimiques volatils dérivés de plantes pour le développement de nouveaux pièges appâtés pour attirer toutes les tsé-tsé des 3 sous-genres. Il est possible que les mouches tsé-tsé font un usage parcimonieux de chimiostimuli (par exemple 1-octène-3-ol qui est présent dans les odeurs d'hôte autant que les substances volatiles de plantes) pour trouver hôte ou abris sous les plantes.

En utilisant l'electroantennographie, les réponses sensorielles aux produits volatils couramment trouvés dans les plantes ainsi qu'aux odeurs d'hôtes ont été enregistrées chez des mouches tsé-tsé des trois habitats africains. Les produits comprennent des monoterpènes, des sesquiterpènes et des C6-alcohols et aldehydes (GLVs). Nous avons démontré que les espèces de glossines des trois habitats africains perçoivent les composants végétaux volatils d'une manière similaire, dont plusieurs à des seuils de détection similaires à l'1-octène-3-ol. Pour les études comportementales, la L-térébenthine est particulièrement interéssante, car il s'agit d'un mélange de terpènes simples avec un seuil de détection bas, avec le limonène et l'α-pinène comme composants principaux, accompagnés de monoterpène ocimène et d'aromatiques *p*-cymène. La L-térébenthine est relativement facile à obtenir, peu coûteuse et peut être produite directement en Afrique.
Les mouches tsé-tsé ayant un vol rapide, nous avons filmé les réactions des *G. pallidipes*, *G. brevipalpis* et *G. swynnertoni* dans une soufflerie en utilisant un système d'enregistrement 3D. En combinant l'effet de sensibilisation du CO_2 avec les composés d'essai, nous avons pu augmenter le nombre de mouches activées par dix et donc détecter des différences dans les réponses comportementales des mouches tsé-tsé aux traitements. Nos résultats montrent que les mouches tsé-tsé répondent à une série de composants végétaux simples et à leurs mélanges. Les composants de plantes individuels avec le meilleur potentiel d'activation parmi les composants testés étaient le S-(-)-limonène et le *p*-cymène. Les meilleurs résultats ont été obtenus avec un mélange monoterpène comprenant la L-térébenthine montrant un très bon effet activateur sur *G. pallidipes*, *G. swynnertoni* et *G. brevipalpis* induisant également beaucoup de mouches à effectuer une recherche locale à l'extrémité de la chambre de vol. À la dose optimale testée (10 μg/min), la L-térébenthine était comparable à l'haleine humaine en termes d'activation et d'induction de comportement de recherche locale par *G. pallidipes* et *G. swynnertoni*. Dans notre étude, nous n'avons constaté aucune différence entre les réactions comportementales des mâles et des femelles *G. pallidipes* à l'haleine humaine, le CO_2 présenté seul et au l'1-octène-3-ol, des produits généralement associés à des hôtes, mais nous pouvions détecter des différences entre les réactions comportementales des mâles et des femelles à des composants volatils de plantes (limonène, le *p*-cymène et L-térébenthine). Comme

les *G. brevipalpis* sur le part de pondre répondaient seulement à la L-térébenthine mais pas à l'haleine humaine on pourrait spéculer que les composants volatils de plantes non seulement transportent des informations sur les hôtes, mais indiquent aussi des sites de larviposition ou de repos, qui sont d'une importance plus grande pour les femelles en raison de leur plus grands besoins énergétiques en vol comparé aux mâles.
Pour évaluer si les composants volatils de plantes pouvaient être utiles sur le terrain, à savoir accroître l'attractivité des pièges ou des cibles, nous avons testé si les composants de plantes attiraient les mouches tsé-tsé vers une cible visuelle et nous avons donc introduit une sphère bleue phthalogène dans la soufflerie pour tester à la fois des stimuli visuels et olfactifs. Nous avons montré que la L-térébenthine en présence de CO_2 augmente le temps et la distance passés en vol autour d'une cible et affecte également la hauteur relative de vol vers la cible et lors de la recherche locale autour d'elle. En présence de L-térébenthine, le vol vers la cible a été plus orienté qu' avec le CO_2 seul et la recherche locale a eu lieu à une plus faible altitude.

Les résultats présentés dans cette thèse montrent que les mouches tsé-tsé non seulement perçoivent des composants volatils de plantes, mais que ces vecteurs de maladie, en particulier les femelles, sont attirés par les terpènes et les mélanges de terpènes simples présentés avec et sans cible visuelle à des concentrations de 1000 à 10'000 fois inférieures au niveau de CO_2 naturel. De manière générale, ce travail suggère que les composants volatils de plantes sont prometteurs quant é leur intégration dans les techniques de contrôles sémiochimiques pour les mouches tsé-tsé.

mots-clé : Ecologie sensorielle, mouches tsé-tsé, *Glossina*, *G. pallidipes*, *G. swynnertoni*, *G. brevipalpis*, *G. fuscipes fuscipes*, *G. palpalis gambiensis*, éléctrophysiologie, comportement, soufflerie, système d'enregistrement 3D

Zusammenfassung

Tsetse-Fliegen (Diptera, Glossinidae) unterteilen sich in 31 Arten und Unterarten, welche in drei Untergattungen eingeteilt werden. Ihre Verbreitung ist zur Zeit auf ein Gebiet südlich der Sahara begrenzt, wo sie drei bevorzugte Habitate besiedeln: Savanne, Uferzonen von Flüssen und Seen sowie Waldregionen. Beide Geschlechter sind rein haematophag und können während ihrer Blutmahlzeit Trypanosomen übertragen, welche bei Menschen die Schlafkrankheit und bei Tieren Nagana auslösen. Geschätzte 60 Millionen Menschen und 50 Millionen Tiere sind in den 20 betroffenen afrikanischen Ländern ständig in Infektionsgefahr, was zu enormen ökonomischen Verlusten führt.

Auf kurze Distanz finden Tsetse-Fliegen ihre Wirte durch visuelle Signale wie Kontrast, Form und Farbe und auf längere Distanz durch olfaktorische Stimuli. Bedingt durch ihren Ovoviviparismus ist die Populationsdynamik der Tsetse-Fliegen durch ein langsames intrinsisches Wachstum charakterisiert, wodurch Kontrollmassnahmen eine Population in wenigen Jahren auslöschen können. Die wichtigsten in Fallen verwendeten Köder sind 1-Octen-3-ol, 3-*n*-Propylphenol, *p*-Cresol und Aceton.

Pflanzen stellen nicht nur die Lebensräume und Verstecke für die Tsetse-Fliegen, sondern dienen den Weibchen auch als sonnengeschützte Ablageplätze für ihre Larven. Bisher wurde der Fähigkeit der Fliegen Unterschlüpfe unter der Vegatation zu finden, wenig Beachtung entgegengebracht. Berücksichtigt man die Rolle der hausnahen Ruheplätze, müssen flüchtige Pflanzenstoffe für die Entwicklung neuer Köder zum Anlocken der Tsetse-Arten aus allen drei Untergattungen betrachtet werden. Möglicherweise erlaubt die sparsame Benutzung von Chemostimuli (wie z. B. 1-Octen-3-ol, welches Bestandteil von Wirt- und Pflanzendüften ist) den Tsetse-Fliegen Wirte wie auch Verstecke unter Pflanzen zu finden.

Anhand von Elektroantennogrammen wurden die sensoriellen Antworten auf flüchtige Produkte, welche üblicherweise in Pflanzen und Wirten vorhanden sind, bei Tsetse-Fliegen aus allen drei Habitatstypen gemessen. Zu den Produkten gehörten Monoterpene, Sesquiterpene und Grün-Blattduftstoffe (GLVs). Wir konnten zeigen, dass Tsetse-Arten aus allen afrikanischen Habitaten flüchtige Pflanzenstoffe in ähnlicher Weise wahrnehmen. Einige dieser Produkte weisen eine ähnlich tiefe Detektionsschwelle auf wie 1-Octen-3-ol. Von speziellem Interesse für die Verhaltensstudien ist L-Terpentin, eine einfache Mischung aus Terpenen mit einer niedrigen Detektionsschwelle. Seine Hauptbestandteile sind Limonen und α-Pinen mit dem Monoterpen Ocimen und dem Aromaten *p*-Cymen. Zu seinen Vorteilen gehört, dass es relativ einfach und günstig direkt in Afrika produziert werden kann.

Da Tsetse-Fliegen sehr schnelle Flieger sind, filmten wir das Verhalten von *G. pallidipes*, *G. brevipalpis* und *G. swynnertoni* mit einem 3D-Aufnahmeverfahren in einem Windkanal. Indem wir den Sensibilisierungseffekt von CO_2 mit den Testprodukten kombinierten, konnten wir die Anzahl der aktivierten Fliegen um ein zehnfaches erhöhen, und so Unterschiede in ihrem Verhalten auf Behandlungen feststellen. Unsere Resultate zeigen, dass Tsetse-Fliegen auf eine Reihe von flüchtigen Pflanzenstoffen sowie ihren Mischungen reagieren. Die einfachen Pflanzenprokdukte mit dem grössten Aktivierungspotential waren S-(-)-Limonen und *p*-Cymen. Die besten Resultate in Bezug auf Aktivierung, Flug- und Suchverhalten am Ende des Windkanals von *G. pallidipes*, *G. brevipalpis* und *G. swynnertoni* wurden mit der Monoterpenmischung L-Terpentin erhalten. Die Wirkung von L-Terpentin in seiner optimalen getesteten Dosis (10μg/min) war in Bezug auf sein Aktivierungspotential und als Auslöser des Suchverhaltens am Ende des Windkanals von *G. pallidipes* und *G. swynnertoni* vergleichbar mit der Wirkung menschlichen Atems.

In unserer Untersuchung wurden keine Unterschiede im Verhalten von männlichen und weiblichen Fliegen bei menschlichem Atem, CO_2 und 1-Octen-3-ol, also Produkten, die normalerweise mit den Wirten in Verbindung gebracht werden, festgestellt. Jedoch konnten Unterschiede in ihrem Verhalten zu flüchtigen Pflanzenstoffen (Limonen, *p*-Cymen und L-Terpentin) festgestellt werden. Da tragende *G. brevipalpis* auf L-Terpentin,

nicht jedoch auf menschlichen Atem reagierten, könnte spekuliert werden, dass flüchtige Pflanzenstoffe nicht nur Informationen zu Wirten, sondern auch zu Larvablege- und Ruheplätzen tragen. Diese beiden Informationen sind für tragende Weibchen aufgrund ihres grösseren Energieverbrauchs beim Fliegen von grösserer Bedeutung als für Männchen.
Zum evaluieren, ob flüchtige Pflanzenstoffe im Feld von Nutze sein könnten, z. B. zum Erhöhen der Attraktivität der Fallen, testeten wir, ob diese Pflanzenprodukte die Fliegen zusätzlich zu einem visuellen Signal anziehen könnten. Dazu führten wir eine enzianblaue Kugel in den Windkanal ein und konnten so die Kombination von visuellen und olfaktorischen Stimuli testen. Wir konnten zeigen, dass L-Terpentin in Kombination mit CO_2 nicht nur die Zeit und die Flugdistanz vergrösserten, die die Fliegen um die Kugel herum verbrachten, sondern auch die relative Flughöhe und die Höhe des Suchverhaltens um die Kugel herum beeinflussten. Im Beisein vom L-Terpentin waren die Flüge und die Höhe des Suchverhaltens, verglichen mit CO_2 alleine, tiefer.

Die Resultate dieser Doktorarbeit zeigen, dass Tsetse-Fliegen nicht nur flüchtige Pflanzenstoffe wahrnehmen, sondern dass insbesondere die Weibchen, von Terpenen und einfachen Terpenmischungen angezogen werden, und dies sowohl im Beisein von visuellen Stimuli wie auch ohne. Sie reagieren auf diese Stoffe schon in Mengen, die 1000 bis 10'000 mal kleiner sind als die natürlichen CO_2-Mengen. Schliesslich zeigt diese Arbeit eine Möglichkeit zur Integration der flüchtigen Pflanzenstoffe in die bestehenden Tsetse-Kontrolltechniken auf.

Schlagworte : Sensoriellen Ökologie, Tsetse-Fliege, *Glossina*, *G. pallidipes*, *G. swynnertoni*, *G. brevipalpis*, *G. fuscipes fuscipes*, *G. palpalis gambiensis*, Elektrophysiologie, Verhalten, Windkanal, 3D video Tracking

1. General Introduction

1.1. The tsetse flies *Glossina sp.*

1.1.1. Description and life cycle of tsetse flies

Tsetse flies (Diptera, Glossinidae, *Glossina spp.*) are medium-sized (0.5 - 2cm) typical dipteran with a relatively slender body, that can be distinguished from other flies by several anatomical characteristics:

Wings: when resting, tsetse flies fold their wings completely one on top of the other.

Proboscis: the long proboscis extends directly forward and is attached by a distinct bulb to the bottom of the head.

Arista: the hairs of the arista are branched. Additionally only the front side of the arista has hairs.

Wing cell: the discal medial cell of the wing has a characteristic hatchet shape.

Amongst medically important vectors of disease tsetse flies present a unique life cycle, known as adenotrophic viviparity. The female fertilizes one egg at a time and retains the larva in the uterus to allow it to develop internally, nourishing it through specialised 'milk' glands until mature. The mature larva is deposited in shaded moist soil or sand where the larva rapidly burrows into the substrate to pupate. Depending on temperature, the adult flies emerge after 20 to 45 days. Thus each female produces only one offspring at a time, and can produce up to 12 offspring at intervals of about 9-10 days during their typical adult lifespan of 2-3 month [106]. This results in a low intrinsic rate of population growth and therefore a high vulnerability to control techniques. Tsetse flies are daytime active and contrary to other blood-sucking Diptera, they are purely haematophagous and able to take blood meals from a wide range of mammals and reptiles depending

on their ecology [92, 113, 182]. The main hosts of the different tsetse species belong to the Bovidae (bush-buck, kudu, eland, buffalo and cattle), the Suidae (warthog, bushpig, red river hog, the giant forest hog and the domestic pig), man, and, especially for the palpalis group, reptiles (monitor lizard, crocodile) [136]. Finding a host is critical for survival and reproduction, and interestingly, host choice is apparently influenced by the host selected for the first blood meal [13]. Peridomestic resting sites, within the reach of the tsetses' preferred hosts, not only provide suitable micro-climatic conditions facilitating flies' longevity but also provide the opportunity for feeding repeatedly on the same host, which is important in the establishment of its vectorial capacity [36, 116]. The total daily time spent in flight is relatively short, with bursts of activity at dawn and dusk [16, 17]. The daily activity pattern is to ~80% under the control of an endogenous circadian clock, and only to ~20% directly controled by temperature while light directly influences the daily rhythm mainly by its effects at low intensity [20]; humidity only marginally influences the activity pattern [20].

1.1.2. Systematic and distribution

The distribution of the 31 tsetse species and subspecies (Glossinidae) covers most of Africa between the Sahara and the Kalahari Desert. Species numbers are highest in the wetter central and western parts of the Continent (Table 1.1, Figures 1.1, 1.2, 1.3). Historically tsetse flies are divided into three subgenera based on morphological criteria [123] with different habitat requirements: the *morsitans* flies prefer open savannah woodlands, the *palpalis* flies riverine vegetation and *fusca* flies forested habitats (1.2). This grouping was also confirmed by genetic and allozyme variation [57–60], but more recent genetic research [31] suggests the formation of a forth monospecific subgenus, *Machadomyia* out of the *morsitans*-group, which also shows sufficiently distinct genitalia from the other members of this group [41].

***fusca*-group** The typical habitats of the *fusca*-group species are West African forests, ranging from relatively dry forest islands and moist gallery forests in the savannah to dense and wet rain forests [88, 90, 106]. *Glossina brevipalpis* is found in East and

Table 1.1.: **Summary of the ecological zones in Africa and their characteristics.** Adapted from Jahnke (1982) and Kristjanson et al. (1999) [86, 100]

	Ecological Zones				
Characteristics	Arid	Semi-arid	Sub-humid	Humid	Highlands
Area (1000km^2)	8327	4050	4858	4137	990
Rainfall (mm)	< 500	$500 - 1000$	$1000 - 1500$	> 1500	variable
Moisture index	> 36	$20 - 36$	$0 - 20$	≤ 0	variable
Growing days	0 - 90	90 - 180	180 - 270	270 - 365	variable
Tsetse-infested area (%)	12.5	50.3	68.2	89.7	19.7
Cattle (%)	21.3	30.8	22.2	6.0	19.7
Cattle[1] in 1999 (%)	17.8	31.6	21.3	3.8	25.6
Cattle[1] (%) in tsetse-infested area	6.0	28.6	60.6	90.4	21.6
predominant Tsetse group	*Morsitans*	*Morsitans* *Palpalis*	*Palpalis* *Fusca*	*Fusca* *Palpalis*	None

[1] Source: Kristjanson et al. 1999 [100]

southern Africa inhabiting dense thickets leading to a patchy and localized distribution [115]. An exception is the distribution of *G. longipennis*, which lives, due to its pupal adaptation to dry conditions [55], in one of the driest habitats inhabited by tsetse flies consisting of dry deciduous *Commiphora* and *Acacia* bush in eastern and central Kenya, southern Somalia and Ethiopia (Figure 1.1).

***palpalis*-group** The habitats of the palpalis flies occur in the drainage systems leading to the Atlantic or the Mediterranean Ocean, extending from the wet mangrove and lowland rainforests to the drier savannah areas, where they are restricted to the ecoclimate of the watercourses or islands of gallery forests (Figure 1.2) [106]. Many *palpalis* species prefer peridomestic conditions and maintain close associations with villages [10, 11, 101]

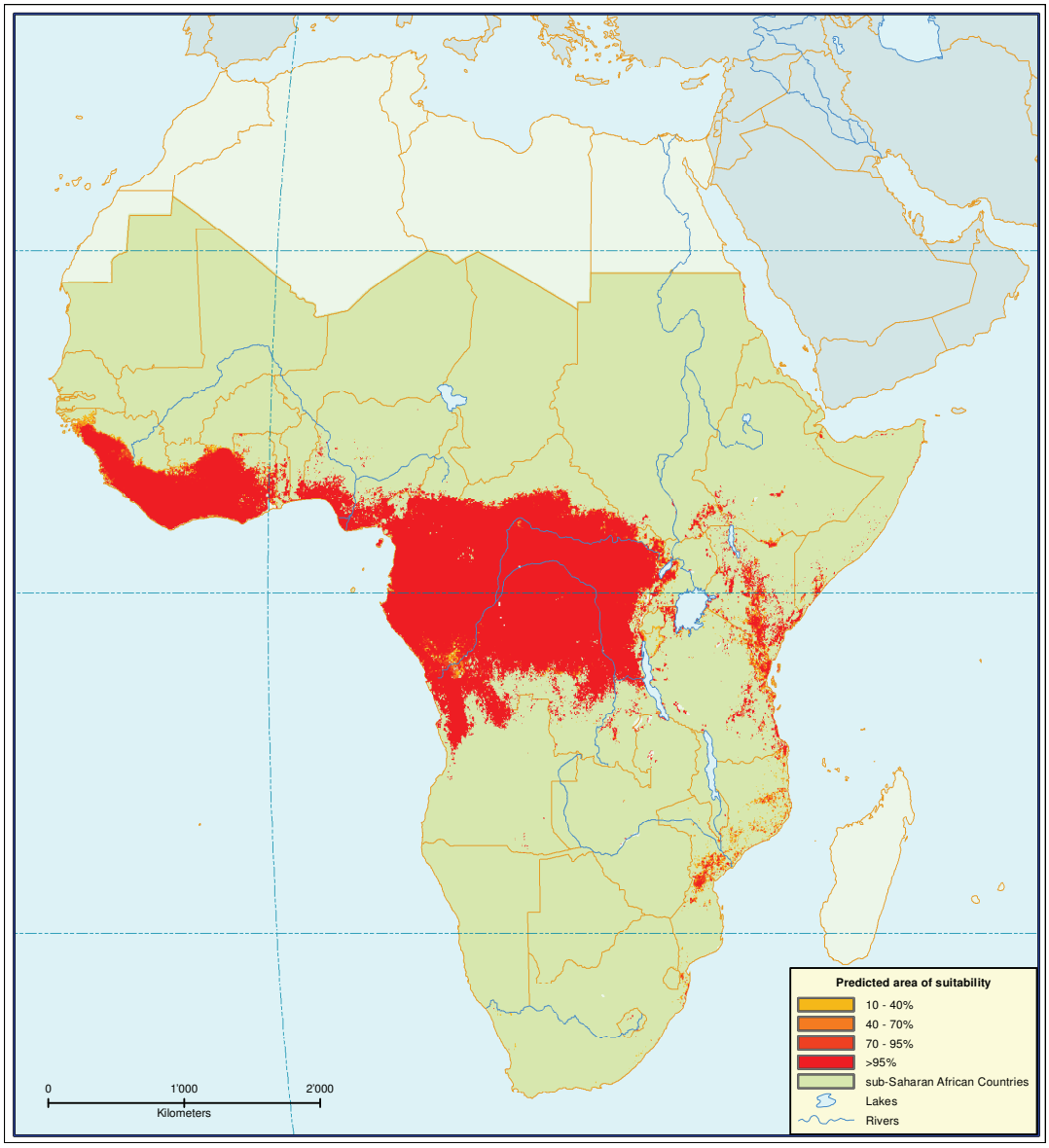

Figure 1.1.: **Predicted areas of suitability for *fusca*-group, subgenus *Austenina* in Africa (1999).** Data provided by the Programme against African Trypanosomiasis Information System PAAT-IS (http://www.fao.org/ag/paat-is.html, [30]). The map represents the probability of environmental suitability for tsetse flies. The map is a Robinson projection with a central meridian at -60 °.

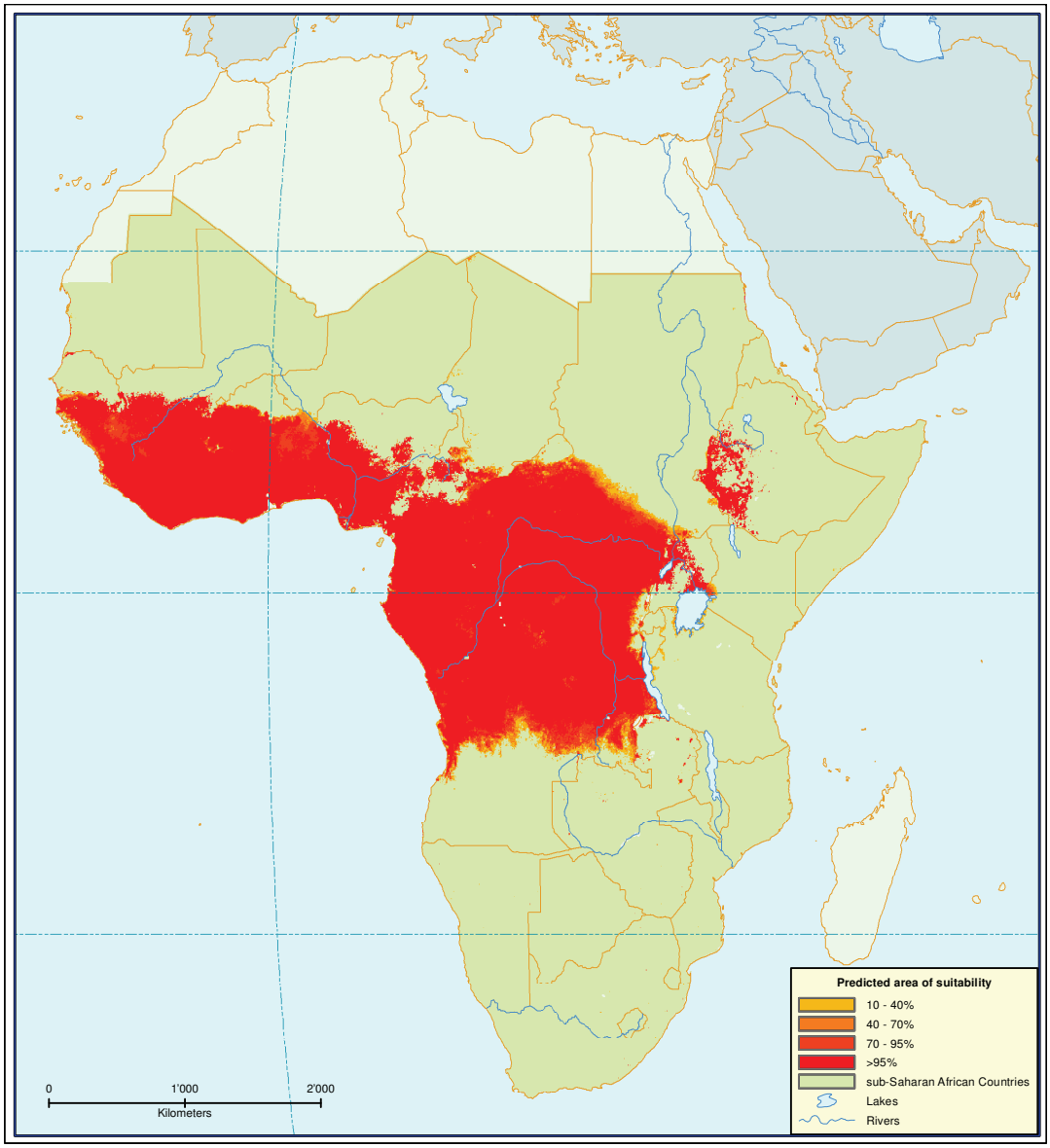

Figure 1.2.: **Predicted areas of suitability for *palpalis*-group, subgenus *Nemorrhina* in Africa (1999).** Data provided by the Programme against African Trypanosomiasis Information System PAAT-IS (http://www.fao.org/ag/paat-is.html, [30]). For details see legend to Figure 1.1

Table 1.2.: **List of *Glossina* species arranged according to the three species groups.** Species in bold were used in experiments.

Palpalis-group	**Morsitans-group**
Glossina caliginea (Austen, 1911)	*Glossina austeni* (Newstead, 1912)
***Glossina fuscipes fuscipes* (Newstead, 1911)**	*Glossina longipalpis* (Wiedemann, 1830)
Glossina fuscipes martinii (Zumpt, 1935)	*Glossina morsitans centralis* (Machado, 1970)
Glossina fuscipes quanzensis (Pires, 1948)	*Glossina morsitans morsitans* (Wiedemann, 1850)
Glossina pallicera pallicera (Bigot, 1891)	*Glossina morsitans submorsitans* (Newstead, 1911)
Glossina pallicera newsteadi (Austen, 1929)	***Glossina pallidipes* (Austen, 1903)**
Glossina palpalis palpalis (Robineau-Desvoidy, 1830)	***Glossina swynnertoni* (Austen, 1923)**
***Glossina palpalis gambiensis* (Vanderplank, 1911)**	
Glossina tachinoides (Westwood, 1850)	
Fusca-group	
***Glossina brevipalpis* (Newstead, 1911)**	*Glossina nashi* (Potts,1955)
Glossina fusca congolensis (Newstead and Evans, 1921)	*Glossina nigrofusca hopkinsi* (Van Emden, 1944)
Glossina fusca fusca (Walker, 1849)	*Glossina nigrofusca nigrofusca* (Newstead, 1911)
Glossina fuscipleuris (Austen, 1911)	*Glossina severini* (Newstead, 1913)
Glossina frezili (Gouteux, 1987)	*Glossina schwetzi* (Newstead and Evans, 1921)
Glossina haningtoni (Newstead and Evans, 1922)	*Glossina tabaniformis* (Westwood, 1850)
Glossina longipennis (Corti, 1895)	*Glossina vanhoofi* (Henrard, 1952)
Glossina medicorum (Austen, 1911)	

***morsitans*-group** The typical habitats of the *morsitans*-group species are open savannah woodlands, like the 'miombo', 'mopane' and 'doka' woodlands where *Brachytegia sp.* and *Julbernardia sp.*, *Colophospermum mopane* and *Isoberlinia sp.*, respectively, dominate the vegetation. They can also be found in *Acacia-Commiphora* vegetation and occupy secondary shrubs, thickets and islands of forests, along with an abundance of wild life (Figure 1.3)[106].

1.1.3. Trypanosomiasis, its implications and control

Trypanosomiasis Trypanosomiasis, is a vector-borne parasitic disease, occurring in both sub-Saharan Africa and Central and Southern America. In Africa the disease is also known as sleeping sickness (human African trypanosomiasis, HAT) or Nagana (cattle) and in America as Chagas disease. In both cases the parasites are protozoans belonging

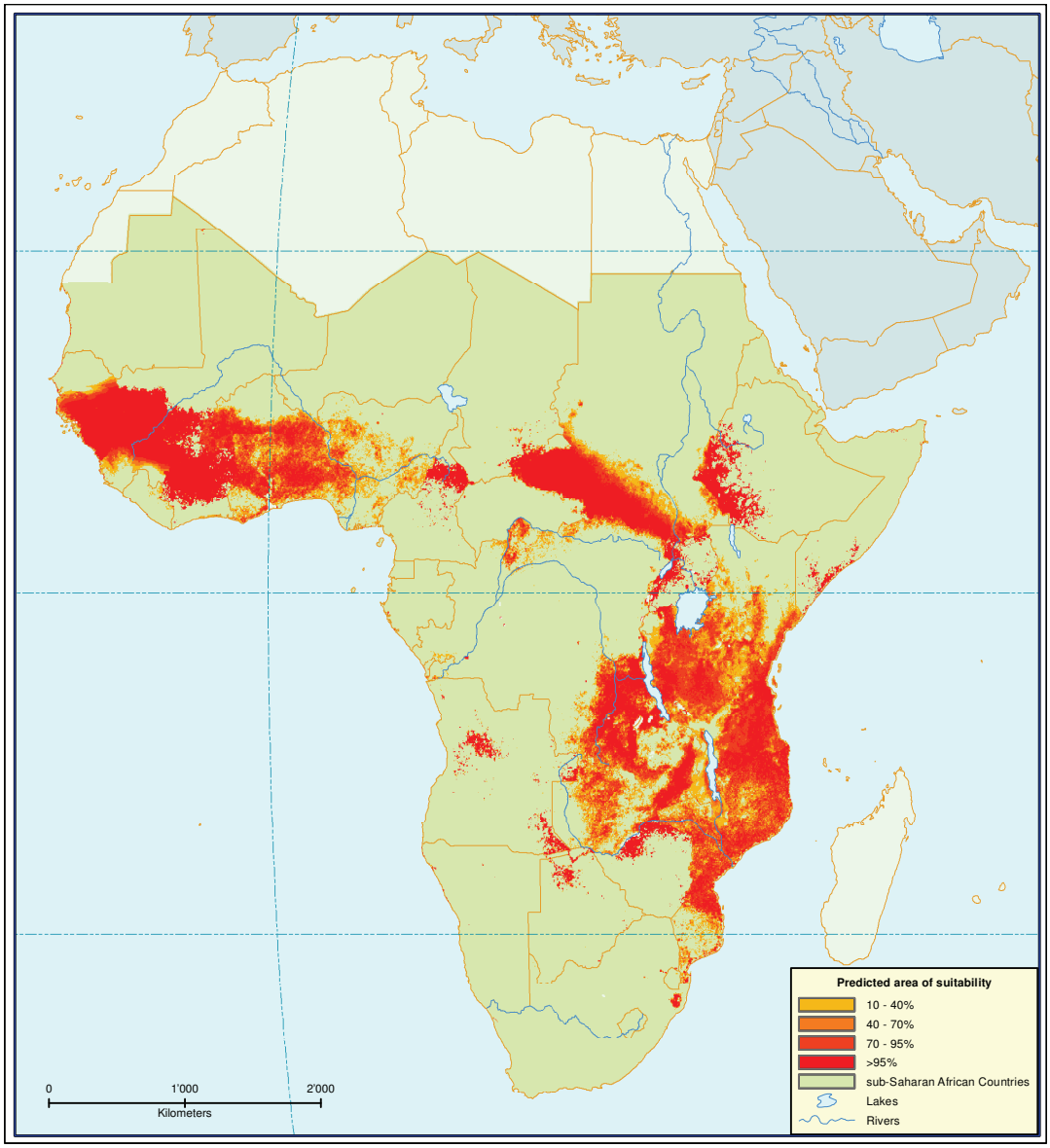

Figure 1.3.: **Predicted areas of suitability for *morsitans*-group, subgenus *Glossina s.s.* in Africa (1999).** Data provided by the Programme against African Trypanosomiasis Information System PAAT-IS (http://www.fao.org/ag/paat-is.html, [30]). For details see legend to Figure 1.1

to the genus Trypanosoma, *T. brucei rhodensiense* and *T. b. gambiense* causing HAT and *T. cruzi* causing Chagas disease, and are transmitted by feeding insect vectors, by the tsetse flies in Africa and by triatomine bugs in America. Some forms of Trypanosoma, e.g. *T. evansi* and *T. vivax* can also be transmitted mechanically by biting flies [74]. In Africa there have been several epidemics of human trypanosomiasis over the last centuries (Figure 1.4): one between 1896 and 1906 mostly in Uganda and the Congo Basin, one in 1920 in several African countries and one beginning in 1970 in Busoga, Uganda is ongoing showing a decline since 1997 due to continued control efforts of the national control programs, bilateral cooperation, NGO's and the WHO. In 2009 the number of cases reported has dropped below 10'000 for the first time in 50 years [183] and some countries are now reported HAT free (Figure 1.5, [149]).

The first stage of the disease, the haemolymphatic phase, is characterized by fever, headaches, joint pains and itching. The second stage, the neurological stage, begins when the parasite crosses the blood-brain barrier and invades the central nervous system. The typical symptoms of this stage are confusion, sensory disturbances, poor coordination and disturbance of the sleep cycle. Without treatment, sleeping sickness is fatal. Treatment is notoriously difficult considering toxicity and protocol complexity of the drugs actually available for treatment requiring specially trained staff for administration. Furthermore, WHO reported that parasite resistance to existing drugs has appeared in recent years [51].

Animal African trypanosomiasis (AAT) is caused by a wider number of trypanosome species with higher prevalence. Trypanosome infections that threaten livestock are over 100 to 150-fold higher in *G. morsitans* than the trypanosome infections that cause human trypanosomiasis [89]. AAT is most important in cattle but can cause serious losses in pigs, camels, goats, and sheep. Infection of cattle results in subacute, acute, or chronic disease characterized by intermittent fever, anemia, occasional diarrhea, and rapid loss of condition and often terminates in death [74]. According to Ford [50, 137] the impact of animal trypanosomiasis was so profound that it influenced the migration routes of cattle-owning tribes into the continent to avoid the *G. morsitans* "fly-belts". Tsetse flies not only influenced the migration routes of tribes, but also influences food

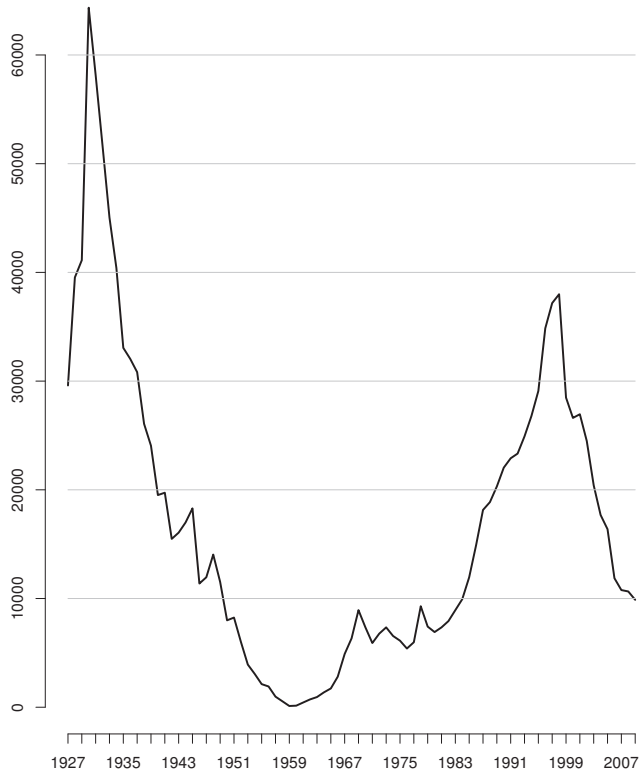

Figure 1.4.: **Reported cases of human African trypanosomiasis between 1927 and 2009.** Continued control efforts since the 1930ies led to near extinction of human trypanosomiasis by 1960. Conflicts and the belief that HAT had disappeared prevented further control efforts and led to new epidemics starting around 1970. Since the renewal of the control efforts from 1997 by WHO, fewer HAT cases are detected annually. Adapted from [150, 151]

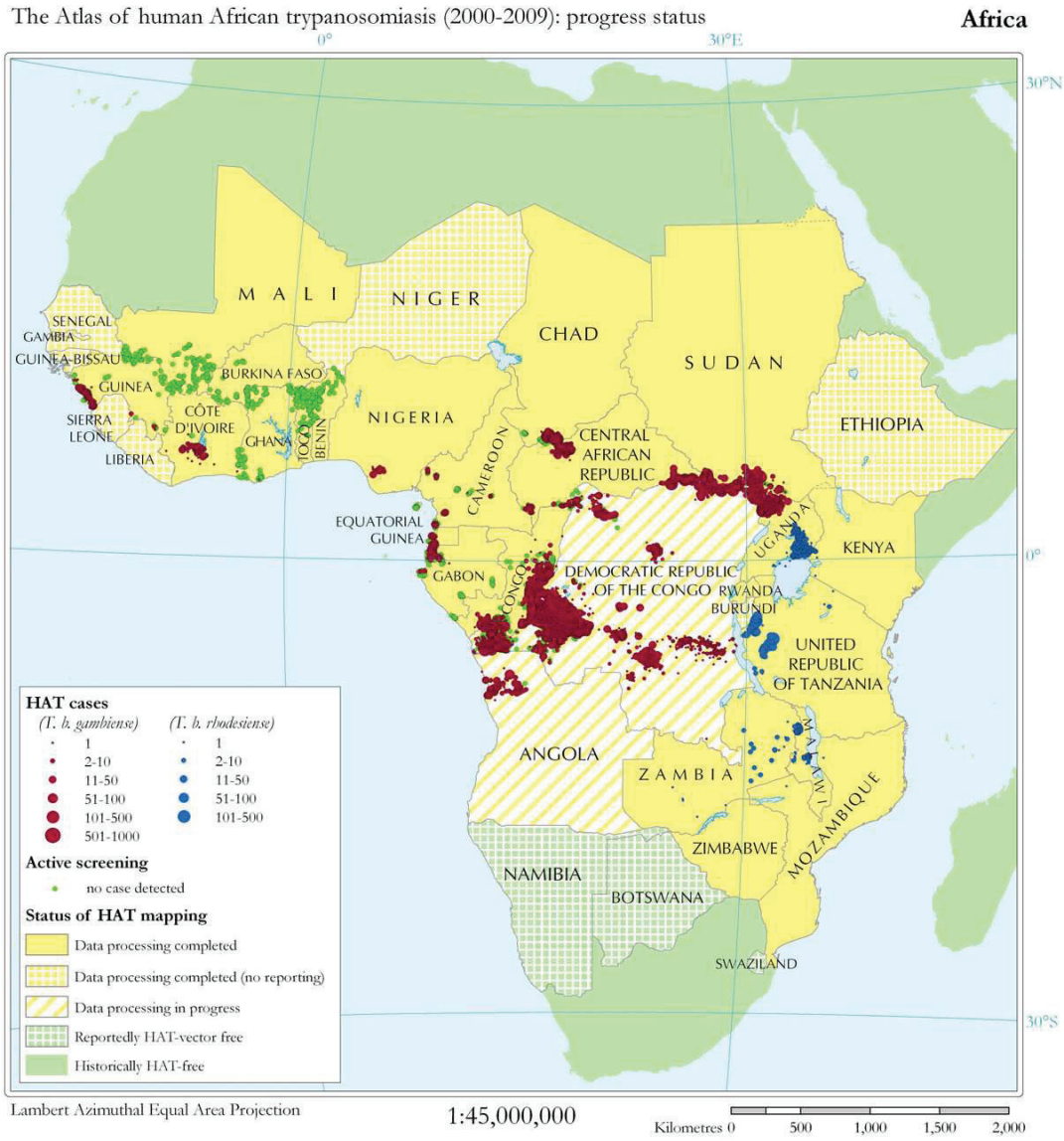

Figure 1.5.: **Distribution of reported HAT cases from 2000 to 2009.** Source: HAT atlas Simarro [149]

production, natural-resource utilization and the pattern of human settlement throughout sub-Saharan Africa [83]. Studies based on data of cattle distribution per district estimate that of the 150 million cattle in sub-Saharan Africa only 48 million are located in tsetse infested areas [100]. An earlier review by Hursey and Slingenbergh [83] estimated a much lower cattle density in tsetse infested areas with only 10 out of 165 million cattle in such zones. Cattle densities are highest in the moister subhumid areas, except where tsetse are present. In these tsetse infested areas cattle are maintained at the periphery in less favorable conditions (Figure 1.6, [83]).

Economic importance of HAT and AAT It is estimated that about 60 million people are continuously at risk in the 20 countries where active transmission of trypanosomiasis is considered to take place, but that only 3 to 4 million of these people are covered by surveillance. Despite the high number of HAT cases reported (Figure1.4) WHO estimates that these represent only 10-15% of the actual number of infected individuals thought to be around 300,000-500,000 HAT cases and 66,000 deaths annually [29, 110] with 100,000 people remaining disabled after a Trypanosoma infection. In villages in certain foci, the prevalence is higher than 70-80% [183]. In 2002 HAT caused the loss of approximately 1.5 million disability-adjusted life years (DALYs) resulting in estimated costs of about US$ 25.5 million [109]. Compared to malaria and AIDS there are relatively few HAT cases, but the disease is still considered a major public health risk. Because of the specific epidemiology of the disease, in the absence of effective surveillance, epidemic proportions are rapidly reached with a high case fatality rate [148]. The treatment of human African trypanosomiasis costs $300-500 per patient and the costs of prevention and control are still very high. Since 2000 and 2001 public-private partnerships between WHO, Sanofi-Aventis and Bayer HealthCare eased the situation by the creation of a WHO surveillance team, providing support to endemic countries in their control activities and the supply of drugs free of charge [183].
FAO has estimated that removal of trypanosomiasis could double livestock production and thus markedly increase cultivation levels in Sub-Saharan Africa. A study by Budd [22] estimated that annual losses in agricultural production are in the region of £3

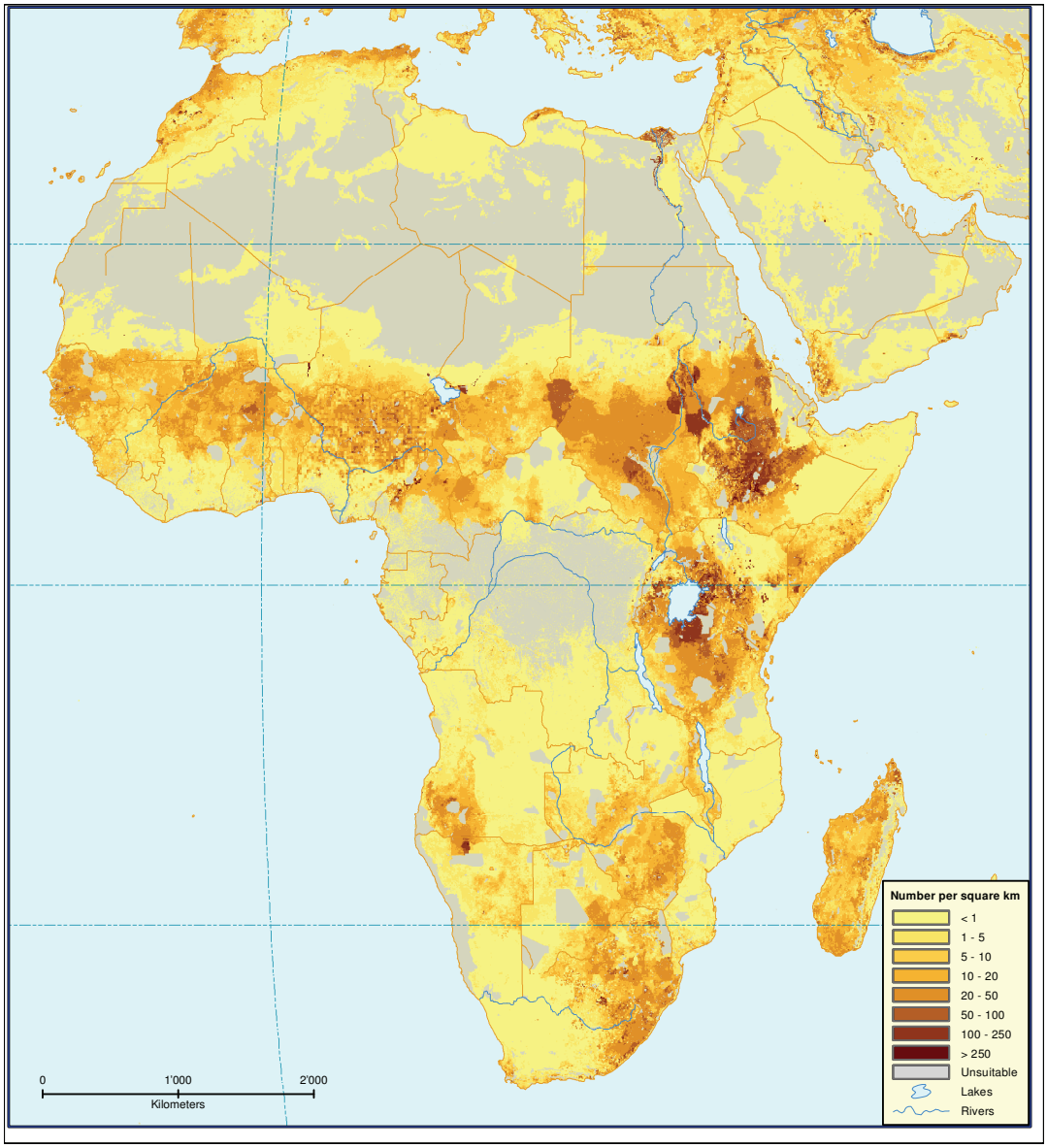

Figure 1.6.: **Cattle densities in Africa.** Data from PAAT-IS (http://www.fao.org/ag/paat-is.html, [30]

billion while the Programme Against African Trypanosomiasis (PAAT) estimates the loss in cattle production alone to be 1 to 1.2 billion US$. The indirect losses due to tsetse infestation are more difficult to estimate, but the establishment and maintenance of mixed-farming system, an important factor in agricultural expansion, in areas with a trypanosomiasis prevalence of more than 30% is virtually impossible [83]. Kristjanson et al. [100] estimate that in sub-Saharan Africa an increase in cattle population of 62% is feasible, which would yield an extra annual agricultural income of US$ 4500 million in the long run [22].

Control of trypanosomiasis Control of sleeping sickness is based on two different approaches. Firstly surveillance, control and treatment of trypanosomiasis, and secondly vector control. The transmission of trypanosomiasis involves four interacting organisms: the human host, the insect vector, the pathogenic parasite, and domestic and wild animal reservoirs. A reduction of the numbers of vectors should lead to significantly reduced transmission and hence contribute to the elimination of HAT and the sustainability of control efforts. Without immigration or reinvasion, sustained elimination of ~4% of the population would lead to extinction [76]. In the past, vector control has involved bush clearing (tsetse habitat destruction), elimination of wild animals (tsetse reservoir hosts), and extensive insecticide ground spraying. These interventions have been abandoned for ecological, environmental or health concerns relating to residual insecticides. Currently, vector control is achieved through sequential aerial spraying (SAT), ground spraying, insecticide-treated targets or insecticide-treated animals as live baits, the use of traps and the sterile insect technique (SIT). First attempts at controlling tsetse populations by mass trapping was on the island of Principe in the early 1900s using sticky traps carried on the backs of plantation workers [35]. Since then trapping techniques have greatly evolved by development of designs that mimic the fly's perception of their host, generally using blue and black cloth in monoconical or biconical shaped traps, thus funnelling the attracted flies upwards into a cone at the top of the devices. Targets are screens of suspended blue and black cloth impregnated with a biodegradable pyrethroid insecticide that flies pick up when they land on the target (Figure 1.7; [165]).

Figure 1.7.: **Example of a biconical trap (a) and a target (b) used to capture and control tsetse flies.**

1.2. Sensory ecology

All organisms depend on the ability to obtain and react appropriately to information from the environment. Sensory ecology deals with the understanding how organisms acquire sensory information and how they use this information to interact with their environment. Typical questions addressed in insect sensory ecology are: which visual and olfactory cues are used to find food, mates, oviposition sites, or shelter. Haematophagous insects are confronted with the problem that they have to locate their host hidden within an array of plants and other animals all emmiting olfactory and visual signals confusing the host signals. Some invertebrates rely on olfaction as a principal sense and their olfactory system evolved great sensitivity and specificity enabling them to identify minimal concentrations of essential chemical compounds, as is the case of *Bombyx mori* where a single pheromone molecule is sufficient to elicit a response in the male olfactory neuron [96]. Other invertebrates, e. g. diurnal haematophagous insects like the tsetse fly, combine visual and olfactory information to find an appropriate host [54, 169].

1.2.1. Sensory organs

Sensory organs transduce internal and external stimuli into electrical signals, feeding them into the central nervous system. There are several types of sensory receptor organs that can be classified according to their morphology and function. Mechanoreceptors monitor body or appendage orientation in space, serve as wind-speed indicators, perceive vibrations and proprioceptive information. There are thermo-, hydro-, and infrared receptors. Light receptors, like simple dermal light receptor cells, ocelli and compound eyes, detect the presence or absence of light and form images. Chemoreceptors can be divided into gustatory and olfactory receptors. While gustatory receptors perceive molecules in solution and are often concentrated on mouthparts, ovipositor and tarsi, olfactory receptors perceive airborne chemicals and are most often concentrated on the antennae.

Vision

Structure of the compound eye The compound eyes are composed of multiple functional units, the ommatidia. Each ommatidium is composed of the dioptic structures (cornea and cone), the photosensitive cells (retinular cells with the rhabdomere), and the shielding cells (pigment cells surrounding retinula cells and cone). In the apposition eyes of most diurnal insects the rhabdomere extends from the cone to the basement membrane and there is little to no movement of the pigment in the shielding cells so that light strikes the photosensitive pigment only when entering axially and is effectively shielded from light straying from adjacent ommatidia. The eye of the tsetse fly [75, 163] is very similar to those of other higher Diptera with the exception that the number of surrounding secondary pigment cells is unusually high at 16-18 cells. The unusually thick facet lenses with a weakly curved outer surface and a strongly convex inner surface give rise to to the characteristic striped reflections of tsetse eyes by total internal reflection in *G. morsitans morsitans* (Figure 1.8; [75]).

Tsetse fly responses to colour Colour vision is the ability to discriminate between two wavelengths of light, and has been extensively reviewed in Briscoe and Chittka [21]. Compound eyes of many insects are known to be sensitive to UV, blue and green wavelengths. Tsetse flies respond to strong contrasts of an object against the background [18, 54], but also appear to employ colour information. Spectral sensitivity curves of *G. m. morisitans* indicate a peak in the ultraviolet (350-365nm), a broad peak in the blue-green (450-550nm) and a secondary peak in the red (~625nm) [63, 65]. Green and Flint [66] recorded the behavioural responses of tsetse to 53 colours in the field and suggested that trap effectiveness depended mainly on reflectivity in four different wavelength bands, blue-green and red were correlated positively while ultraviolet and green-yellow-orange were correlated negatively with trap score. The best trap colour was royal blue, strongly reflecting blue-green but very little ultraviolet and green-yellow-orange, followed by black and white [66].

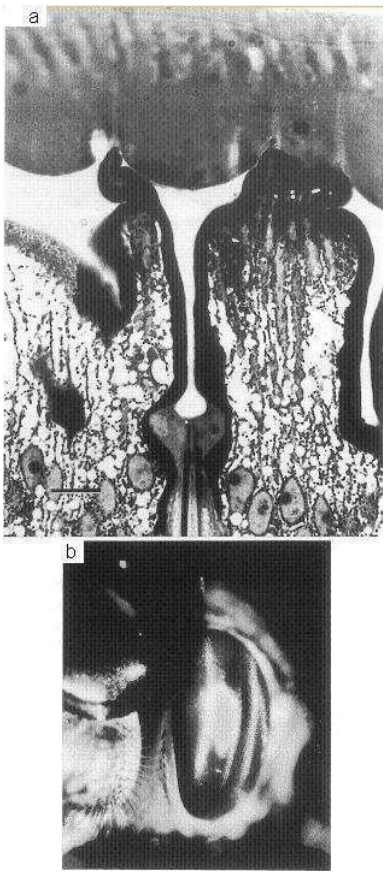

Figure 1.8.: **a) Radial section through the cornea and distal retina and b) the striped reflection pattern observed with oblique illumination of the eye of *G. morsitans morsitans*.** The radial section shows the thick corneal lens, curved inner surface, the typical dipteran open rhabdom arrangement and some secondary pigment cell nuclei. Scale bar: 10μm. Reprinted from the Journal of Insect Physiology, Vol 35, Hardie R., Vogt K. and Rudolph A., The compound eye of the tsetse fly (*Glossina morsitans morsitans* and *Glossina palpalis palpalis*), 423-431, Copyright (1989) [75], with permission from Elsevier (License number 2714260065545; Jul 22, 2011.)

Olfaction

Structure of the olfactory receptor cells Chemoreceptor neurons are bipolar (usually two to five) within a sensillum with the cell bodies located peripherally near the stimulus site (Figure 1.9). Characteristically, the dendrite of an olfactory receptor cell comprises a large inner segment connected by a narrow ciliary segment to a smaller outer process extending to the tip of the sensillum [122]. The external cuticular structure takes either the form of a hair (sensilla trichoidea), a plate (sensilla placodea), a peg (sensilla basiconica) or a peg-in-a-pit (sensilla coeloconica) and tends to be thin-walled with an inflexible cuticular socket. The cuticular walls contain multiple pores with microtubules leading from the inner sensillar wall of the pore inward, often making direct contact with the dentritic endings. The axons of olfactory receptor neurons (ORNs) are usually small and form the antennal nerves generally passing directly into the antennal lobe in the deutocerebrum without synapsing [96, 122].

Signal transduction Chemosensory neurons convert the information about quality, strength and duration of a chemical stimuli into electrical responses. Kaissling [93] postulated the following six steps as part of the process of semiochemical detection:

1) **Adsorption** of an odour molecule by sensory hairs on the antennae
2) **Penetration** of the molecule through pores in the setal wall
3) **Binding** of the molecule and transport to the sensory nerve endings
4) **Membrane alteration,** probably opening of K^+ and Ca^{2+} channels
5) **Receptor potential generation,** a graded potential, followed by spike generation in the axon hillock region
6) **Inactivation** of the odour molecule and removal in the sensillar lymph.

After entering through wall pores of the sensillum the mostly hydrophobic volatile molecules are bound to water-soluble odorant binding proteins (OBPs), which transport the volatile molecule through the aqueous sensillar lymph to the olfactory receptor cells (ORs). The OBPs not only are carriers of the odour molecules, but also appear to make a first selection of olfactory information [107] and protect the molecules from degradation [176]. The interaction of the odour molecules with the ORs triggers intra-

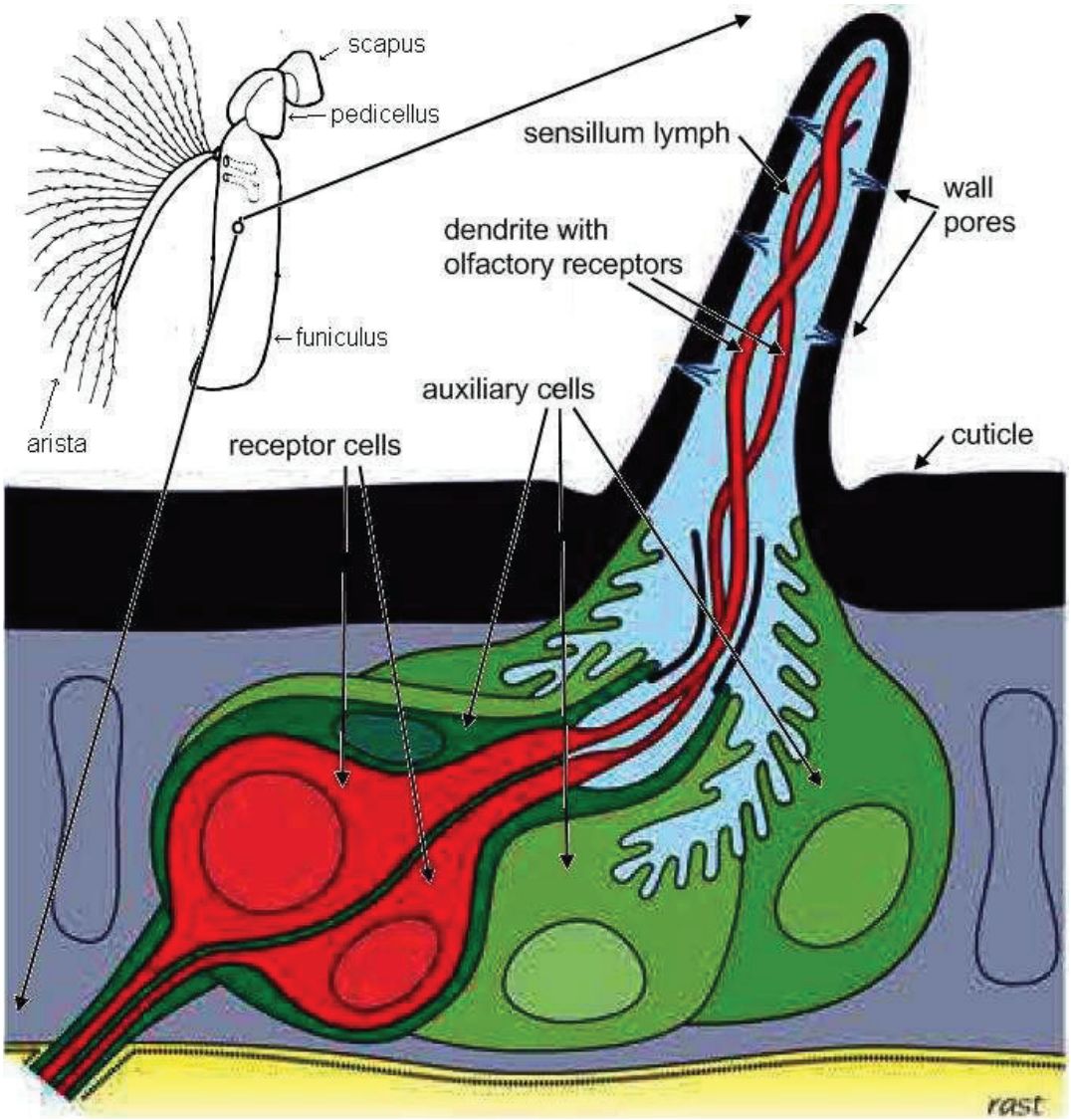

Figure 1.9.: **Schematic diagram of an olfactory sensillum trichoideum with two receptor cells (red) and three auxiliary cells (green).** To the left a schematic diagram of a tsetse fly antenna. Adapted from Kaissling [96] and Pollock [136].

cellular G protein-coupled reaction cascades resulting in the opening of ion channels in the plasma membrane. The influx of K^+ and Ca^2+ ions from the sensillar lymph into the ORs cause a depolarization of the membrane potential. When the potential exceeds a certain threshold, the graded response is changed into an all-or-none event, the action potential (AP). The amplitude of the AP of one ORN may not change with the strength of the odour signal, but the frequency of APs does, either augmenting in an excitatory neuron or decreasing in an inhibitory neuron. The action potentials are propagated without decrement along the axons to the antennal lobe where the ORs converge on glomeruli. Each glomerulus receives information from only one class of olfactory receptor neuron expressing one OR, resulting in a specific pattern of activated glomeruli in the antennal lobe for a particular odour [85]. This odor-specific map is then decoded in higher brain centers to induce a behavioural response.
The electroantennogram (EAG) introduced by Schneider [145] allows the measurement of the electrophysiological response of insect antennal receptor cells to stimulation with an odour (equivalent to the olfactogram in vertebrates). The EAG can be used to readily test compounds or their mixtures as olfactory stimulants and to investigate stimulus-response characteristics of insect olfactory receptor cells. The EAG is derived from the receptor potentials of a population of activated olfactory receptor cells across the antenna and is measured in mV of depolarization (DC). When the antenna is set up at the outlet from a gas chromatographic column it can be used to locate biologically active constituents in odour extracts that are separated on the column. A second method of recording from olfactory sensilla of insects is by single sensillum recordings (SSR), where the potential change of the sensillum lymph versus the hemolymph, that constitutes the receptor potentials (DC), and resulting action potentials (AC) of single receptor cells are measured [94].

Stimulus volatility Compounds used for olfactory chemical communication need to be volatile in order to readily travel in turbulent air to transport the actual signaling components to an insect olfactory receptor cell. The volatility of a compound depends on its molecular weight, polarity, and structure of the molecule and is expressed in vapour

pressure [99]. Molecules with a high molecular weight and high polarity have low vapour pressures while apolar molecules with low molecular weights are highly volatile [147]. The volatility of a molecule not only affects the evaporation rate but also its dilution in air and therefore the time the signal persists in the environment. Without any air movement, small molecules have diffusivity speeds in air of approximately $2 \times 10^{-}5m^2/s$ [44], a value that decreases for larger molecules. It is estimated that characteristic diffusion distances in air are 7cm in one minute, 2.6m in a day and 50m in a year, making clear that diffusion is a slow process, not suitable for transmission over long distances [44]. However, air movements greatly affect the transport of volatile compounds and wind carries them readily over large distances in discrete packages of odour molecules forming an odour plume [117–119].

1.2.2. Host detection by tsetse flies

Different sensory modalities such as olfaction and vision intervene depending on the distance between the tsetse fly and its host. Since chemical cues are carried by wind it is estimated that olfactory perception is extended to 60-120m downwind from the host [169] whereas within a range of less than 20m host visual cues predominate [54]. Since the beginning of the 20th century when researchers revealed the critical importance of olfaction in host seeking behaviour by tsetse flies [56] efforts were made to identify the attractive chemical cues emanating from hosts with a view to improve the efficiency of visual traps [167, 168]. Host odours originate in 3 different ways: in breath, sweat and excretions (urine and faeces). CO_2 alone, present in host breath, attracts *G. pallidipes* [139]. Further studies showed that CO_2 elicits upwind anemotaxis in *G. morsitans morsitans* [32, 132]. *G. morsitans morsitans* and *G. pallidipes* are attracted in the field by other volatile organic compounds (VOCs) present in breath such as acetone and 1-octen-3-ol [23, 73]. These substances also increase the number of upwind turns made by *G. morsitans morsitans* in wind tunnel experiments [131] and affects the direction of flight in the field and in wind tunnel experiments [25, 180]. The odour of whole bovine rumen fluid attracted *G. pallidipes*, inducing responses in the wind tunnel, whereas the acidic fraction of the rumen fluids induced only low response levels [79]. In contrast to

breath, the role of ox sebum in the sensory ecology of tsetse flies is unclear. Sebum produced variable results in catching *G. morsitans morsitans* in Zimbabwe and in inducing landing responses on a $1m^2$ black cloth target [179, 181]. Tsetse flies respond positively to aged bovine urine [128]. Indeed, a mixture of *p*-cresol and 3-*n*-propyl phenol, isolated from urine, act synergistically to attract tsetse flies [27, 130]. These 2 substances, in addition of 1-octen-3-ol and acetone, are constituents of a blend of chemicals that enhance catches of visual traps and targets: traps baited with a blend of these chemicals are highly effective for species found in woodland savannah (*Glossina* subgenus) [23, 64, 73, 91, 152], and were recently found to be effective for species found in lowland rainforests and gallery forests (*Austenina* subgenus) [23, 153] as well as for riverbank and lakeshore lowland rain forest species (*Nemorrhina* subgenus) [111, 127, 138, 152].

1.3. The role of plants in the life of tsetse flies

Plants not only provide the habitat of *fusca* and *palpalis* spp. (see 1.1.2) but they also provide shelter in all biotopes tsetses occupy, permitting flies to mature, digest and gestate under appropriate conditions. Without the use of shelter the fat reserves of the flies are depleted faster and the surviving generations are characterized by individuals of smaller size [134, 135]. Plants also provide females with sunlight-protected places for larviposition, as the larvae need humid but not damp soil to pupate [28, 166]. Indeed, the degree of vegetation cover has been related to survival and population densities of different tsetse species [140].

Little attention has been paid to the flies' propensity for finding suitable cover under vegetation until now. Keeping in mind the role of peridomestic resting sites, it is important to consider volatile chemicals derived from plants for the development of new odour-baited traps to attract tsetse from all 3 subgenera. A first attempt to investigate the perception of plant compounds by tsetse flies from all 3 subgenera was made in this laboratory by Syed and Guerin (2004) [157] who showed that tsetse flies perceive mono- and sesquiterpenes, lipoxidation products and aromatics from both leaf and flower extracts of *Lantana camara* with the major chemostimuli for the antennal receptor

cells being 1-octen-3-ol and β-caryophyllene. Further, volatiles from these extracts such as 1-octen-3-ol alone, 1-octen-3-ol in combination with β-caryophyllene, and these two compounds together with (+)-camphor, (+)-α-terpineol, (-)-bornyl acetate, eugenol and citral in a 7-component mixture were attractive in a wind tunnel to *G. pallidipes*, *G. brevipalpis* and *G. f. fuscipes*. It is possible that the tsetse flies make parsimonious use of chemostimuli (e.g. 1-octen-3-ol present in both host odours and plant volatiles) for host finding and shelter under plants. Further, S. Mohottalage identified chemostimuli in essential oils of 11 plants for *G. pallidipes* [114] using the EAG linked to a gas chromatograph to locate the biologically active constituents. The EAG-active compounds on *G. pallidipes* antennae were grouped according to their chemical structure: 12 monoterpene hydrocarbons including limonene, 31 oxygenated monoterpenes, 19 sesquiterpene hydrocarbons, 15 oxygenated sesquiterpenes, 11 phenolic compounds, 9 oxygenated long and short chain hydrocarbons, and the bicyclic β-diketone.

1.3.1. Green leaf volatiles

A major group of compounds released by all higher green plants are six-carbon alcohols, aldehydes and esters produced through the lipoxygenase pathway, together with other short-chain aliphatic acids [146]. They are generally referred to as green leaf volatiles (GLVs) and smelled by man as grass-like odours. Despite the fact that the compounds are produced by many plant species, the quantitative differences of GLVs in plant odour bouquets can convey specific information to the arthropods [173]. GLVs are known to attract phytophagous insects to their host plants as well as to attract parasitoids to plants infested by phytophagous species [42]. Steullet and Guerin [155] found olfactory receptor cells responding to hexanal and (E)-2-hexenal on the tarsi in *Amblyomma variegatum* while (Z)-3-hexenal and (E)-2-hexenal did not evoke any responses from olfactory cells in basiconic and grooved-peg sensilla of *Triatoma infestans* nymphs [69]. Moreover Syed and Guerin [157] recorded EAG responses from flies from all three tsetse fly habitats to (E)-2-hexenal.

1.3.2. Terpenes and their derivatives

Terpenes represent the most predominant group of chemostimuli from plants and are known to have a wide range of functions throughout the Plant and the Animal kingdom. They are produced as secondary metabolites and act as defensive substance in plants and animals [2, 80], are used by plants to deter herbivores or to inform conspecifics of attack [80, 97, 141], or to attract natural enemies of phytophagous insects [12, 80, 97]. The latter largely exploit terpenes for host seeking. Plant hormones as well as the steroid hormones of mammals are derivates of terpenes [80]. Terpenes have a unique structure and consist of an integral number of isoprene units. They are classified according to their number of these 5-carbon units into monoterpenes (C-10), sesquiterpenes (C-15), diterpenes (C-20), triterpenes (C-30), tetraterpenes (C-40) and polyterpenoids (>40C). Terpenes are the main components of the essential oils of plants and are used in the cosmetic and perfume industries. Terpenes are defined to be primary derivatives of isoprenic units with a biological function whereas terpenoids are secondary or condensed mevalonate metabolites with additional groups, such as more complicated derivatives of sesqui-, di- and triterpenes. One of the main sources of terpenoids in industry are pine species, largely available in conifer plantations that serve as new habitats of several tsetse species [62, 162]. Pine as well as cocoa and coffee plantations represent an important economic resource in Africa and have been invaded by teneral tsetse flies [126] and are also used as peridomestic breeding sites by *G. f. fuscipes* [125]. Terpenes are also known to be attractive to several haematophagous insects such as mosquitoes [14] and sand flies [43], to be perceived by triatomine bugs [67] and to be present in the urine and breath of humans [133] and ruminants [87, 154], known tsetse hosts. Harraca et al. [79] identified a range of volatile compounds in bovine rumen fluids including carboxylic acids (unbranched and branched C2 – C7 acids), terpenes (limonene, camphor, β-caryophyllene, iso-caryophyllene, β-selinene, β-farnesene and germacrene D), *p*-cresol and indoles (indole and skatole) that induced EAG responses from *G. pallidipes*, *G. f. fuscipes* and *G. brevipalpis*.

1.3.3. Research interest and outline of the thesis

Tsetse flies are an important vector of disease throughout sub-Saharan Africa (Section 1.1.3), and use visual as well as olfactory cues to locate hosts (Section 1.2.2). Control methods include baited traps and targets. The most used bait is a mixture of two phenols and 1-octen-3-ol, sometimes in combination with acetone. Major drawbacks of these baits are insufficient effects on several key HAT vector species, mainly riverine tsetse, the high toxicity of the two phenols and the difficult and expensive synthesis of 3-*n*-propylphenol [38, 164]. We have some evidence that volatile plant compounds are perceived by tsetse flies [114, 157] and could be used in a parsimonious way during host finding and the localization of larviposition and resting sites [106, 120].

The aim of this thesis was to improve our knowledge of odour perception in tsetse flies, its implication in their flight behaviour and the identification of new semiochemicals derived from plant secondary metabolism. A practical outcome of this thesis would be the development of new, relatively inexpensive and environmentally safer baits based on secondary plant compounds that could be directly produced on site in Africa.

First, I had to provide evidence that tsetse flies are capable to perceive volatile plant compounds. In chapter 2 we recorded electroantennogram responses from tsetse fly antennae in species from all three habitats to identify possible candidates for behavioural assays.

Secondly, after the identification of candidate plant compounds, the behavioural responses of tsetse flies to these volatile compounds were recorded in a wind tunnel (chapter 3). In addition, behavioural experiments were made to study the effects of mixtures of volatile plant compounds and to study the effects of volatile plant compounds on the attractiveness of a visual target.

2. Sensory responses of tsetse flies *Glossina spp.* to plant compounds

2.1. Introduction

Olfaction plays a role in long range host finding in tsetse flies. They are estimated to be able to detect host odours up to 120m downwind of the target [169]. A variety of traps and targets has been designed for tsetse flies, based on shape, colour perception and contrast with the background [64, 168]. The development of odour baits improved the efficacy of traps and targets significantly by increasing the attractiveness and trap entry of these devices [33, 54, 73, 165, 167]. The major olfactory stimulants used in traps for tsetse flies are mixtures of acetone, 1-octen-3-ol, 3-*n*-propylphenol and *p*-cresol, originating from oxen [27, 73, 82, 127, 138, 172]. These currently used odour attractants do not consistently increase the catches of most of the *palpalis* and *fusca* group species, but work well for most of the *morsitans* species [64, 121, 127, 138, 153]. Plant volatiles might play a role in tsetse sensory ecology, being present in host odours, as well as to provide tsetse flies with cues to find their resting and larviposition sites. What cues, apart from visual ones, tsetse flies use to find such sites remains unclear, but the perception of temperature, humidity and plant odours could well be of importance. Research on the perception of plant derived volatile chemicals by tsetse flies has been sparse, concentrating mainly on host derived chemicals [52–54, 73]. To date Syed and Guerin [157] and Mohottalage [114] have been the only investigators to systematically investigate the perception of plant derived volatiles by tsetse flies. Plant derived volatiles are of a ubiquitous nature, found in the tsetse fly habitats as well as in host odours and

could represent products able to modify the behaviour of tsetse flies. Furthermore, they could be easily produced at low costs by distillation in African countries. We decided to investigate the perception of plant compounds, mainly low molecular weight products, including monoterpenes and green-leaf volatiles with a view to identifying new chemical stimulants for tsetse species.

2.2. Material and Method

Biological material *G. pallidipes* pupae were supplied by the International Atomic Energy Agency (IAEA, Vienna, Austria), *G. p. gambiensis* by the Centre International de Recherche-Développement sur l'Elevage en Zone Subhumide (CIRDES, Bobo-Dioulasso, Burkina Faso), *G. f. fuscipes* by the Department of Entomology of the Slovak Academy of Science (Bratislava, Slovakia) and *G. brevipalpis* pupae by the Agricultural Research Council of the Onderstepoort Veterinary Institute in Pretoria (South Africa). Flies were kept in a climate chamber (10h light, 25°C, 85% R.H., 14 h dark, 22°C, 85% R.H.). Pupae were held at 90% R.H. in plastic boxes until emergence and every 24 hours sexes were separated into rectangular cotton netting cages (1mm mesh, 25x15x15cm) and 2 to 4 day-old teneral individuals were used for EAG and GC-EAG recordings.

Electrophysiological recordings The tsetse fly antenna was mounted as in Guerin and Visser [71] and EAG responses were either recorded as such or the antennal preparation was used as a biological detector linked to a gas chromatograph (GC-EAG; [58]). In this case the EAG responses were recorded in tandem with the flame ionisation detector of the GC (5300, Carlo Erba Instruments) to locate biologically active constituents in odour extracts and in mixtures of volatiles. The identity of the compounds was verified using gas chromatography-mass spectrometry (GC-MS) (Faculty of Science, Analytical Service, University of Neuchâtel) and by comparison of Kovat's retention indices of biologically active constituents of test products compared to those of standards. The identity of the constituents of therebenthine esserect (Givaudan, Switzerland) were identified by R. Clery (Givaudan, Switzerland). Two apolar capillary columns were used for

the analyses of the terpene and green leaf volatile mixtures by GC-EAG and by GC-MS (for details of compounds analysed see next paragraph): a ZB 5 capillary column (L 30 m, ID 0.32, film thickness 0.25μm, Phenomenex, USA) was used for the analysis of mixtures of volatiles by GC-EAG with *G. pallidipes* and *G. brevipalpis* antennae and a DB 5 capillary column (L 15 m, ID 0.25 mm, film thickness 0.1μm, BGB Analytik, Switzerland) for the analysis of L-turpentine by GC-EAG with the antennae of *G. f. fuscipes* and *G. p. gambiensis*. For EAG recordings without the GC, the vapours of test substances were delivered from a 5ml polypropylene syringe (BD Plastipak., Spain) containing a filter paper strip (0.8cm x 3cm) impregnated with 10μl of a solution containing the test substance at 10ng/μl, 100ng/μl, 1μg/μl and 10μg/μl in dichloromethane (DCM, analytical grade $\geq$99.8%, Merck). Puffs (1ml/sec) of odour were injected into a humidified air stream (95% RH, 1m/sec) passing over the fly's antenna. The EAG responses to test products were normalised using an air puff (1ml/sec) passing over a filter paper strip impregnated with 10μl of a solution containing 1-octen-3-ol (50:50 R/S racemic, >97% pure, Merck, Germany) at 100ng/μl in DCM as described in Harraca et al. [79].

Dose response curve The GC-EAG responses were quantified and normalised using the response to a pulse of 1-octen-3-ol (see above) at the beginning and the end of each recording and calculated using the following equation:

$$d_x + \left[\left(\frac{d_e - d_s}{t_e - t_s}\right) * (t_x - t_s)\right]$$

with d = depolarisation in mV, t = time of depolarisation, x = compound tested, s and e = pulse of 1-octen-3-ol at the beginning and the end of the recording [78]. The EAG responses were normalised using a pulse of 1-octen-3-ol (see above) at the beginning and the end of each recording, consisting of a series of test compounds at one dose and calculated using the following equation:

$$\frac{d_{x1} + d_{x2}}{d_e + d_s} * 100$$

with d = depolarisation in mV, $x1$ = compound tested, first pulse, $x2$ = compound tested, second pulse, s and e = pulse of 1-octen-3-ol at the beginning and the end of the recording [78]. Normalised EAG dose response curves were then fitted by log-linear regression using the statistical package `R` (Version 2.12.0).

Compounds tested A total of 26 standard compounds of plant origin were tested in the EAG and GC-EAG assays. Detailed information on the single compounds – name, mass formula, CAS number, and origin – are summarized in Table 2.1. L-Turpentine was from Sigma-Aldrich, Switzerland, therebenthine esserect from Givaudan, Switzerland. Additionally, essential oils from *Pinus silvestris, Pinus pumiliones, Callitris colmellaris, Angelica archangelica, Abies alba, Thuja sp.* (from local stores) and from Tea tree oil (from Sigma-Aldrich, CH), and 3 terpene mixtures including lime dienes (10% in triethylcitrate (T.E.C.) from Givaudan), dipentene 10 and dipentene 38D (from D.R.T., France) were also tested. Furthermore, 2 phenols, *p*-cresol (> 98%, Fluka, CH) and 3-*n*-propylphenol (> 99%, Ubichem Research Ltd, Hungary), known to be behaviourally active for several tsetse species, were tested on *G. swynnertoni*.

Table 2.1.: **List of compounds tested in electrophysiological assays.**

Class	Compound	MF	CAS number	Provider
monoterpenes	α-pinene	$C_{10}H_{16}$	80-56-8	Givaudan, CH
	β-pinene	$C_{10}H_{16}$	127-91-3	Givaudan, CH
	camphene	$C_{10}H_{16}$	79-92-5	Givaudan, CH
	ocimene	$C_{10}H_{16}$	13877-91-3	Givaudan, CH
	myrcene, >90%	$C_{10}H_{16}$	123-35-3	Givaudan, CH
	dipentene	$C_{10}H_{16}$	138-86-3	Givaudan, CH
	γ-terpinene	$C_{10}H_{16}$	99-85-4	Givaudan, CH
	terpinolene	$C_{10}H_{16}$	586-62-9	Givaudan, CH
	S-(-)-limonene, ≥97%	$C_{10}H_{16}$	5989-54-8	Fluka, CH
	3-carene, ≥99%	$C_{10}H_{16}$	13466-78-9	Fluka, CH
aromatics related to monoterpene	*p*-cymene	$C_{10}H_{14}$	99-87-6	Givaudan, CH
	p-cymene, >95%	$C_{10}H_{14}$	99-87-6	drt, France
	diphenyl methane	$C_{13}H_{12}$	101-81-5	Givaudan, CH
monoterpene alcohols	terpineol pure	$C_{10}H_{18}O$	8000-41-7	Givaudan, CH
	tetrahydro myrcenol	$C_{10}H_{22}O$	18479-57-7	Givaudan, CH
polyunsaturated hydrocarbon	undecatriene	$C_{11}H_{18}$	16356-1-9	Givaudan, CH
sesquiterpenes	longifolene	$C_{15}H_{24}$	475-20-7	Givaudan, CH
	β-caryophyllene	$C_{15}H_{24}$	87-44-5	Givaudan, CH
green leaf alcohols	1 hexanol, >99%	$C_6H_{14}O$	111-27-3	Fluka, CH
	Z-3-hexenol, >98%	$C_6H_{12}O$	928-96-1	Fluka, CH
	E-2-hexenol, >95%	$C_6H_{12}O$	928-95-0	Fluka, CH
	1 heptanol, >99%	$C_7H_{16}O$	111-70-6	Fluka, CH
	1-octen-3-ol, >97%	$C_8H_{16}O$	3391-86-4	Fluka, CH
green leaf aldehydes	hexanal, >99%	$C_6H_{12}O$	66-25-1	Fluka, CH
	E-2-hexenal, >99%	$C_6H_{10}O$	6728-26-3	Sigma-Aldrich, CH
	Z-3-hexenal	$C_6H_{10}O$	6789-80-6	Oril SA, France
green leaf ester	Z-3-hexen-1-yl-acetate, >98%	$C_8H_{14}O_2$	3681-71-8	Sigma-Aldrich, CH

2.3. Results

2.3.1. GC-EAG analysis of L-turpentine(Sigma) and therebenthine esserect (Givaudan)

The compounds in L-turpentine were identified as α-pinene, β-pinene, camphene, *p*-cymene, 3-carene, and limonene (Table 2.2, Figure 2.1). While *G. brevipalpis* antennae showed EAG responses to α- and β-pinene, camphene, and limonene the antennae of *G. pallidipes* responded consistently only to limonene (Figure 2.1). Similar EAG responses were recorded for *G. brevipalpis* and *G. pallidipes* to therebenthine esserect. Compared to L-turpentine, therebenthine esserect contains not only monoterpenes, but also sesquiterpenes, terpene alcohols, -oxides, and -esters (Table 2.3). To detect compounds present in low quantities a ten times higher amount than for L-turpentine was injected. At these higher doses, *G. pallidipes* antennae responded to α- and β-pinene, limonene, and β-caryophyllene and humulene in therebenthine (Figure 2.2). *G. brevipalpis* antennae responded additionally to α-phellandrene, *p*-cymene, α-copaene, and δ-cadinene (Figure 2.2).

2.3.2. GC-EAG analysis of green-leaf volatiles

The EAG responses of *G. pallidipes* and *G. brevipalpis* to seven green-leaf volatiles (C_6-aldehydes, -alcohols, and an ester), 1-heptanol and 1-octen-3-ol were recorded (Figures 2.3 and 2.4). Products tested are listed in Table 2.1. Of the aldehydes only E-2-hexenal consistently evoked EAG responses from both species at about 50% of the response to 1-octen-3-ol at the same source dose. At the lowest dose tested no other aldehyde or the ester evoked EAG responses from either *G. pallidipes* and *G. brevipalpis*. Of the alcohols 1-heptanol evoked an EAG response from *G. pallidipes* and *G. brevipalpis* at about 30-60% of the response to 1-octen-3-ol at the same source dose while E2-hexenol evoked only a small response and 1-hexanol and Z3-hexenol none.

Table 2.2.: **Compounds and their relative abundance present in L-turpentine (Sigma-Aldrich, see M&M, N = 3)**. Kovat's retention indices established for standards and for compounds present in L-turpentine. Relative abundance [%] established by gas-chromatography.

	Kovat's retention index		
Compound	**standard**	**L-turpentine**	[%]
α-pinene	880	883	54.33
camphene	895	899	1.80
β-pinene	924	924	29.83
3-carene	954	955	2.55
S-(-)-limonene	975	975	11.07
		total identified	99.57

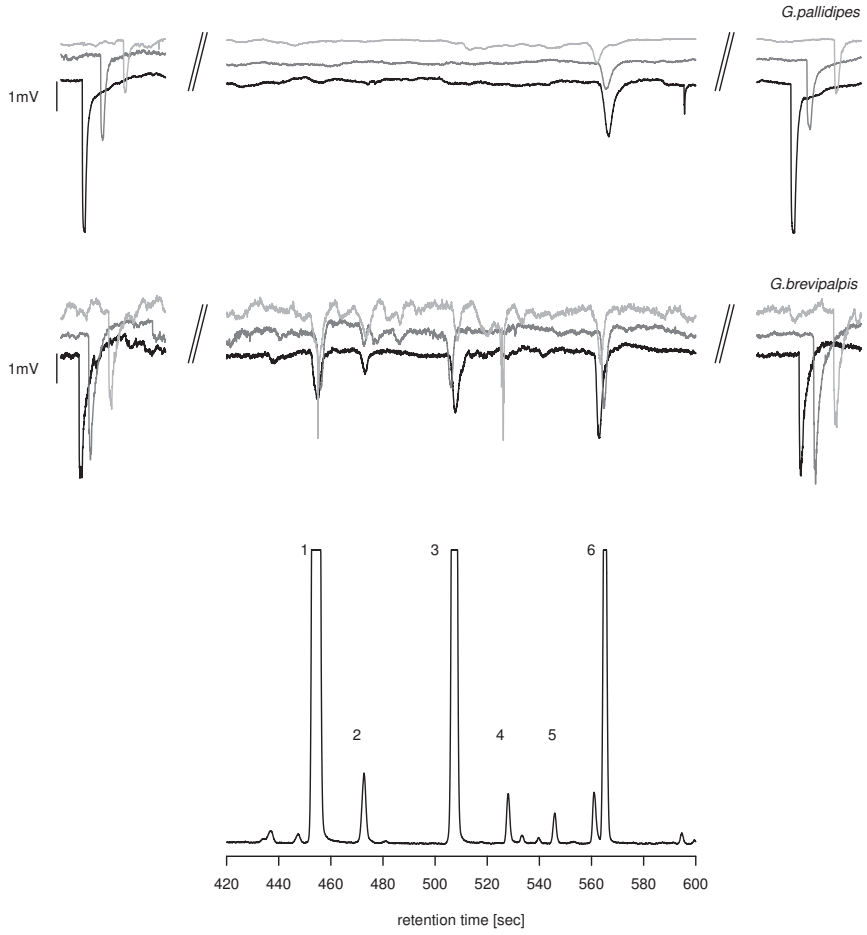

Figure 2.1.: **GC-EAG analysis of L-turpentine with the antennae of *G. pallidipes* and *G. brevipalpis* as biological detectors.** The upper traces are the EAG responses of each of three *G. pallidipes* and *G. brevipalpis* antennae, and the lower one the FID trace of the gas chromatograph. At the beginning and the end of the GC-EAG recording the EAG responses to a puff of air over 1μg of 1-octen-3-ol on a filter paper strip in a stimulus syringe were recorded as a reference. Peaks identified are 1) α-pinene, 2) camphene, 3) β-pinene, 4) *p*-cymene, 5) 3-carene and 6) limonene.

Table 2.3.: **Compounds and their relative abundance present in therebenthine esserect**. Identifications by R. Clery (Givaudan, Switzerland) by GC-MS. Relative abundance (% RPA) and retention times (RT) established by gas-chromatography.

Compound	**RT**	**% RPA**	**Compound**	**RT**	**% RPA**
tricyclene	19.65	0.06	(Z)-pinocarveol	35.13	0.07
α-thujene	19.93	0.03	borneol	37.06	0.09
α-pinene	20.54	72.77	terpinen-4-ol	37.85	0.03
α-fenchene	21.32	0.04	p-cymen-8-ol	38.27	0.06
camphene	21.47	0.87	α-terpineol	38.75	0.88
thuja 2,4(10)-diene	21.82	0.04	estragol	39.23	0.06
β-pinene	23.45	14.11	bornyl acetate	45.4	0.11
myrcene	24.26	0.81	α-cubebene	49.79	0.12
α-phellandrene	25.31	0.04	α-longipinene	50.02	0.13
1,4-cineole	26.05	0.03	α-copaene	51.61	0.19
α-terpinene	26.19	0.03	longifolene	53.71	1.04
limonene	27.08	3.23	caryophyllene	54.5	2.07
γ-terpinene	29.21	0.03	humulene	56.61	0.25
terpinolene	31.41	0.61	α-muurolene	59.32	0.04
α-pinene oxide	32.18	0.04	δ-cadinene	60.68	0.23
α-fenchol	33.26	0.07	caryophyllene epoxide	64.42	0.16
				total identified	98.34

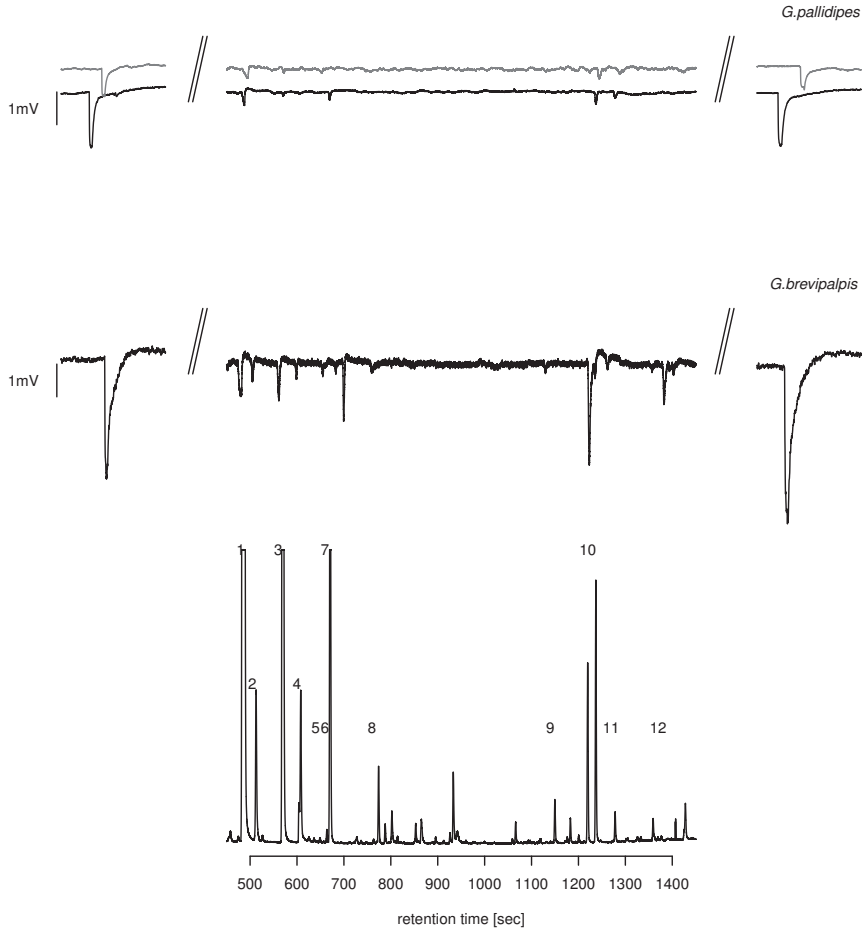

Figure 2.2.: **GC-EAG analysis of therebenthine esserect with the antennae of *G. pallidipes* and *G. brevipalpis* as biological detectors.** The upper traces are the EAG responses of *G. pallidipes* and *G. brevipalpis*, and the lower one the FID trace of the gas chromatograph. At the beginning and the end of the GC-EAG recording the EAG responses to a puff of air over 1μg of 1-octen-3-ol on a filter paper strip in a stimulus syringe were recorded as a reference. EAG-active constituents identified are 1) α-pinene, 2) camphene, 3) β-pinene, 4) myrcene, 5) α-phellandrene, 6) *p*-cymene, 7) limonene, 8) terpinolene, 9) α-copaene, 10) β-caryophyllene, 11) humulene and 12) δ-cadinene.

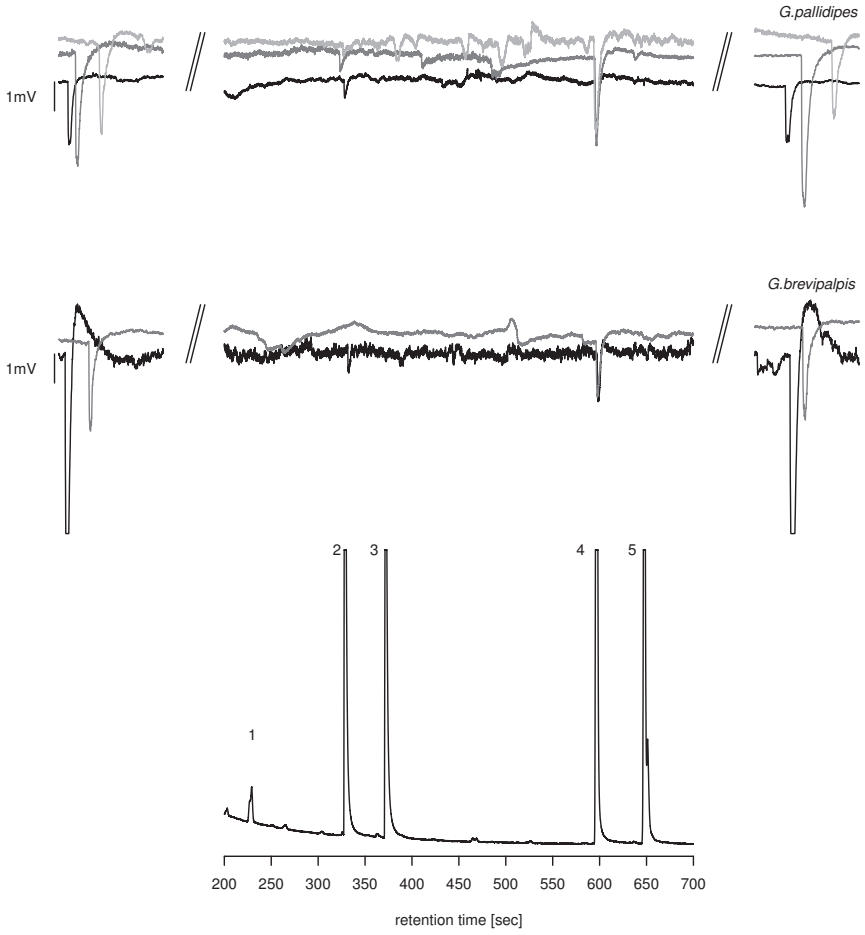

Figure 2.3.: **GC-EAG analysis of a mixture of 4 green leaf volatiles (GLVs) and 1-octen-3-ol with the antennae of *G. pallidipes* and *G. brevipalpis* as biological detectors.** The upper traces are the EAG responses of *G. pallidipes* and *G. brevipalpis*, and the lower one the FID trace of the gas chromatograph. At the beginning and the end of the GC-EAG recording the EAG responses to a puff of air over 1μg of 1-octen-3-ol on a filter paper strip in a stimulus syringe were recorded as a reference. Peaks correspond to 1) Z-3-hexenal, 2) E-2-hexenal, 3) 1-hexanol, 4) 1-octen-3-ol and 5) Z-3-hexen-1-yl-acetate.

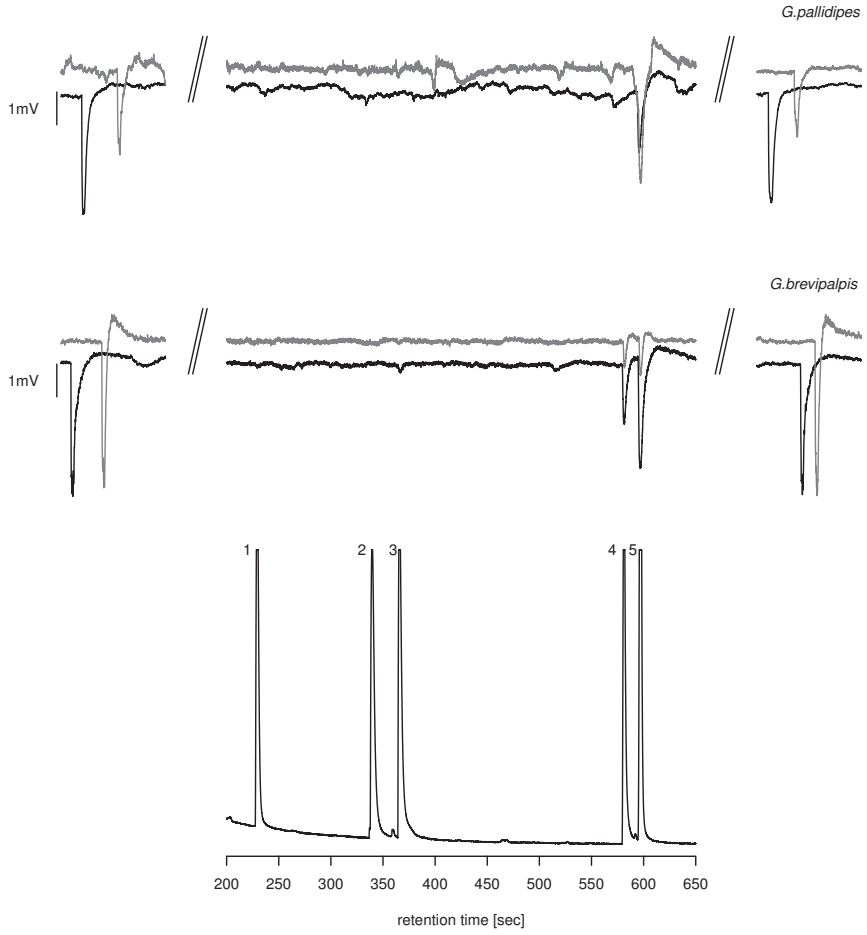

Figure 2.4.: **GC-EAG analysis of a mixture of 3 green leaf volatiles (GLVs), 1-heptenol and 1-octen-3-ol with the antennae of *G. pallidipes* and *G. brevipalpis* as biological detectors.** The upper traces are the EAG responses of *G. pallidipes* and *G. brevipalpis*, and the lower one the FID trace of the gas chromatograph. At the beginning and the end of the GC-EAG recording the EAG responses to a puff of air over 1μg of 1-octen-3-ol on a filter paper strip in a stimulus syringe were recorded as a reference. Peaks correspond to 1) hexanal, 2) Z-3-hexenol, 3) E-2-hexenol, 4) 1-heptanol and 5) 1-octen-3-ol.

2.3.3. Dose dependent EAG-responses of tsetse spp. to volatile plant compounds

EAG-responses of the *palpalis*-group flies *G. f. fuscipes* and *G. p. gambiensis* to plant volatiles

EAG-responses to turpentines, monoterpenes present in turpentines, and to β-caryophyllene EAG-responses were recorded to all products at source doses of 100ng, 1μg, 10μg and 100μg (Figure 2.5). At lower source doses (100ng and 1μg) only the responses to L-turpentine were above the response threshold to the solvent for *G. f. fuscipes* and *G. p. gambiensis*, with a response approaching that to 1-octen-3-ol at the same source dose (Figures 2.5a&b). The overall responses were the same for both *G. f. fuscipes* and *G. p. gambiensis* with the best response to L-turpentine at 1 and 10μg source doses. Equivalent responses were recorded to the monoterpenes at 100μg source dose and the weakest response was to β-caryophyllene. Generally, the EAG depolarisations of *G. f. fuscipes* were stronger (in mV) than the responses of *G. p. gambiensis* .

EAG-responses to monoterpenes, longifolene, diphenyl methane, and undecatriene These 11 substances were only tested using the EAG responses of *G. f. fuscipes* antennae (Figure 2.6). Only ocimene approached the response to 1-octen-3-ol at the 1μg source dose. The responses to the other 10 terpenes at low source doses (100ng to 1μg) were at the level of the response threshold to the solvent but evoked stronger EAG responses at higher source doses with the exception of the lime diene mixture, undecatriene and diphenyl methane that only evoked weak responses. The responses to ocimene, myrcene, dipentene, γ-terpinene, terpinolene and terpineol at high source doses (10μg to 100μg) are comparable to the responses of the monoterpenes (α- and β-pinene, camphene and *p*-cymene; Figure 2.5a).

EAG-responses to mixtures of terpene hydrocarbons and alcohols These 8 products were only tested using the EAG responses of *G. f. fuscipes* antennae (Figure 2.7). Of

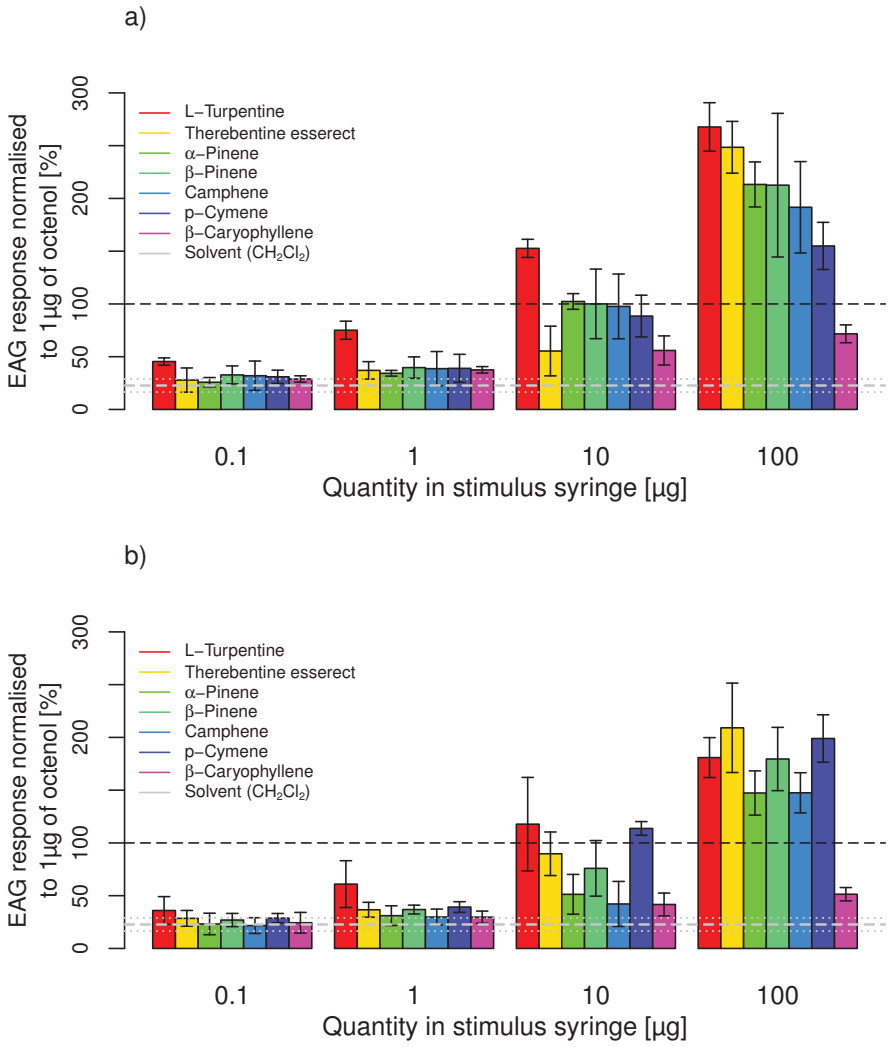

Figure 2.5.: **Electroantennogram responses of *G. f. fuscipes* (a) and *G. p. gambiensis* (b) to turpentines, to monoterpenes present in turpentine (α-pinene, β-pinene, camphene, p-cymene) and to β-caryophyllene delivered from a stimulus syringe containing 100ng to 100μg doses on filter paper (N=3 for each species).** The EAG responses of *G. f. fuscipes* and *G. p. gambiensis* were recorded to air puffs of the compounds diluted in dichloromethane (DCM) and applied at 4 different doses ranging from 100ng to 100μg in the stimulus syringe. Responses were normalised with respect to the response to an air puff over 1μg of 1-octen-3-ol in the stimulus syringe used as reference at the start and end of each recording period. The response to pure DCM (gray dotted line) provides an estimation of the response threshold. The black dotted line indicates the response amplitude to 1-octen-3-ol at a 1μg source dose (100%).

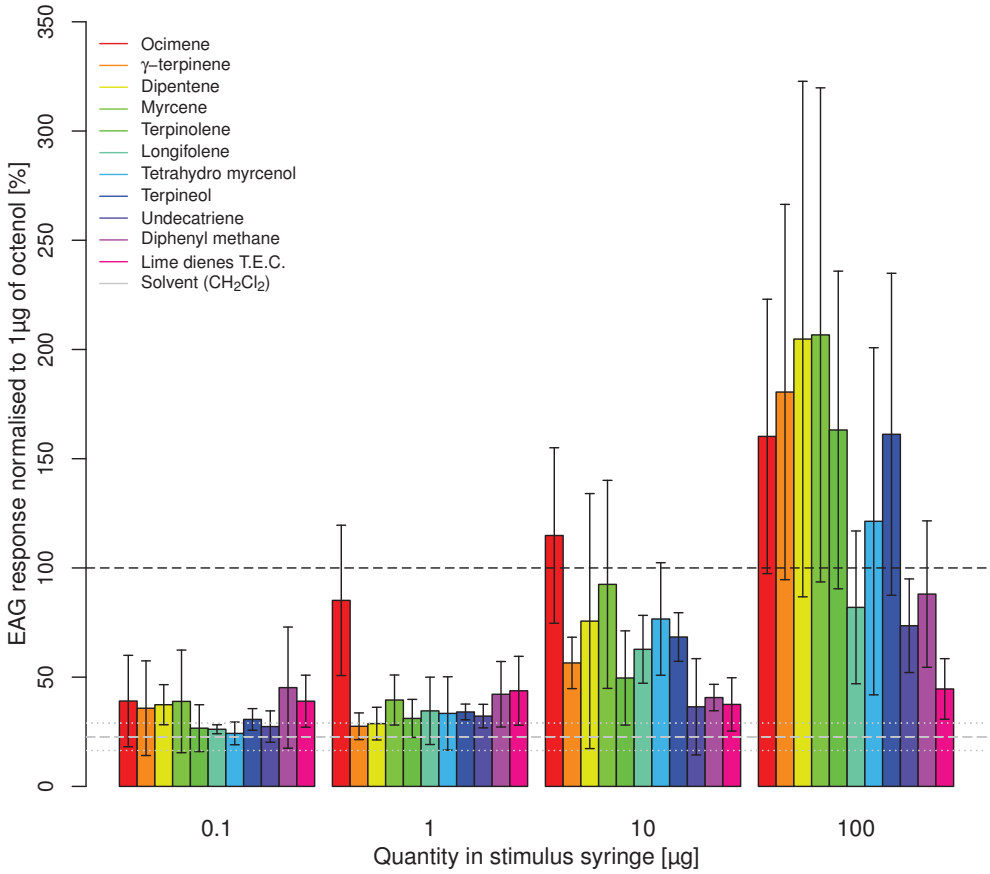

Figure 2.6.: **Electroantennogram responses of *G. f. fuscipes* to monoterpenes (ocimene, myrcene, dipentene, γ-terpinene, terpinolene and lime diene 10%), monoterpene alcohols (terpineol and tetrahydro myrcenol), sesquiterpene (longifolene), diphenyl methane and undecatriene delivered from a stimulus syringe containing 100ng to 100μg doses on filter paper (N=3).** For further details see legend to Figure 2.5

these products only *p*-cymene and "geraniol-98" reached 50% of the level of the EAG response to 1-octen-3-ol at the same source dose. At higher source doses (10μg to 100μg) "geraniol-98" only evoked weak responses while "essence de pin", "dipentene-10", "citronellol-70" and "cineols-80" evoked stronger EAG responses with "cineols-80" at the 100μg source dose evoking 4 times the response to 1-octen-3-ol at a 1μg source dose.

EAG-responses of the *morsitans*-group flies *G. pallidipes* and *G. swynnertoni* to plant volatiles

EAG-responses of *G. swynnertoni* to turpentine, monoterpenes, sesquiterpenes, and terpene alcohols The normalised EAG responses of *G. swynnertoni* to monoterpenes, sesquiterpenes, terpene alcohols and to L-turpentine were only half the response level shown by *G. f. fuscipes* or *G. p. gambiensis* but the strongest response was again to L-turpentine (Figure 2.8). The responses to the monoterpenes were only clearly above the response threshold to the solvent at high source doses (10μg to 100μg) with the strongest responses to ocimene, myrcene, terpinolene, γ-terpinene and camphene. These substances, along with the aromatic hydrocarbon *p*-cymene, evoke EAG responses comparable to L-turpentine at the same source dose. Only at the highest dose tested (100μg) did the responses reach the level of 1-octen-3-ol at 1μg source dose with the exception of 10μg L-turpentine that approached the response level of 1-octen-3-ol at 1μg source dose.

EAG-responses of *G. pallidipes* to turpentine and essential oils The EAG responses of *G. pallidipes* to seven essential oils and L-turpentine were tested (Figure 2.9). The normalised EAG responses to the essential oils were only above the response threshold to the solvent at higher source doses (10μ to 100μg) with the exception of the response to *Abies alba* and *Thuja sp.* essential oils where the response at 1μg already exceeded the response threshold. At the highest source dose (100μg) all essential oils reached the response level of 1-octen-3-ol at 1μg source dose with the exception of *Callitris colmellaris* oil which only evoked weak responses. The normalised EAG response to L-

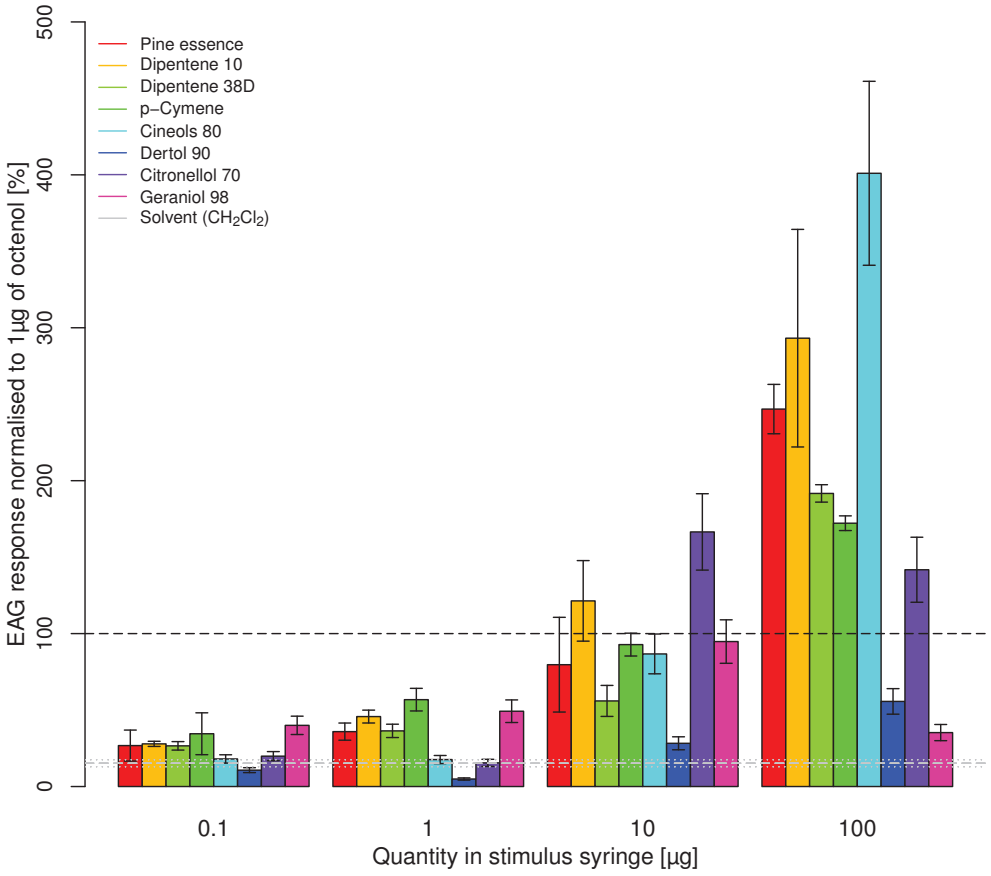

Figure 2.7.: **Electroantennogram responses of *G. f. fuscipes* to *p*-cymene and mixtures of terpene hydrocarbons and alcohols delivered from a stimulus syringe containing 100ng to 100μg doses on filter paper (N=2).** For further details see legend to Figure 2.5

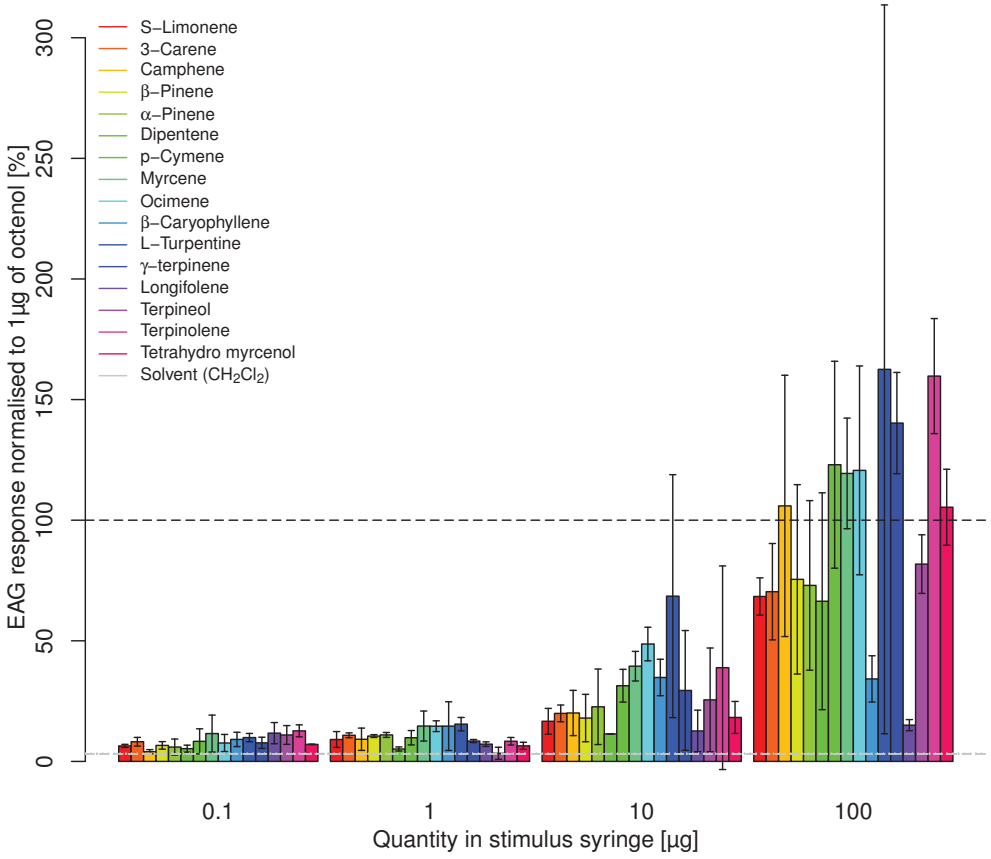

Figure 2.8.: **Electroantennogram responses of *G. swynnertoni* to L-turpentine, monoterpenes, sesquiterpenes, terpene hydrocarbons and alcohols delivered from a stimulus syringe containing 100ng to 100μg doses on filter paper (N=2).** For further details see legend to Figure 2.5

turpentine was equal to that of 1-octen-3-ol at the same source dose (1μg) and already reached 50% of the response to 1-octen-3-ol at 1μg source dose at a tenfold lower source dose (Figure 2.9).

A direct comparison of 1-octen-3-ol and L-turpentine reveals that the responses of *G. pallidipes* to L-turpentine are comparable to the responses to 1-octen-3-ol over the whole dose range (10ng to 100μg; Figure 2.10, Table 2.4). Dose response curves were fitted to the responses of *G. pallidipes* to 1-octen-3-ol and L-turpentine (Figure 2.10). The x-intercept, indicating the detection threshold, the slope, indicating how the response augments with increasing source dose, and the coefficients of determination (R^2) are shown in Table 2.4. The coefficients of determination explain how much of the variance is explained by the model, revealing that ±95% of the variance in the dose response curves of *G. pallidipes* to 1-octen-3-ol and L-turpentine are explained by the log-linear regression model, with the significance level indicating that the log-linear regression is a valuable model. However, the fitted regressions have to be interpreted with caution as it is difficult to fit a non-linear dose response relationship to only three (L-turpentine Nr.2 and 1-octen-3-ol) to four points (L-turpentine Nr.1). The calculated x-intercepts indicated detection thresholds at 0.5ng for 1-octen-3-ol and 10-30ng for L-turpentine. Yet neither of these detection thresholds nor the slopes differed significantly (Table 2.4).

Table 2.4.: **Responses as a function of dose of *G. pallidipes* to L-turpentine and 1-octen-3-ol (log-linear regression, $p < 0.5$; *).** Details of the log-linear regression fitted to the data presented in Figure 2.10. A low x-intercept indicates a low detection threshold for the compound while the slope indicates how the response augments with higher doses.

Compound	**x-intercept [μg]**	**slope**	**R^2**
L-turpentine Nr.1	0.02713	74.549	0.9551*
L-turpentine Nr.2	0.00916	203.980	0.9443*
1-octen-3-ol	0.00047	30.616	0.9569*

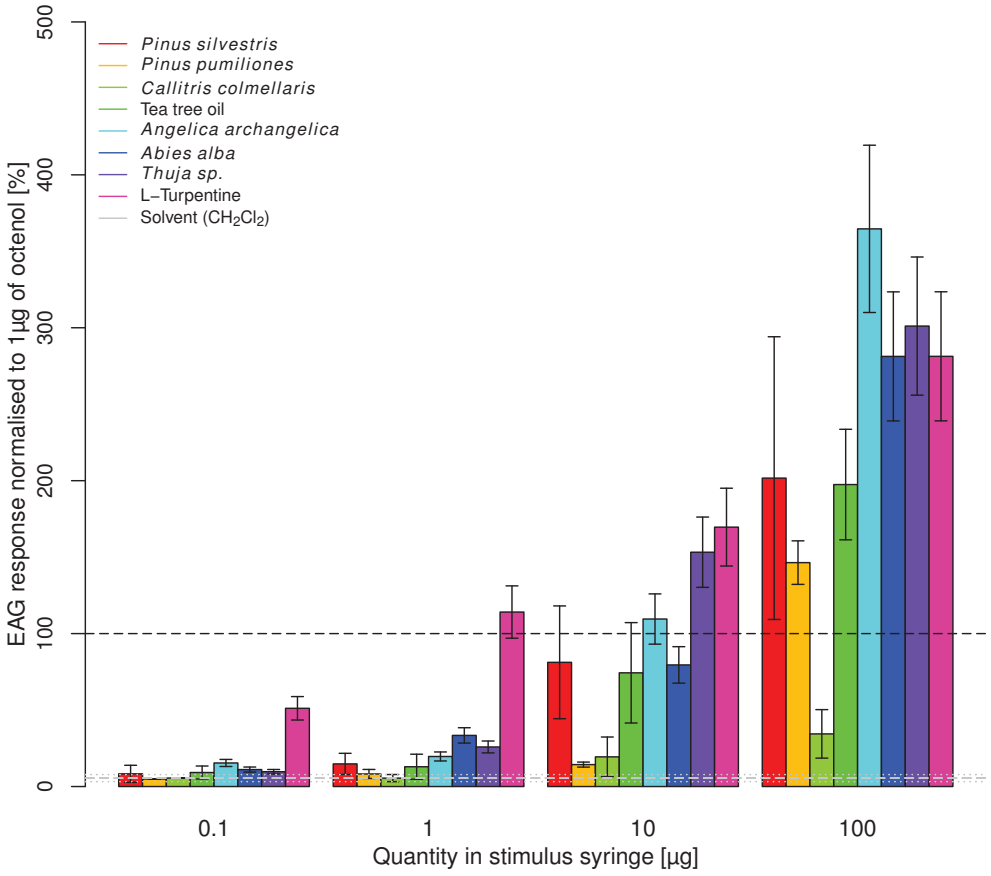

Figure 2.9.: **Electroantennogram responses of *G. pallidipes* to L-turpentine and 7 essential oils delivered from a stimulus syringe containing 100ng to 100μg doses on filter paper (N=2).** For further details see legend to Figure 2.5

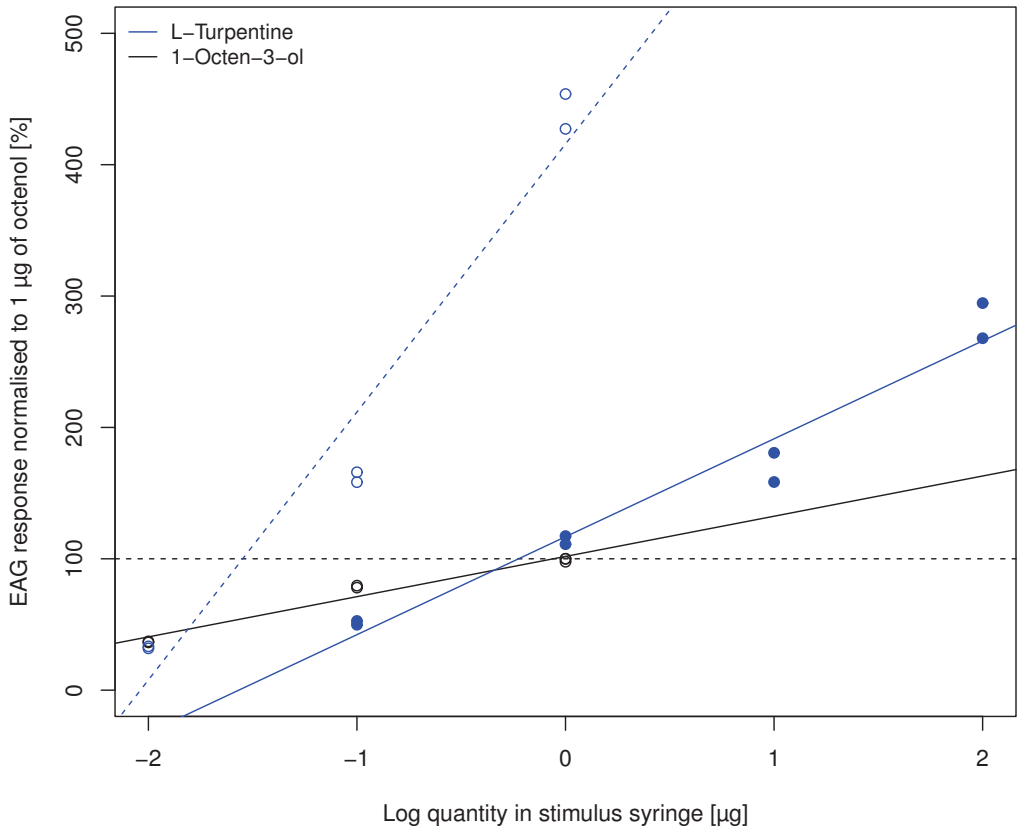

Figure 2.10.: **Relative electroantennogram responses of *G. pallidipes* to L-turpentine and 1-octen-3-ol delivered from a stimulus syringe containing 10ng to 100μg doses on filter paper (N=2).** The EAG responses were recorded to air puffs of the compounds diluted in dichloromethane (DCM) and applied at 5 different doses ranging from 10ng to 100μg in the stimulus syringe. Responses were normalised with respect to the response to an air puff over 1μg of 1-octen-3-ol in the stimulus syringe used as reference at the start and end of each recording period. The black dotted line indicates the response amplitude to 1-octen-3-ol at a 1μg source dose (100%). The blue dotted line represents the EAG results of *G. pallidipes* to L-turpentine in a second recording series (N=2).

Response as a function of dose for *G. pallidipes* responding to limonene, α-pinene, β-pinene, E2-hexenal, Z3-hexenol and 1-octen-3-ol For limonene, α-pinene, β-pinene, E2-hexenal, Z3-hexenol and 1-octen-3-ol a dose response curve was established by GC-EAG (Figure 2.11, Table 2.5). Limonene and 1-octen-3-ol show the lowest response threshold at ~1.5ng, followed by α-pinene, E2-hexanal and Z3-hexenol with detection thresholds between 4 and 6ng. The highest response threshold was recorded for β-pinene at 28ng (Table 2.5). With regard of the slope of the regression lines three groups can be recognized: 1-octen-3-ol with E2-hexenal, limonene with Z3-hexenol, and $\alpha-$pinene with β-pinene with the lowest slope (Table 2.5). Only the log-linear regressions for 1-octen-3-ol and E2-hexanal are significant, even though limonene, Z3-hexenol and β-pinene have equal or higher R^2 values. This could indicate that the log-linear regression is not suited for the analysis of the dose response curves of these products or that the number of points is insufficient. The results of these log-linear regressions have therefore to be interpreted with caution.

Table 2.5.: **Responses as a function of dose of *G. pallidipes* to volatile plant compounds (log-linear regression, $p < 0.5$). A low x-intercept indicates a low detection threshold for the compound. The compounds are sorted in order of ascending x-intercept, from top to bottom. Only the log-linear regressions fitted to 1-octen-3-ol and E2-hexenal are significant (*).**

Compound	x-intercept [ng]	slope	R^2
limonene	1.355	54.05	0.378
1-octen-3-ol	1.808	89.14	0.299*
α-pinene	4.123	14.80	0.195
E2-hexenal	5.640	89.15	0.647*
Z3-hexenol	6.537	52.75	0.592
β-pinene	28.609	33.53	0.695

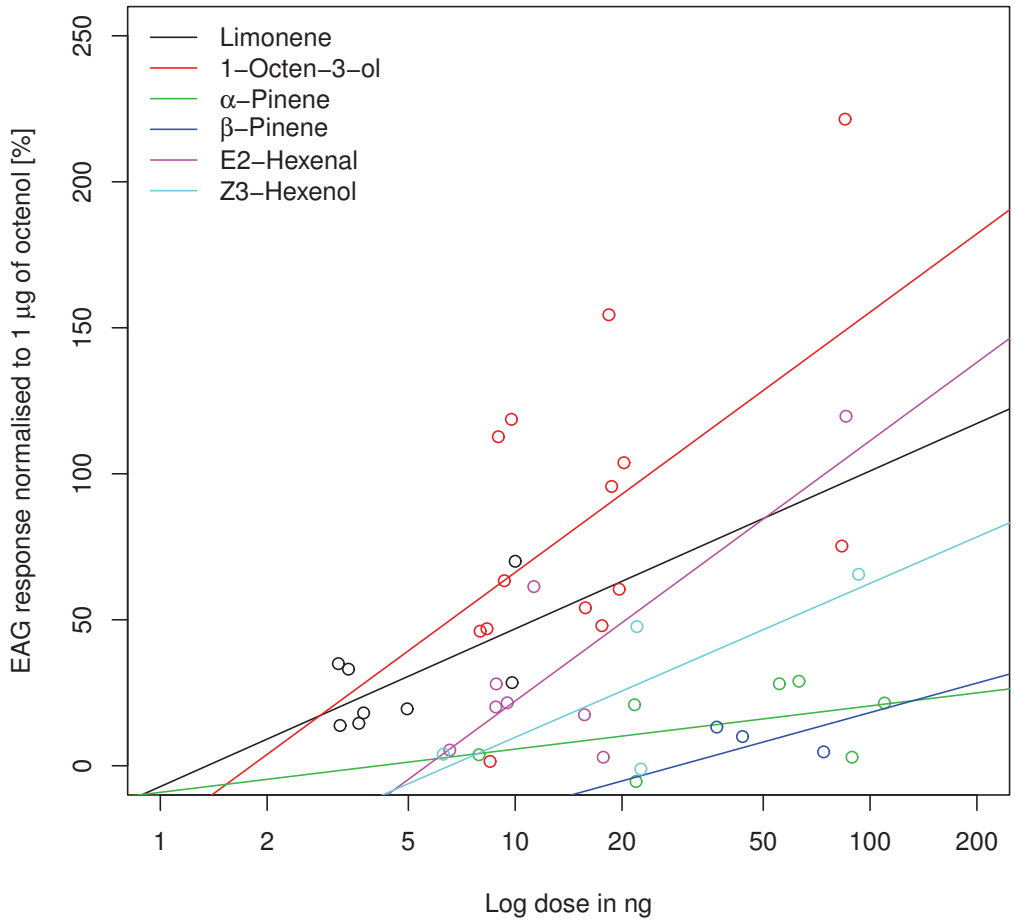

Figure 2.11.: **Responses as a function of dose for *G. pallidipes* EAG responses to six volatile plant compounds relative to 1μg of 1-octen-3-ol.** Details to the log-linear regressions can be found in Table 2.5.

2.3.4. Dose dependent EAG-responses of *G. swynnertoni* to 3-*n*-propylphenol, 1-octen-3-ol and *p*-cresol (POC)

As reference for the interpretation of the EAG responses of *G. swynnertoni* to terpenes, the EAG responses to the known tsetse attractants 3-*n*-propylphenol, 1-octen-3-ol and *p*-cresol were recorded. The responses to *p*-cresol and 3-*n*-propylphenol were generally weak, with only the response to *p*-cresol at the highest source dose (100μg) reaching the level of the EAG response to 1-octen-3-ol at 1μg source dose (Figure 2.12). However all EAG responses to these known tsetse attractants at doses at and above 1μg were above the response threshold to the solvent.

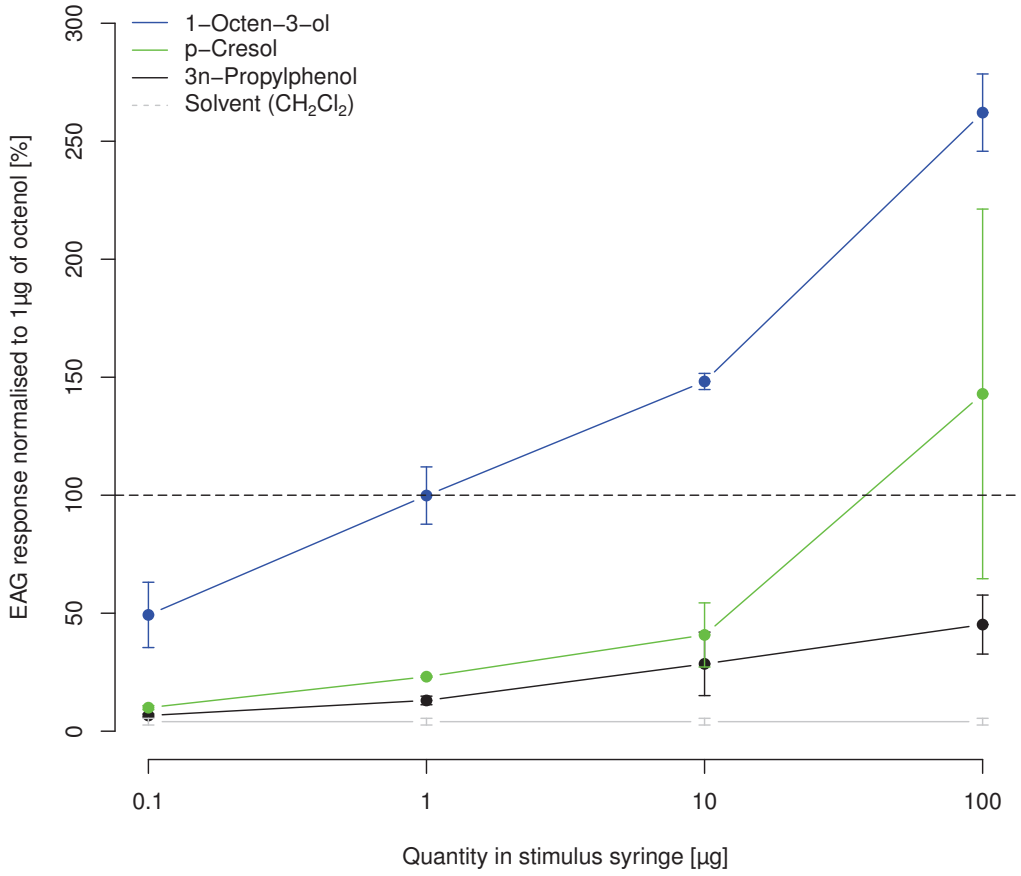

Figure 2.12.: **Electroantennogram responses of *G. swynnertoni* to 3-*n*-propylphenol, 1-octen-3-ol and *p*-cresol delivered from a stimulus syringe containing 10ng to 100μg doses on filter paper (N=2).** For further details see legend to Figure 2.5

2.4. Discussion

Common plant products, including mono- and sesquiterpenes and some green-leaf volatiles are perceived by tsetse spp. The compounds and their relative amounts present in L-turpentine (Sigma) and therebenthine esserect (Givaudan) depend on the origin and the degree of purification of the products. These products are of particular interest, as they are relatively easily obtained, are inexpensive and can be produced directly in Africa. L-turpentine (Sigma) is a highly purified extract from *Pinus* species and contains only $C_{10}H_{16}$ products, while therebenthine esserect is extracted from *Pistacia terebinthus*. Both products contain the monoterpenes α- and β-pinene, limonene and camphene, with α-pinene being the main component followed by β-pinene and limonene. Therebenthine esserect (Givaudan) contains, additionally, other monoterpenes, sesquiterpenes, terpene alcohols, esters and oxides.

Both tsetse species tested, *G. pallidipes* and *G. brevipalpis*, showed EAG responses to the same set of products, with *G. brevipalpis* generally showing higher EAG responses in mV. The difference in the EAG response is either due to an effect of the measurement method, with *G. brevipalpis* having bigger antennae than *G. pallidipes* making it easier to measure from, or it reflects a real difference in sensitivity. This could account for the fact that *G. brevipalpis* additionally showed EAG responses to some compounds that are only present in very small amounts in therebenthine esserect like humulene and δ-cadinene. The riverine tsetse sp. *G. f. fuscipes* and *G. p. gambiensis* also respond to limonene present in L-turpentine (Appendix B.1), but even to a lower extent (in mV) than *G. pallidipes*. The lowest detection threshold is for L-turpentine and therebenthine esserect in all species tested, namely *G. pallidipes*, *G. swynnertoni*, *G. f. fuscipes* and *G. p. gambiensis*. Generally the simpler mixture, L-turpentine, shows a slightly better response, suggesting that either the ratio of the main products is better in L-turpentine than in therebenthine esserect, or that therebenthine esserect contains compounds that reduce the EAG responses of tsetse spp. L-turpentine has an EAG detection threshold approaching that of 1-octen-3-ol by *G. pallidipes*, being only tenfold higher. However, this comparison has its limits as L-turpentine is a mixture of products. Further we have seen that there are large differences in the EAG responses between individual flies. This

may be due to different conditions between the experiments, e.g. slightly higher room temperature or a slightly longer time between the preparation of the test syringe and the actual test, both conditions allowing a higher amount of volatilized product to be injected into the air stream generating a higher EAG potential. Generally these factors are normalised by a higher number of flies tested. It is known for several insect species that not only the compounds are important but also their relative ratios in mixtures is crucial for sensory detection and the behavioural response. This has been shown to be true for phytophagous insects, like *Decemlineata leptinotarsa* [173], and a variety of moth species responding electrophysiologically and behaviourally to pheromones [46, 81].
Looking at the monoterpenes that evoked EAG responses we can see that tsetse flies responded best to the monoterpenes limonene, ocimene, myrcene and the related aromatic *p*-cymene. Of these, limonene approaches the detection threshold of 1-octen-3-ol. Interestingly, limonene is also one of the main compounds present in both L-turpentine and therebenthine esserect, consistently evoking EAG responses. The main monoterpenes tested all have relatively similar vapour pressures ranging from 1.5 to 3.5 mmHg at 25°C with the exception of ocimene that has a lower vapour pressure of 0.8 mmHg. These values are still much higher than the values for the known tsetse attractants 1-octen-3-ol (0.53 mmHg), *p*-cresol (0.11 mmHg) and 3-*n*-propylphenol (0.045 mmHg). This comparison is especially important when comparing the EAG responses of *G. swynnertoni* to the monoterpenes with its EAG responses to POC. *G. swynnertoni* responded only weakly to *p*-cresol and 3-*n*-propylphenol compared to the monoterpenes and 1-octen-3-ol. Considering the much lower vapour pressure of the phenols we can assume that we underestimate the EAG responses to the phenols compared to the monoterpenes. To solve this problem one should compare the products using GC-EAG, where each product is injected at a known amount. Nevertheless the EAG results already provide indications of the doses to be used in wind tunnel and field studies as there the different vapour pressures are reflected in the release rates from the dispenser. To our knowledge the main tsetse attractants 1-octen-3-ol, *p*-cresol, 3-*n*-propylphenol, and the terpenes have never been tested before by EAG for *G. swynnertoni*.
Green-leaf volatiles are present in all green plants and serve as attractants for both phy-

tophagous and parasitoid insects [42, 173]. E2-hexenal has already been described to elicit EAG responses from tsetse flies from all three habitats in Africa [157], and was, in this study, the only C_6-aldehyde that elicited EAG responses from *G. pallidipes* and *G. brevipalpis* even at low doses. At high doses, a second aldehyde, hexanal, evoked EAG responses from *G. pallidipes* (see Annex B.2). Hexanal and E2-hexenal only differ by the presence of a double bond in the C_6-chain of the latter. The dose response curves for E2-hexenal and 1-octen-3-ol show the same trend for *G. pallidipes*, with the exception of 1-octen-3-ol having a lower detection threshold. E2-hexenal is a known attractant for many insect species from different families with different nutritional requirements, acting as a kairomone, allomone and pheromone [42, 47, 173]. Of the alcohols, next to the well known tsetse attractant 1-octen-3-ol, 1-heptanol evoked EAG responses while the C_6-alcohol, 1-hexanol, did not evoke any EAG response at the same source dose and only very weak responses at high doses. This is surprising as the hydrocarbons hexane, heptane and octane all evoke similar EAG responses (Annex B.3, [78]).
We have shown that tsetse species from all three African habitats perceive volatile plant compounds, several at detection thresholds similar to 1-octen-3-ol. Of main interest for behavioural studies are L-turpentine, as it is a simple terpene mixture with a low detection threshold, with main components limonene and α-pinene, along with the monoterpene ocimene and aromatic *p*-cymene. Further studies are required to investigate systematically the sesquiterpenes present in therebenthine esserect. β-caryophyllene has already been shown to evoke EAG and behavioural responses from tsetse species from all three African habitats [156, 157], and its isomer humulene evoked GC-EAG responses from *G. brevipalpis* in this study. These products might be of interest because of their lower vapour pressure, potentially acting over a different volatility range than the fast evaporating monoterpenes. The toothbrush tree *Salvadora persica* would be an interesting plant to investigate. The toothbrush tree or miswak is used as a natural toothbrush and has been extensively studied due to its antimicrobial effects [3]. Phytochemical investigation revealed that it contains oleic, linolic, and stearic acids. Among the compounds identified are esters of fatty acids and of aromatic acids, some terpenoids and four benzylamides [1, 98]. The major components from the essential oil of the toothbrush tree stem

have been identified as 1,8-cineole, humulene, β-pinene, and 9-epi-(E)-caryophyllene [5] while the essential oil of the leaves contained mainly benzyl nitrile, eugenol, thymol, isothymol, eucalyptol, isoterpinolene, and β-caryophyllene [4]. The surroundings of this bush has been reported as a main larviposition site for some tsetse spp. [105]. Is there a specific plant compound in the tree or a compound in the soil surrounding it that triggers the aggregation of tsetse to larviposit?

3. The influence of volatile plant compounds on the flight behaviour of tsetse flies *Glossina spp.*

3.1. Introduction

In GC-EAG and EAG recordings several compounds derived from plants were identified as chemical stimulants for tsetse flies (see Chapter 2). In particular, tsetse flies showed a low detection threshold for L-turpentine, comparable to that of 1-octen-3-ol, with its key components limonene and α-pinene. However, EAG responses alone do not allow conclusions concerning the behavioural effects of products. To address this question behavioural experiments, in our case in a wind tunnel, were made. At first, the effects of *Pinus sylvestris* essential oil, ethanol and acetone on the behaviour of tsetse flies were tested on their own in the wind tunnel. Ethanol and acetone are two solvents, acetone a known attract for tsetse flies in the field [112, 158, 170] and wind tunnel [131], while there are indications that the essential oil of *P. sylvestris*, a main source for terpenes, is a chemical stimulant for tsetse flies [84]. Each of these products activated less than 10% of the flies tested. At such low response levels comparisons of effects of plant volatiles are unrealistic and difficult to determine statistically. CO_2 has been shown to attract tsetse flies in the field and in laboratory wind tunnels and has been shown to sensitise mosquitoes to skin odours [37, 49]. Therefore CO_2 at a low dose close to its detection level by tsetse flies [49, 68, 70], was added to the test products, increasing the behavioural responses tenfold (see Table 3.2), rendering them amenable

to statistical analysis. The volatile chemostimuli identified in Chapter 2 (terpenes and green leaf volatiles) occur throughout the plant kingdom and are also found in the breath of hosts, mainly in ruminants. So far, little is known about the effects of plant volatiles on the behaviour of tsetse flies. We were therefore interested in the effects of single volatile plant compounds (α-pinene, limonene, *p*-cymene, 1-octen-3-ol and E-2-hexenal), simple monoterpene mixtures (L-turpentine, α-pinene plus limonene) and mixtures of L-turpentine with either 1-octen-3-ol or E-2-hexenal on the behaviour of tsetse flies. We expected a better performance of the simple monoterpene mixtures compared to single compounds and hoped for a synergistic effect of the known tsetse attractant 1-octen-3-ol with L-turpentine. Further, we were interested if male and female tsetse fly behaviour is affected to the same extent by volatile plant compounds, and the effect feeding could have on the behaviour induced by chemostimuli. Due to the higher dependence of female tsetse flies on plants, using them not only as resting sites as males do but also as larviposition sites, we expected a higher effect of volatile plant compounds on female behaviour. We expected teneral flies to show a behavioural response to a broader spectrum of chemostimuli than fed flies as it is known that tsetse flies prefer the same host after a first blood meal [6, 61, 178] and also due to the probably higher appetance of starved teneral flies compared to starved fed flies. Finally, it is important to know whether volatile plant compounds are able to induce behaviours in tsetse flies but also if the chemical stimulants can induce flight towards and around a visual stimuli, taking into account the importance of vision in tsetse fly sensory ecology.

3.2. Material and Method

Biological material Pupae were obtained and maintained, and sexes separated as in section 2.2. *G. pallidipes* and *G. swynnertoni* received defibrinated bovine blood-meals through a silicon membrane [102, 104] at 7 day intervals from day 3 after emergence, while *G. brevipalpis* received blood-meals at 9 day intervals from day 4 after emergence. Teneral *G. pallidipes* and *G. swynnertoni* tested in the wind tunnel were starved for 2

days, fed *G. pallidipes* were starved for 5-6 days, while teneral and fed *G. brevipalpis* were starved for 3 and 7-8 days, respectively.

Wind tunnel Tsetse fly behaviour was observed with individual flies in a wind tunnel that provided a near physical mimic of the environment where temperature, humidity, light and wind speed could be manipulated. In the past, wind tunnels had been used successfully to investigate tsetse flies behaviour [32, 49, 72, 79, 132, 142, 157]. The closed circuit wind tunnel (working area: 250cm long, 100x100cm) made of non reflecting glass had a centrifugal ventilator to move humid air ($85 \pm 1\%$ R.H., $26 \pm 0.1°C$) in a laminar flow of 0.6 m/s. Active charcoal filters at either end scrubbed the air of any residual chemicals. At the upwind end of the wind tunnel a static gas mixer served as a plume generator carrying test chemicals leaving an oscillating capillary (piezo electric driven) as an aerosol [45, 144]. The shape of the plume was maintained in a laminar flow generated by aluminum honeycombs (Figure 3.1). High frequency lighting (36 W, 1000 Hz, Philips, The Netherlands) simulated day light with 700 Lux on the floor of the wind tunnel, that was covered with a medium density fiberboard (MDF, 3mm, light brown). On both sides of the wind tunnel light blue vertical bands 7cm in width spaced 7cm apart provided visual cues that enabled tsetse flies to orient (for spectral reflectance of the bands see Appendix A.3). For experiments with a visual target the floor cover was removed and replaced by diffuse red light, consisting of 9000 warm white LED lamps covered with a red filter (E-color plus, #027 medium red, Rosco Laboratories Inc., USA) and frosted glass plates. The target, a glass sphere painted blue (16cm diameter, blue acrylic paint with maximum absorbance at ~460nm and the typical shoulder of phthalogen blue at ~420nm, see Appendix A.3; phthalogen blue, "Gentian blue RAL 5010", Deco Matt, MOTIP DUPLI GmbH, Germany) was placed on a stand at 1m from the upwind end in the centre of the wind tunnel, i.e. equidistant from the floor, top and the sides. A blue sphere has already been used by Gurba et al. [72] and proven to be useful to study the flight behaviour of *G. brevipalpis* in a wind tunnel. The flight of tsetse flies was filmed and recorded by two video cameras (Basler eXcite exA640-120m 656x491pixel, 132 fps, f=4.5mm objectives 59.4x79°, CL-Electronics, Switzerland), placed 87cm above

the wind tunnel centre (distance between cameras 160cm) and linked to 3D software (HALCON software HD) in a computer in order to track the flight of the tsetse flies in 3D. Crow Software (University of Neuchâtel) was used to calculate the x, y, and z coordinates of each flight point. This permitted to determine the location of the flies within the wind tunnel at any given time.

Compounds tested Ethanol (pro Analysi, Merck, Germany) was used as solvent for all compounds tested in the wind tunnel for polar products (e.g. alcohols) but also the terpenes were readily soluble in ethanol. Ethanol has been shown by El-Sayed et al. [45] and Schmidt-Büsser [143] to have no influence on the behaviour of the torticid moth *Eupoecilia ambiguella*. Our tests showed that ethanol did not have an influence on the behaviour of tsetse flies (see Tables 3.2, 3.3, 3.6). L-Turpentine (Sigma-Aldrich, Switzerland) was released at 1μg/min, 10μg/min and 100μg/min, S-(-)-limonene (97% purum, Fluka, Switzerland) at 0.1μg/min and 1μg/min, *p*-cymene ($>$ 95%, drt, France) at 1μg/min, $\alpha-$pinene (Givaudan, Switzerland) at 10μg/min, E-2-hexenal (Sigma-Aldrich, Switzerland) at 1μg/min and 1-octen-3-ol (racemic, $>$ 97% pure, Merck, Germany) at 1ng/min and 10ng/min. Chemostimuli were tested in the presence of CO_2 at a level of 26.96$\pm$2.19 ppm measured at the release cage because of its activating and additive effects with other host products for tsetse [49, 70, 132]. As a positive control human breath delivered from a Tedlar® bag at 5l/min was used. The Tedlar® bag was filled with human breath at least 30min prior to experiments to allow to equalise the temperature of the breath with ambient temperature in order to avoid temperature effects on the odour stimulant. CO_2 was present in human breath at a level of 59.37$\pm$1.77 ppm measured at the release cage.

Behavioural experiments The behaviour of tsetse flies was observed during two daily activity peaks in the first and the last two hours of the photophase [15, 17, 19]. Before the experiments each fly was transferred into a plastic release cage (transparent PVC cylinders 15cm$*$10cm) and allowed to acclimatize to the wind tunnel conditions for 15 (*G. pallidipes* and *G. swynnertoni*) or 30 minutes (*G. brevipalpis*). The cage was then placed horizontally on a stand at 5cm from the downwind end in the centre of the

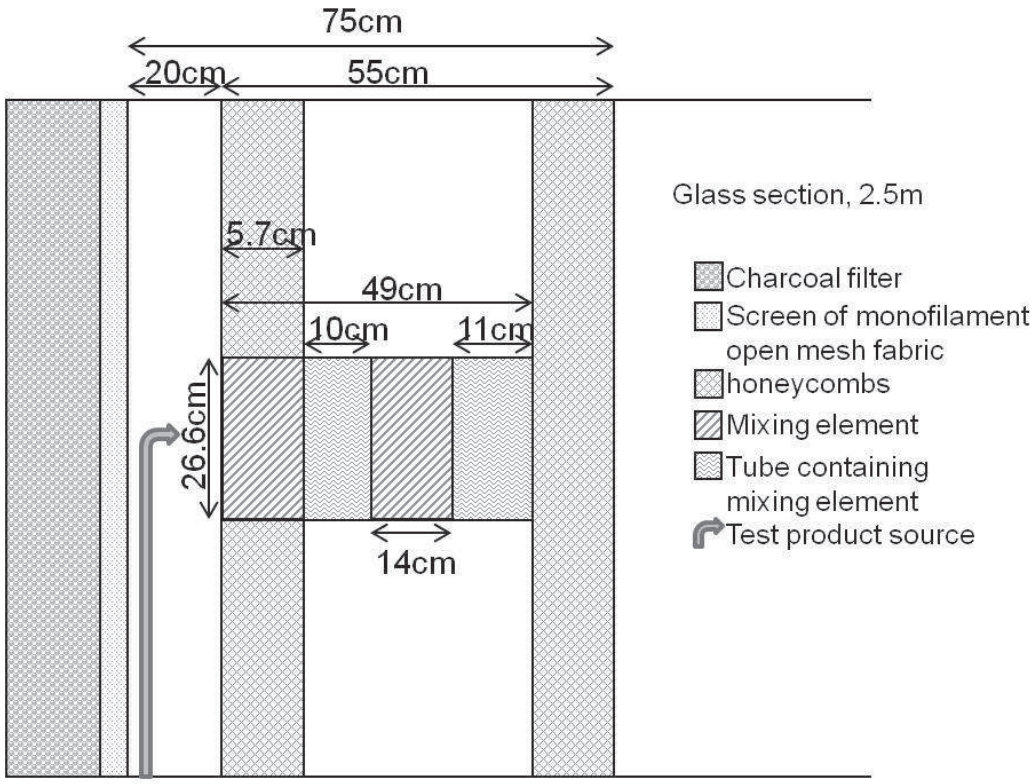

Figure 3.1.: **Profile of the laminar flow and plume generating system of the wind tunnel.** Directly after the charcoal filters (at extreme left) a monofilament open mesh fabric screen (polyethylene terephthalate PETP) of 25% open area (SEFAR**PETEX**®, Sefar AG Switzerland) was installed to induce a laminar flow. A honeycomb layer (aluminium, $^{3}/_{8}$" cell size, 57mm thick, Plascore GmbH&CoKG, Germany) with a hole for the tube containing the mixers was installed. The holder of the tube was not included in the profile for clarity. The elements in the mixer (Sulzer SMV^{TM} static gas mixer, Sulzer Ltd, Switzerland, tube i. d. 26.6cm, length 49cm) were oriented at 90° to each other to allow a mixing of the odour in all directions. The second honeycomb layer (aluminum, $^{3}/_{8}$", 57mm thick) was fixed directly after the tube. All elements with the exception of the laminar flow screen were made of stainless steel (mixer) or aluminum (honeycombs) to prevent contamination. The laminar flow screen equalizes the pressure over the whole wind tunnel section, while the honeycombs are used for aligning the directions of the pressure vectors, resulting in a laminar flow. Mosquito netting (high density polyethylene (HDPE)) was stretched across the downwind side of the second honeycomb to provide contrast for tracking the flies. This screen (not shown) was changed regularly to minimize effects of pollution.

wind tunnel, i.e. equidistant from the floor, roof and the sides. The door of the release cage was slowly lifted and the fly was successively exposed for 1 min to odour-free air (negative control) and then for 1 min to the test odour. A webcam installed at the downwind end of the wind tunnel was used to observe the flies in the release cage.

Flight parameters The following behavioural categories were recorded: 'activation', 'exit', 'passing 1/4 WT', 'passing the midline', 'passing 3/4 WT' and arriving at the 'source'. Definitions of the behavioural categories are provided in Table 3.1. For the behavioural category 'activation', in addition to the behaviour itself the time from stimulus onset to activation of the fly was also measured. For experiments with a visual target additional parameters were analysed: distance covered by the insect, ground velocity of the upwind flight and the flight around the target, and time spent and distance covered in a zone around the target (±30cm from the center of the target in x and y directions, ±50cm in z direction) [34]. Additionally, the spatial point pattern of the upwind flight and the flight around the target was analysed by kernel smoothed intensity estimates.

Statistical analysis The behavioural responses of tsetse flies to volatile plant compounds were compared to the solvent and positive control (human breath) by fitting a general linear model (GLM) with a logit link function to the responses using the statistical package `R` (version 2.12.0). Time-event analyses were performed using survival statistics with a proportional hazards (Cox) regression model using the `R` package `survival`. To compare the effect of different doses of L-turpentine on the behavioural responses of the tsetse flies and the effect of volatile plant compounds on the behavioural responses of male and female flies, odds ratios were calculated. The odds ratio compares the odds of two groups, e.g. the ratio of the probability that females pass through the midline of the wind tunnel to the probability that they do not pass the midline of the wind tunnel compared with the ratio of the probability that males pass through the midline of the wind tunnel to the probability that they do not. Odds ratio values vary between zero and infinity, where an odds ratio of 1 indicates no difference, a value lower than 1 a negative effect and values higher than 1 a positive effect. For analyses of the spatial flight pattern around the target the `R` package `spatstat` was used [8, 9].

Table 3.1.: **Description of the behavioural categories recorded for tsetse flies in the wind tunnel.**

Behavioural category	**Description**
Activation	The fly turns into the wind and starts walking inside the release cage.
Exit	The fly takes off from the release cage.
Passing 1/4 WT	The fly passes through the first quarter of the wind tunnel.
Passing Midline	The fly passes through the midline of the wind tunnel.
Passing 3/4 WT	The fly passes through the third quarter of the wind tunnel.
Source	The fly has crossed 2m20cm of the wind tunnel and conducted a local search by performing at least one circle within 30cm of the upwind end of the wind tunnel.

3.3. Results

3.3.1. The effect of CO_2 on the activation of tsetse flies

Adding CO_2 at a low dose of ~26ppm to a test substance increases the percentage of activated flies by tenfold (Table 3.2), irrespective of the nature of the test substance or the feeding status of the flies. The higher percentage of responding flies allows a better comparison of the behavioural categories following activation, as the possibility that subsequent behaviours occur is increased. With teneral *G. pallidipes*, CO_2 on its own was tested at ~26ppm and ~80 ppm, activating 48% and 32% of the flies, respectively (N=100 each, Table 3.3). CO_2 on its own at 26 and 80ppm induced, respectively, only 17% and 9% of tsetses to pass through the first quarter of the wind tunnel and only 3, respectively, 2% of the tested flies performed a local search at the upwind end of the wind tunnel (Table 3.3). Fed and subsequently starved *G. pallidipes* showed a reduced response to CO_2 on its own: only ~20% of the flies were activated, and only 3% passed through the first quarter of the wind tunnel while none were induced to conduct a local search at the upwind end of the wind tunnel (N=100, Table 3.6).

3.3.2. Behavioural responses of teneral *G. pallidipes* to individual volatile plant compounds

The responses of 1400 teneral *G. pallidipes* were recorded to 14 treatments (Table 3.3). Treatments included CO_2 at two doses and EtOH in the presence of CO_2 as negative and solvent control, human breath delivered form a Tedlar® bag as positive control, the terpenes α-pinene and *p*-cymene released at 1 and 10μg/min respectively, the alcohol 1-octen-3-ol at 1 and 10ng/min, the green-leaf-volatile E-2-hexenal at 1μg/min and the terpene mixture L-turpentine at three release rates ranging from 1μg/min to 100μg/min all in the presence of CO_2. As described previously CO_2 at ~26ppm on its own activated ~48% of the flies, induced 37% to exit the release cage, 17% to pass through the first quarter of the wind tunnel, 8% to pass the midline of the wind tunnel and only 3% to conduct a local search at the upwind end of the wind tunnel. Ethanol in the presence

Table 3.2.: **The effect of CO_2 on activation of *G. pallidipes* .** N is the number of flies tested (teneral, fed and subsequently starved flies pooled), release rates of *Pinus sylvestris* oil are pooled; Activation [%] refers to the percentage of activated flies. Values accompanied by different letters are significantly different (GLM(logit), $p < 0.05$)

Substance	**N**	**Activation [%]**
CO_2 at ~26ppm	172	36.63^a
Ethanol 10000μg/min	49	4.08^b
Ethanol 10000μg/min + CO_2	200	44.50^a
Pinus sylvestris oil at 1 to 100μg/min release rates	72	6.94^b
Pinus sylvestris oil at 1 to 100μg/min release rates + CO_2	21	61.90^a
Acetone 10000μg/min	49	4.08^b
Acetone 10000μg/min + CO_2	171	44.44^a

of CO_2 (solvent control) was comparable ($p > 0.05$) to CO_2 on its own, with the only difference that it induced more flies (13%) to pass the midline. CO_2 at ~80ppm activated slightly fewer flies (32%) but induced as many flies to pass through the first quarter, cross the midline and pass through the third quarter of the wind tunnel and to reach the source zone as CO_2 on its own at ~26ppm or in combination with EtOH ($p > 0.05$). Of the test treatments S-(-)-limonene released at 0.1μg/min, E-2-hexenal at 1μg/min and 1-octen-3-ol at 1 and 10ng/min were all comparable ($p > 0.05$) with the negative and solvent controls in terms of number of flies activated and induced to exit the release cage, to pass through the first quarter, the midline and the third quarter of the wind tunnel and to conduct a local search within the upwind end of the wind tunnel (Table 3.3). It took S-(-)-limonene released at 0.1μg/min, E-2-hexenal and 1-octen-3-ol at 1ng/min slightly longer to activate flies than the negative (CO_2 at 26ppm) and solvent controls ($p > 0.05$; Figure 3.2). A second group of treatments, *p*-cymene released at 1μg/min, S-(-)-limonene at 1μg/min, L-turpentine at 100μg/min and 1-octen-3-ol at 10ng/min activated between 56 and 69% of the flies, induced ~25% to pass through the first quarter of the wind tunnel, between 15-20% to pass the midline of the wind tunnel and ~6% to conduct a local search at the upwind end of the wind tunnel ($p < 0.05$ compared to positive control; $p > 0.05$ compared to negative and solvent control except for *p*-cymene activating and inducing exit and 1-octen-3-ol at 0.001μg/min inducing exit($p < 0.05$); Table 3.3). Of these four substances S-(-)-limonene at 1μg/min was the best treatment activating 69% of the flies ($p < 0.01$), inducing 60% of the flies to exit the release cage ($p < 0.05$), 26% to pass through the first quarter ($p > 0.05$), 15% to pass through the midline ($p > 0.05$), 10% through the third quarter of the wind tunnel and 9% to conduct a local search at the upwind end of the wind tunnel ($p > 0.05$). Higher doses of 1-octen-3-ol (0.1 and 1μg/min) activated and induced flight behaviour by significantly less flies than the lower doses of this substance (Table C.1). A third group of treatments included α-pinene released at 10μg/min and L-turpentine at 1 and 10μg/min activating 59 (ns), 68 and 69% of the flies, inducing 49 (ns), 60 and 62% to exit the release cage, inducing 38, 34 and 39% to pass the first quarter of the wind tunnel, 30, 22 (ns) and 27% to pass the midline, 18, 15 (ns) and 14% (ns) to pass the third quarter of the wind tunnel and 14,

9 (ns) and 10% (ns) of the flies to conduct a local search in the source zone (Table 3.3; ns = not significant compared to negative control). Neither of these test treatments approaches the level of the response to breath, which activated flies to a greater extent and significantly faster (Cox proportional hazard model, p< 0.05, Figure 3.2) than all other treatments. Breath also induced significantly more flies to undertake the recorded behavioural categories with the exception of flies passing the midline and arriving at the source for α-pinene presented at 10μg/min (Table 3.3). Other than breath, only L-turpentine at 10μg/min and S-(-)-limonene at 1μg/min activated flies significantly faster than the solvent control (Cox proportional hazard model, p< 0.05, Figure 3.2).

3.3.3. Comparison of teneral *G. pallidipes* male and female behavioural responses to individual plant volatiles

The responses of male and female teneral *G. pallidipes* to volatile plant compounds were comparable (p> 0.05; Table 3.4), with some exceptions, within the treatments that induced at least 10% of the flies to pass through the midline and 5% to reach the source zone (S-(-)-limonene released at 0.1 and 1μg/min, *p*-cymene at 1μg/min, 1-octen-3-ol at 0.01μg/min, L-turpentine at 1, 10 and 100μg/min and human breath). S-(-)-limonene at 1μg/min induced more females than males to pass through the midline and the third quarter of the wind tunnel and to conduct a local search at the upwind end of the wind tunnel (odds ratios 3.24, 2.55 and 3.91, but not significant due to too few flies responding (N = 2 - 11); Table 3.4). The responses of female and male *G. pallidipes* to L-turpentine released at 1 and 100μg/min were comparable (p> 0.05) with the exception of the behavioural parameter 'reaching the source zone' where the odds ratios were 3.91 and 0.24, respectively (p> 0.05; Table 3.4). The greatest differences between female and male responses were recorded to L-turpentine at 10μg/min where this treatment induced significantly more females than males to pass through the first quarter, midline and the third quarter of the wind tunnel (odds ratios 2.57, 2.26, 2.88, GLM(logit), $p < 0.05$ as a high number of flies responded (N = 4 - 25); Table 3.4). In addition, females show a nearly 900% higher chance to conduct a local search in the source zone of the wind

Table 3.3.: **Behavioural responses of teneral *G. pallidipes* to L-turpentine, S-(-)-limonene, *p*-cymene, α-pinene, E-2-hexenal, 1-octen-3-ol and to ethanol (solvent control) each in the presence of CO_2 at 26ppm, to CO_2 alone at ~26ppm (negative control) and at ~80ppm, and to human breath delivered from a Tedlar® bag (positive control);** N=100 for each treatment. Asterisks ('*') indicate a significant difference from the solvent control, stars ('$\star$') a significant difference from the positive control, (GLM(logit);'$^{*}_{\star}$' $p < 0.05$; '$^{**}_{\star\star}$' $p < 0.01$; '$^{***}_{\star\star\star}$' $p < 0.001$). Treatments in bold induced ≥10% of flies to reach the source zone.

Substance and release rate	**Activation** [%]	**Exit** [%]	**Passing** 1/4 **WT** [%]	**Passing Midline** [%]	**Passing** 3/4 **WT** [%]	**Source** [%]
Ethanol 10000μg/min	$51_{\star\star\star}$	$37_{\star\star\star}$	$19_{\star\star\star}$	$13_{\star\star\star}$	$7_{\star\star\star}$	$4_{\star\star\star}$
CO_2 ~26ppm	$48_{\star\star\star}$	$37_{\star\star\star}$	$17_{\star\star\star}$	$8_{\star\star\star}$	$4_{\star\star\star}$	$3_{\star\star\star}$
CO_2 ~80ppm	$32^{**}_{\star\star\star}$	$18^{**}_{\star\star\star}$	$9^{*}_{\star\star\star}$	$4^{*}_{\star\star\star}$	$3_{\star\star\star}$	$2_{\star\star\star}$
S-(-)-Limonene 0.1μg/min	$50_{\star\star\star}$	$37_{\star\star\star}$	$18_{\star\star\star}$	$12_{\star\star\star}$	$10_{\star\star\star}$	$6_{\star\star\star}$
S-(-)-Limonene 1μg/min	$69^{**}_{\star}$	$60^{**}_{\star}$	$27_{\star\star\star}$	$15_{\star\star\star}$	$10_{\star\star\star}$	$9_{\star\star}$
$\alpha-$Pinene 10μg/min	$58_{\star\star\star}$	$49_{\star\star\star}$	$38^{**}_{\star}$	30^{**}	$18^{*}_{\star}$	14^{*}
p-Cymene 1μg/min	$65^{*}_{\star\star}$	$53^{*}_{\star\star\star}$	$26_{\star\star\star}$	$20_{\star\star\star}$	$11_{\star\star\star}$	$7_{\star\star}$
E-2-hexenal 1μg/min	$38_{\star\star\star}$	$20^{**}_{\star\star\star}$	$12_{\star\star\star}$	$6_{\star\star\star}$	$4_{\star\star\star}$	$2_{\star\star\star}$
1-octen-3-ol 0.001μg/min	$39_{\star\star\star}$	$24^{*}_{\star\star\star}$	$14_{\star\star\star}$	$9_{\star\star\star}$	$2_{\star\star\star}$	$1_{\star\star\star}$
1-octen-3-ol 0.01μg/min	$56_{\star\star\star}$	$42_{\star\star\star}$	$23_{\star\star\star}$	$17_{\star\star\star}$	$9_{\star\star\star}$	$5_{\star\star\star}$
L-Turpentine 1μg/min	$68^{*}_{\star}$	$58^{**}_{\star\star}$	$34^{*}_{\star\star}$	$22_{\star\star}$	$15_{\star\star}$	$9_{\star\star}$
L-Turpentine 10μg/min	$69^{**}_{\star}$	$62^{***}_{\star}$	$39^{**}_{\star}$	$27^{*}_{\star}$	$14_{\star\star}$	$10_{\star\star}$
L-Turpentine 100μg/min	$59_{\star\star\star}$	$46_{\star\star\star}$	$27_{\star\star\star}$	$18_{\star\star\star}$	$10_{\star\star\star}$	$5_{\star\star\star}$
Human breath delivered from Tedlar® bag 5l/min	82^{***}	76^{***}	54^{***}	43^{***}	33^{***}	25^{***}

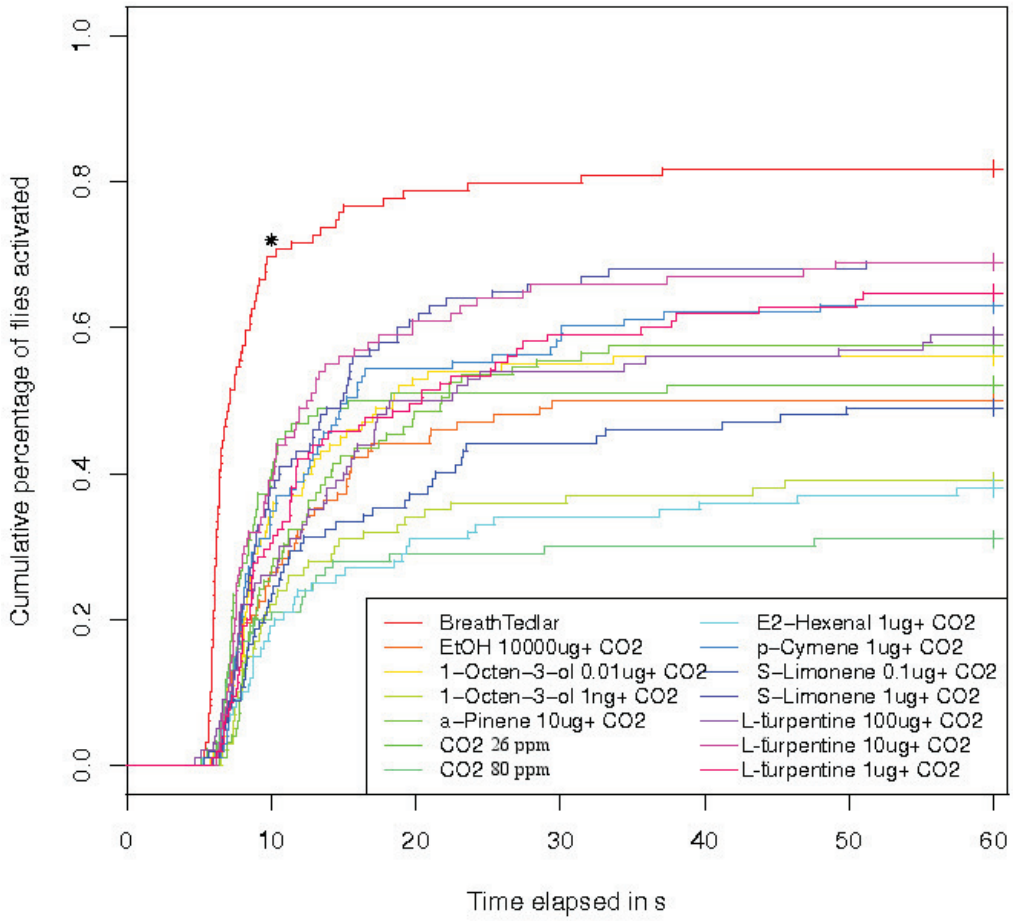

Figure 3.2.: **Cumulated percentage of teneral *G. pallidipes* activated over a time period of 60s to L-turpentine, S-(-)-limonene, *p*-cymene, α-pinene, E-2-hexenal, 1-octen-3-ol and to ethanol (solvent control) each in the presence of CO_2 at 26ppm, to CO_2 alone at 26ppm (negative control) and at 80ppm and to human breath delivered from a Tedlar® bag (positive control).** Breath activated flies significantly faster than any other treatment (Cox proportional hazard model; $p<0.05$). Of the test treatments L-turpentine released at 10μg/min and S-(-)-limonene at 1μg/min activated flies significantly faster than the solvent control (Cox proportional hazard model; $p<0.05$). N=100 for each treatment.

tunnel than males when stimulated with L-turpentine at 10μg/min (odds ratio 10.8, GLM(logit), $p < 0.05$, Table 3.4).

3.3.4. Behavioural responses of teneral *G. pallidipes* to mixtures of volatile plant compounds

The responses of teneral *G. pallidipes* were recorded to five mixtures of volatile plant compounds to investigate possible interactions between products. Two mixtures of α-pinene and S-(-)-limonene at two different ratios were tested to investigate whether these two compounds could account for the attraction to L-turpentine. The analysis of L-turpentine by gas-chromatography revealed a relative abundance of α-pinene of ~55% and of limonene of ~10% (Figure 2.1, Table 2.2). This suggested a ratio of 5:1 of α-pinene to limonene in a first test mixture. A second ratio was established from the fact that α-pinene at 10μg/min and S-(-)-limonene at 1μg/min on their own were already effective, suggesting a 9:1 ratio of these two compounds for another test mixture. Neither of these mixtures succeeded to account for the activity of L-turpentine at 10μg/min in terms of number of flies activated or the induced flight behaviour. On the contrary, both mixtures activated slightly less flies than when α-pinene and S-(-)-limonene were tested on their own. The 5:1 α-pinene and S-(-)-limonene mixture activated the flies significantly slower than L-turpentine at 10μg/min (Cox proportional hazard model, $p<0.05$, Figure D.1).

The two L-Turpentine - 1-octen-3-ol mixtures (ratios 10:0.01 and 10:0.1) were comparable to 1-octen-3-ol at 0.001μg/min and L-turpentine at 10μg/min for all the behavioural criteria recorded (Tables 3.3 and 3.5). Mixtures including 1-octen-3-ol at 0.1μg/min activated flies equally as fast as L-turpentine at 10μg/min, and both of these treatments activated flies slightly faster than the mixture including 1-octen-3-ol at 0.01μg/min (Figure D.1). Adding E-2-hexenal at 1μg/min to L-turpentine at 10μg/min did not influence the efficacy of L-turpentine on its own in terms of numbers of flies activated or numbers of flies induced to pass through to the odour source zone of the wind tunnel, but this treatment activated flies slightly slower than L-turpentine alone (Figure D.1).

Table 3.4.: **Odds ratios for behavioural responses of teneral female and male *G. pallidipes* to L-turpentine, S-(-)-limonene, *p*-cymene, α-pinene, E-2-hexenal, 1-octen-3-ol and to ethanol (solvent control) each in the presence of CO_2 at 26ppm, to CO_2 alone at ~26ppm (negative control) and at ~80ppm, and to human breath delivered from a Tedlar® bag (positive control);** N=50 for each treatment and sex. An odds ratio > 1 indicates that the behavioural response is more likely to occur in females than males, while an odds ratio < 1 indicates the opposite. Values in bold indicate significant differences between females and males (GLM(logit), $p < 0.05$).

Substance and release rate	**Activation** [%]	**Exit** [%]	**Passing 1/4 WT** [%]	**Passing Midline** [%]	**Passing 3/4 WT** [%]	**Source** [%]
Ethanol 10000μg/min	0.923	1.090	0.388	0.255	0.375	1.000
CO_2 ~26ppm	1.379	1.539	1.536	3.273	3.128	5.454e+07
CO_2 ~80ppm	0.832	0.762	0.468	0.320	0.490	0.000
S-(-)-Limonene 0.1μg/min	1.907	**2.627**	1.313	1.000	1.000	1.000
S-(-)-Limonene 1μg/min	0.755	0.846	0.903	3.244	2.550	3.907
$\alpha-$Pinene 10μg/min	0.609	0.669	0.503	0.561	0.577	0.716
p-Cymene 1μg/min	0.768	0.668	0.531	0.351	0.534	0.734
E-2-hexenal 1μg/min	1.185	1.658	1.000	5.444	3.123	9.679e+07
1-octen-3-ol 0.001μg/min	0.919	0.803	1.976	2.136	1.000	4.741e+07
1-octen-3-ol 0.01μg/min	1.000	1.390	1.120	1.536	0.783	0.653
L-Turpentine 1μg/min	2.111	1.941	0.485	0.625	1.170	3.907
L-Turpentine 10μg/min	1.325	1.670	**2.571**	**2.563**	2.875	**10.756**
L-Turpentine 100μg/min	0.921	1.175	0.903	0.762	1.000	0.235
Human breath delivered from Tedlar® bag 5l/min	0.577	0.803	0.724	0.664	0.762	1.379

Table 3.5.: **Behavioural responses of teneral *G. pallidipes* to mixtures of volatile plant compounds;** N=100 for each treatment. Asterisks ('*') indicate a significant difference from the solvent control, stars ('$\star$') a significant difference from the positive control (see Table3.3; GLM(logit),'$^{*}_{\star}$' $p < 0.05$; '$^{**}_{\star\star}$' $p < 0.01$; '$^{***}_{\star\star\star}$' $p < 0.001$).

Substance and release rate	**Activation** [%]	**Exit** [%]	**Passing 1/4 WT** [%]	**Passing Midline** [%]	**Passing 3/4 WT** [%]	**Source** [%]
L-Turpentine 10μg/min + E-2-hexenal 1μg/min	$58_{\star\star\star}$	$42_{\star\star\star}$	$31_{\star\star}$	$25^{*}_{\star\star}$	$16_{\star\star}$	$10_{\star\star}$
L-Turpentine 10μg/min + 1-octen-3-ol 0.01μg/min	$57_{\star\star\star}$	$45_{\star\star\star}$	$30_{\star\star\star}$	$24^{*}_{\star\star}$	$11_{\star\star\star}$	$8_{\star\star}$
L-Turpentine 10μg/min + 1-octen-3-ol 0.1μg/min	$69^{**}_{\star}$	$55^{*}_{\star\star}$	$39^{**}_{\star}$	31^{**}	$13_{\star\star}$	$6_{\star\star\star}$
α-Pinene 9μg/min + S-(-)-limonene 1μg/min	$59_{\star\star\star}$	$48_{\star\star\star}$	$33^{*}_{\star\star}$	$25^{***}_{\star\star}$	$13_{\star\star}$	$8_{\star\star}$
α-Pinene 5μg/min + S-(-)-limonene 1μg/min	$51_{\star\star\star}$	$38_{\star\star\star}$	$24_{\star\star\star}$	$21_{\star\star}$	$10_{\star\star\star}$	$5_{\star\star\star}$

3.3.5. Behavioural responses of fed and subsequently starved *G. pallidipes* to volatile plant compounds

The responses of a total of 1000 fed and subsequently starved *G. pallidipes* were recorded to ten treatments (Table 3.6). Treatments included CO_2 at ~26ppm and EtOH in the presence of CO_2 as negative and solvent control, human breath delivered from a Tedlar® bag as positive control, L-turpentine at three release rates ranging from 1μg/min to 100μg/min, S-(-)-limonene at 0.1 and 1μg/min and *p*-cymene at 1μg/min all in the presence of CO_2 at 26ppm, and L-turpentine released at 10μg/min in the presence of CO_2 at 80ppm. 80ppm CO_2 corresponds approximately to the amount of CO_2 measured at the release cage with human breath delivered from a Tedlar® bag.

For fly activation three groups of treatments can be distinguished in terms of decreasing effect: 1) the positive control and L-turpentine released at 10μg/min in the presence of CO_2 at 26 and 80 ppm (90, 84 and 92% activation, respectively), 2) L-turpentine released at 1 and 100μg/min, S-(-)-limonene released at 0.1 and 1μg/min and *p*-cymene released at 1μg/min causing 55 to 71% activation, and 3) the negative and the solvent control that activated significantly less flies than the other treatments (19 and 31%, respectively, GLM(logit), $p > 0.5$). Human breath delivered from a Tedlar® bag and L-turpentine released at 10μg/min in the presence of CO_2 at 80ppm activated flies significantly faster than all other treatments and the negative and solvent control (Cox proportional hazard model, $p < 0.05$, Figure 3.3). L-turpentine released at 10μg/min in the presence of 26 ppm CO_2 approaches the level of the positive control, activating as many flies as human breath, but not as fast. L-turpentine released at 10μg/min activated flies significantly faster than CO_2 alone, the solvent control, S-(-)-limonene released at either 0.1μg/min or 1μg/min and *p*-cymene but does not differ from L-turpentine released at 1μg/min and 100μg/min (Cox proportional hazard model, $p<0.05$; Figure 3.3). The negative and the solvent control activated flies significantly slower than the other treatments except S-(-)-limonene at 0.1μg/min.

The same grouping of treatments is also visible for the other behavioural criteria, with the exception of flies flying through the first quarter and passing through the midline of the wind tunnel. With CO_2 released at 26ppm there was no difference between L-

turpentine released at 1μg/min in term of flies induced to pass through the first quarter (41%) and the midline (29%) and L-turpentine released at 10μg/min (44 and 34%, respectively, Table 3.6). Both of these L-turpentine treatments were significantly less effective than the positive control in terms of flies induced to pass through the first quarter (69%) and the midline (52%) (GLM(logit), $p < 0.05$, Table 3.6). L-turpentine released at 10μg/min induced as many flies to pass through the third quarter of the wind tunnel (22%) and to reach the source zone (18%) as the positive control (19 and 11%, respectively; $p > 0.05$), and significantly more than the solvent and the negative control ($< 3\%$, GLM(logit), $p < 0.01$). L-turpentine released at 10μg/min in the presence of CO_2 at 80ppm was also as effective as the positive control in term of flies induced to pass through the first quarter (68%), the midline (46%), the third quarter of the wind tunnel (23%) and to reach the source zone (18%, $p > 0.05$; Table 3.6).

3.3.6. Comparison of fed and subsequently starved *G. pallidipes* male and female behavioural responses to plant volatiles

When comparing the effects of the treatments on fed and subsequently starved *G. pallidipes* differences were observed between female and male responses to volatile plant compounds (Table 3.7). The chances of females responding to S-(-)-limonene at 1μg/min, *p*-cymene at 1μg/min and L-turpentine at 10 and 100μg/min were considerably higher than for males responding to these treatments (odds ratios >2, GLM(logit), $p < 0.05$). Adding CO_2 at ~80ppm instead of ~26ppm to L-turpentine served to equilibrate the differences between males and females except for the criteria activation and exit (odds ratios 3.3 (ns) and 3.9 ($p< 0.05$) respectively, Table 3.7). No differences between male and female responses were recorded to human breath, to CO_2, to ethanol, to S-(-)-limonene released at 0.1μg/min and L-turpentine at 1μg/min in the presence of 26ppm CO_2.

Table 3.6.: **Behavioural responses of fed and subsequently starved *G. pallidipes* to L-turpentine, S-(-)-limonene, *p*-cymene and to ethanol (solvent control) each in the presence of CO_2 at 26 ppm unless otherwise stated, to CO_2 alone (negative control) and to human breath delivered from a Tedlar® bag (positive control);** N=100 for each treatment. Asterisks ('*') indicate a significant difference from the solvent control, stars ('$\star$') a significant difference from the positive control, (GLM(logit);'$^{*}_{\star}$' $p < 0.05$; '$^{**}_{\star\star}$' $p < 0.01$; '$^{***}_{\star\star\star}$' $p < 0.001$).

Substance and release rate	**Activation**	**Exit**	**Passing 1/4 WT**	**Passing Midline**	**Passing 3/4 WT**	**Source**
Ethanol 10000μg/min	$31_{\star\star\star}$	$17_{\star\star\star}$	$5_{\star\star\star}$	$5_{\star\star\star}$	$3_{\star\star}$	$1_{\star}$
CO_2 ~26ppm	$19_{\star\star\star}$	$8_{\star\star\star}$	$3_{\star\star\star}$	$2_{\star\star\star}$	$1_{\star\star}$	0
S-(-)-Limonene 0.1μg/min	$49^{**}_{\star\star\star}$	$39^{***}_{\star\star\star}$	$16^{*}_{\star\star\star}$	$12_{\star\star\star}$	$8_{\star}$	7
S-(-)-Limonene 1μg/min	$65^{***}_{\star\star\star}$	$41^{***}_{\star\star\star}$	$15^{*}_{\star\star\star}$	$14^{*}_{\star\star\star}$	$8_{\star}$	5
p-Cymene 1μg/min	$55^{***}_{\star\star\star}$	$47^{***}_{\star\star\star}$	$25^{***}_{\star\star\star}$	$15^{*}_{\star\star\star}$	$10_{\star}$	8
L-Turpentine 1μg/min	$71^{***}_{\star\star}$	$55^{***}_{\star\star\star}$	$41^{***}_{\star\star\star}$	$29^{***}_{\star\star}$	14^{*}	9^{*}
L-Turpentine 10μg/min	84^{***}	75^{***}	$44^{***}_{\star\star\star}$	$34^{***}_{\star}$	22^{***}	18^{**}
L-Turpentine 100μg/min	$64^{***}_{\star\star\star}$	$47^{***}_{\star\star\star}$	$23^{***}_{\star\star\star}$	$19^{**}_{\star\star\star}$	12^{*}	7
L-Turpentine 10μg/min + CO_2 ~80ppm	92^{***}	88^{***}	68^{***}	46^{***}	23^{***}	18^{**}
Human breath delivered from Tedlar® bag 5l/min	90^{***}	86^{***}	69^{***}	52^{***}	19^{**}	11^{*}

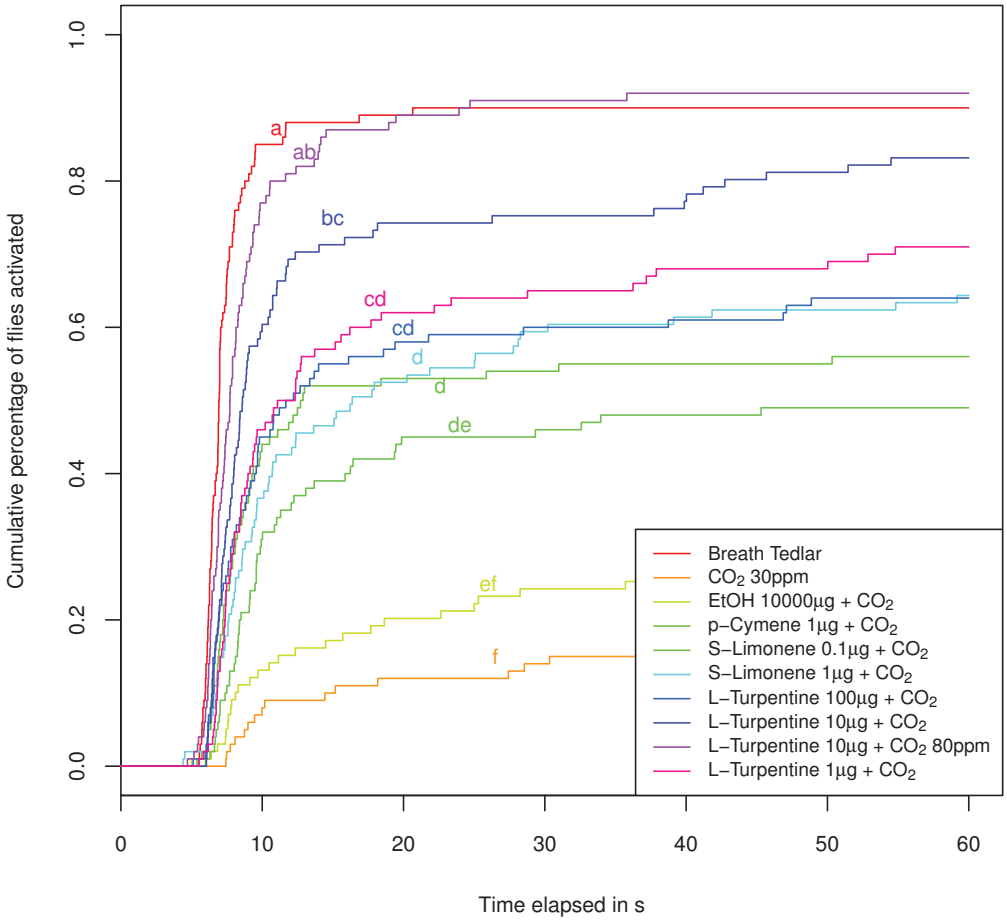

Figure 3.3.: **Cumulated percentage of fed and subsequently starved *G. pallidipes* activated over a time period of 60s by L-turpentine, S-(-)-limonene, *p*-cymene and ethanol (solvent control) each in the presence of CO_2 at 26ppm unless otherwise stated, to CO_2 alone (negative control) and to human breath delivered from a Tedlar® bag (positive control).** The positive control activated flies significantly faster than any other treatment except L-turpentine released at 10μg/min in the presence of CO_2 at 80ppm (Cox proportional hazard model; $p < 0.05$); N=100 for each treatment.

Table 3.7.: **Odds ratios for behavioural responses of fed and subsequently starved female and male *G. pallidipes* to L-turpentine, S-(-)-limonene, *p*-cymene and to ethanol (solvent control) each in the presence of CO_2 at 26ppm unless otherwise stated, to CO_2 alone (negative control) and to human breath delivered from a Tedlar® bag (positive control) as an index of effect;** N=50 for each treatment and sex. An odds ratio > 1 indicates that the behavioural response is more likely to occur in females than males, while an odds ratio < 1 indicates the opposite. Values in bold indicate significant differences between females and males (GLM(logit), $p < 0.05$).

Substance and release rate	**Activation**	**Exit**	**Passing 1/4 WT**	**Passing Midline**	**Passing 3/4 WT**	**Source**
Ethanol 10000μg/min	1.325	1.536	1.532	1.532	2.042	0.000
CO_2 ~26ppm	1.139	0.574	2.042	1.000	0.000	1.000
S-(-)-Limonene 0.1μg/min	1.758	1.287	1.000	1.465	0.574	0.734
S-(-)-Limonene 1μg/min	**2.698**	**4.373**	**8.432**	**7.579**	1.678e+08	9.495e+07
p-Cymene 1μg/min	1.926	2.077	**2.705**	2.538	3.907	2.667
L-Turpentine 1μg/min	1.102	1.275	1.282	1.339	1.000	2.136
L-Turpentine 10μg/min	0.742	0.725	**2.705**	**3.020**	**3.451**	**4.472**
L-Turpentine 100μg/min	**2.923**	1.761	2.250	**3.162**	**5.268**	5.444
L-Turpentine 10μg/min + CO_2 ~80ppm	3.273	**3.917**	1.556	1.381	1.405	1.536
Human breath delivered from Tedlar® bag 5l/min	1.000	1.465	1.325	1.379	0.878	1.227

3.3.7. The effect of the release dose of L-turpentine on the behaviour of fed and subsequently starved *G. pallidipes*

To investigate the effect of the dose of L-turpentine on *G. pallidipes* behaviour odds ratios were calculated (Table 3.8). L-turpentine released at 10μg/min showed a significantly higher probability of activating and inducing flies to exit the release cage than L-turpentine at 1 and 100μg/min (odds ratios 2.14 and 2.95, respectively, GLM(logit), $p < 0.05$). L-turpentine at 10μg/min also showed a significantly higher probability of inducing flies to pass the first, the second and the third quarter of the wind tunnel and to conduct a local search in the odour source zone in the wind tunnel (odds ratios 2.3 - 3.4, GLM(logit), $p < 0.05$, Table 3.8). Adding ~80ppm CO_2 to L-turpentine at 10μg/min augmented the effect at the level of passing the first quarter and the midline of the wind tunnel when comparing the 10 and 1μg/min L-turpentine release rates at the two CO_2 levels used. Furthermore CO_2 at 80ppm significantly increased the chances that flies stimulated with L-turpentine at 10μg/min exited the release cage and passed through the first quarter of the wind tunnel over that observed with L-turpentine at the same release rate but at 26ppm CO_2 (odds ratios 2.23 and 2.36, respectively; GLM(logit), $p < 0.05$, Table 3.8). .

3.3.8. Behavioural responses of *G. swynnertoni* and *G. brevipalpis* to volatile plant compounds

G. swynnertoni responses to volatile plant compounds and human breath

The responses of a total of 80 teneral *G. swynnertoni* were recorded to four treatments (Figure 3.4). Treatments included S-(-)-limonene released at 0.1 and 1μg/min, L-turpentine at 10μg/min in the presence of CO_2 at ~26ppm and human breath delivered from a Tedlar® bag.

L-Turpentine was the best treatment activating (85%) and inducing exit from the release cage (75%), significantly more flies than human breath (55 and 35%, respectively; GLM(logit); $p < 0.05$; Figure 3.4). L-Turpentine induced 30% of the flies to pass through

Table 3.8.: **Odds ratios for behavioural responses of fed and subsequently starved *G. pallidipes* to L-turpentine released at 1μg/min, 10μg/min and 100μg/min in the presence of CO_2 at ~26ppm and to L-turpentine at 10μg/min in the presence of CO_2 at ~80ppm as an index of effect;** N=100 for each treatment. Values > 1 indicate that the behavioural response is more likely in the presence of the test treatment (rows) than the control treatment (columns) while values < 1 indicate the opposite. Values in bold indicate significant differences between the treatments (GLM(logit), $p < 0.05$) .

Control Treatment	L-Turpentine 1μg/min[a]	L-Turpentine 10μg/min[a]	L-Turpentine 10μg/min[b]	L-Turpentine 100μg/min[a]
L-Turpentine 1μg/min[a]:				
Activation		**0.466**	**0.213**	1.377
Exit		**0.407**	**0.183**	1.378
Passing 1/4 WT		0.884	**0.374**	**2.326**
Passing Midline		0.793	**0.479**	1.861
Passing 3/4 WT		0.577	0.545	1.317
Source		0.451	0.483	1.550
L-Turpentine 10μg/min[a]:				
Activation	**2.144**		0.457	**2.953**
Exit	**2.455**		**0.448**	**3.383**
Passing 1/4 WT	1.131		**0.423**	**2.630**
Passing Midline	1.261		0.605	**2.347**
Passing 3/4 WT	1.733		0.944	**2.282**
Source	2.220		1.072	**3.439**
L-Turpentine 10μg/min[b]:				
Activation	**4.697**	2.190		**6.469**
Exit	**5.476**	**2.231**		**7.547**
Passing 1/4 WT	**2.672**	**2.364**		**6.217**
Passing Midline	**2.086**	1.654		**3.881**
Passing 3/4 WT	1.835	1.059		**2.417**
Source	2.071	0.933		**3.209**
L-Turpentine 100μg/min[a]:				
Activation	0.726	**0.339**	**0.155**	
Exit	0.726	**0.296**	**0.133**	
Passing 1/4 WT	**0.430**	**0.380**	**0.161**	
Passing Midline	0.537	**0.426**	**0.258**	
Passing 3/4 WT	0.759	**0.438**	**0.414**	
Source	0.645	**0.291**	**0.312**	

[a] with 26ppm CO_2
[b] with 80ppm CO_2

the first quarter, 20% the midline and 10% the third quarter of the wind tunnel and to conduct a local search within the upwind end of the wind tunnel while human breath induced 15, 10 and 5%, respectively, of flies to do the same. S-(-)-limonene at 0.1μg/min also activated significantly more flies (85%) than human breath and induced 65% to exit the release cage, but none of the flies induced to exit the release cage passed through the first quarter of the wind tunnel. S-(-)-limonene at 1μg/min activated and induced less flies to exit the release cage than at 0.1μg/min but 5% of the flies passed the midline of the wind tunnel. The behavioural criteria 'passing the first quarter of the wind tunnel' to 'source' are of less informative values due to the low numbers of flies tested.

G. brevipalpis responses to volatile plant compounds and human breath

The behavioural responses of teneral, fed and subsequently starved and pregnant *G. brevipalpis* were recorded to L-turpentine released at 1, 10 and 100μg/min, ethanol and acetone each in the presence of CO_2 at 26ppm, CO_2 alone and to human breath (Table 3.9). EtOH and CO_2 alone activated less than 36% of the teneral and fed and subsequently starved *G. brevipalpis* and only 9% of the teneral and 0 fed and subsequently starved flies activated with CO_2 reached the source zone, while with EtOH alone none of the activated flies passed through the third quarter of the wind tunnel. L-Turpentine released at 1, 10 and 100μg/min activated between 63 and 93% *G. brevipalpis*, induced 54 to 84% to exit the release cage and between 7 and 15% reached the source zone. These treatment effects approach the response level to human breath, that activated ~75% of flies, induced exit in 66-73%, with 16-26% reaching the source zone. Acetone activated ~50% of teneral and fed and subsequently starved flies, induced exit in about 40% with 7 to 10% reaching the source zone. In a preliminary test L-turpentine released at 10μg/min and human breath were also tested with pregnant female *G. brevipalpis*. The state of the pregnancy was advanced with a second to third stage larvae, 4 to 3 days prior to larviposition. To prevent early abortion flies were only starved for 3 to 5 days before testing. With human breath no pregnant female was activated while with L-turpentine 25% were activated, exited the release cage and passed through to the third

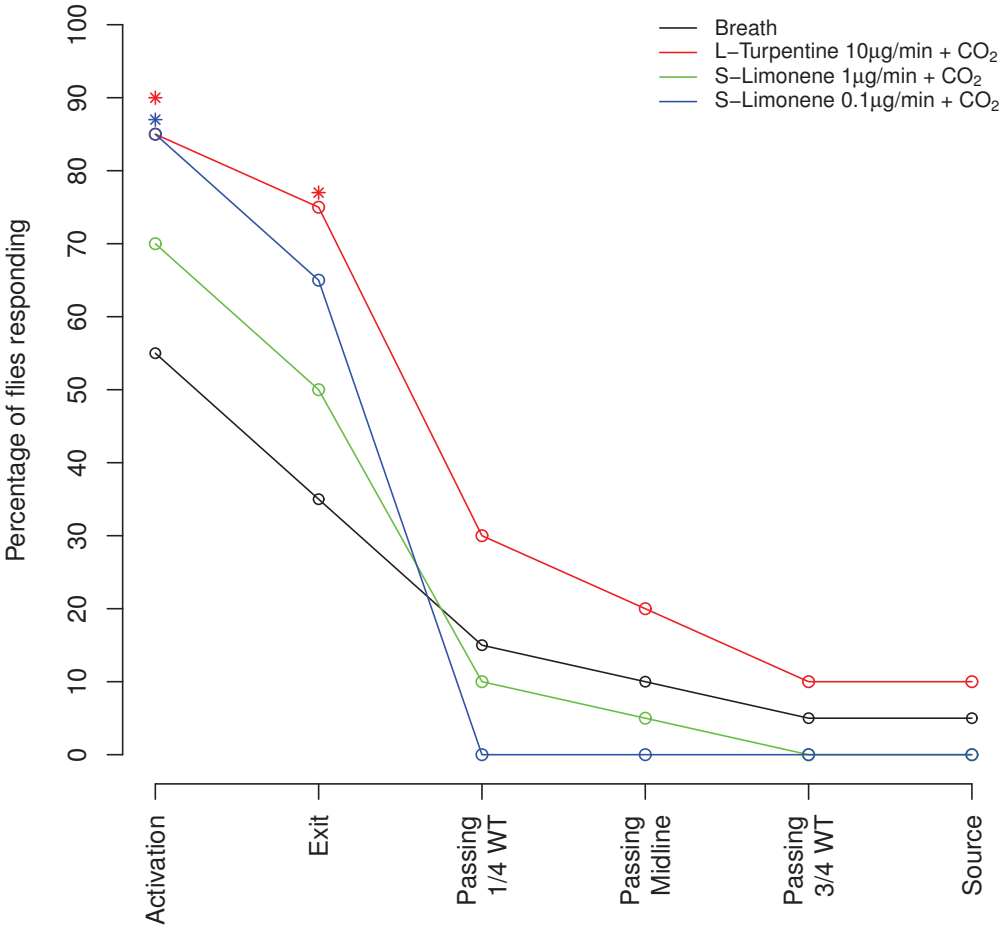

Figure 3.4.: **Behavioural responses of *G. swynnertoni* to L-turpentine and S-(-)-limonene each in the presence of CO_2 at 26ppm and to human breath;** N=20 for each treatment. Asterisks indicate a significant difference from breath (GLM(logit); $p < 0.05$; colours correspond to legend).

quarter of the wind tunnel. One fly (12.5%) performed a local search in the upwind end of the wind tunnel.

Table 3.9.: **Behavioural responses of *G. brevipalpis* to L-turpentine released at 1, 10 and 100μg/min, to acetone, and to ethanol (solvent control) each in the presence of CO_2 at 26 ppm, to CO_2 alone (negative control) and to human breath (positive control);** N=5-19. No statistical analysis was performed due to the low and unequal number of tested flies.

Substance and release rate	**Activation** [%]	**Exit** [%]	**Passing 1/4 WT** [%]	**Passing Mid-line** [%]	**Passing 3/4 WT** [%]	**Source** [%]	**N**
	teneral *G. brevipalpis*						
Ethanol 10000μg/min	16.7	16.7	8.3	8.3	0.0	0.0	12
CO_2 ~26ppm	36.4	18.2	18.2	18.2	18.2	9.1	11
Acetone 10000μg/min	50.0	40.0	40.0	30.0	20.0	10.0	10
L-Turpentine 1μg/min	92.3	84.6	46.2	15.4	15.4	7.7	13
L-Turpentine 10μg/min	63.6	54.5	45.5	27.3	18.2	9.1	11
L-Turpentine 100μg/min	69.2	69.2	61.5	23.1	15.4	15.4	13
Human breath	73.7	73.7	63.2	52.6	31.6	26.3	19
	fed and subsequently starved *G. brevipalpis*						
CO_2 ~26ppm	23.5	17.6	5.9	0.0	0.0	0.0	17
Acetone 10000μg/min	53.80	46.2	23.1	15.4	7.7	7.7	13
Human breath	75.0	66.7	41.7	41.7	16.7	16.7	12
	pregnant *G. brevipalpis* , starved for 3 to 5 days						
L-Turpentine 10μg/min	25.0	25.0	25.0	25.0	25.0	12.5	8
Human breath	0.0	0.0	0.0	0.0	0.0	0.0	5

3.3.9. Behavioural responses of *G. pallidipes* to L-turpentine, CO_2 and a visual target

The flights to and around a visual target of female fed and subsequently starved *G. pallidipes* stimulated with L-turpentine released at 1 and 10μg/min in the presence of CO_2 at ~80pmm, L-turpentine at 10μg/min in the presence of 26ppm CO_2, and CO_2 at 80ppm were analysed. For each treatment 20 flights were analysed. No differences were detected in the velocity of the upwind flights and of the flights around the target between the treatments. Equally, no statistical differences were detected in the total flight distance between the treatments. Flies stimulated with L-turpentine released at 1μg/min in the presence of 80ppm CO_2 covered a significantly longer distance in a zone around the target ($\pm$30cm from the center of the target in x and y directions, $\pm$50cm in z direction) than flies stimulated with either CO_2 alone or L-turpentine at 10μg/min in the presence of 26ppm CO_2 (mean distances were 6, 3.5 and 3m, respectively; $p < 0.05$, Wilcoxon rank-sum test; Figure 3.5). The same differences were recorded for the time spent around the target (mean time spent was 5.5, 3 and 3.5sec, respectively; $p < 0.05$, Wilcoxon rank-sum test; Figure 3.5).

To analyse the spatial flight pattern the the zone around the target was sliced into 10cm disks and the flight intensities in these disks were estimated by taking the co-ordinates of flight points recorded within each disk and applying kernel smoothing to these coordinates (Figures 3.6 to 3.9). Above 60cm from the floor only very few points were recorded and therefore those disks were excluded from analysis. The highest flight intensities were estimated between 20 and 40cm, below the sphere used as target, with the highest intensities downwind of and at the sides of the target. With L-turpentine released at 10μg/min in the presence of 26ppm CO_2 and with CO_2 alone a high flight intensity between 40 and 50cm from the floor directly at the surface of the sphere was estimated, corresponding to flies landing and walking on the sphere (Figures 3.6 and 3.9). With CO_2 alone the flights of *G. pallidipes* were less concentrated around the target, but spaced out more equally (Figure 3.9). The highest total flight intensity was recorded for L-turpentine released at 1μg/min in the presence of 80ppm CO_2, followed by L-turpentine at 10μg/min with 80 ppm CO_2, L-turpentine at 10μg/min with 26ppm CO_2

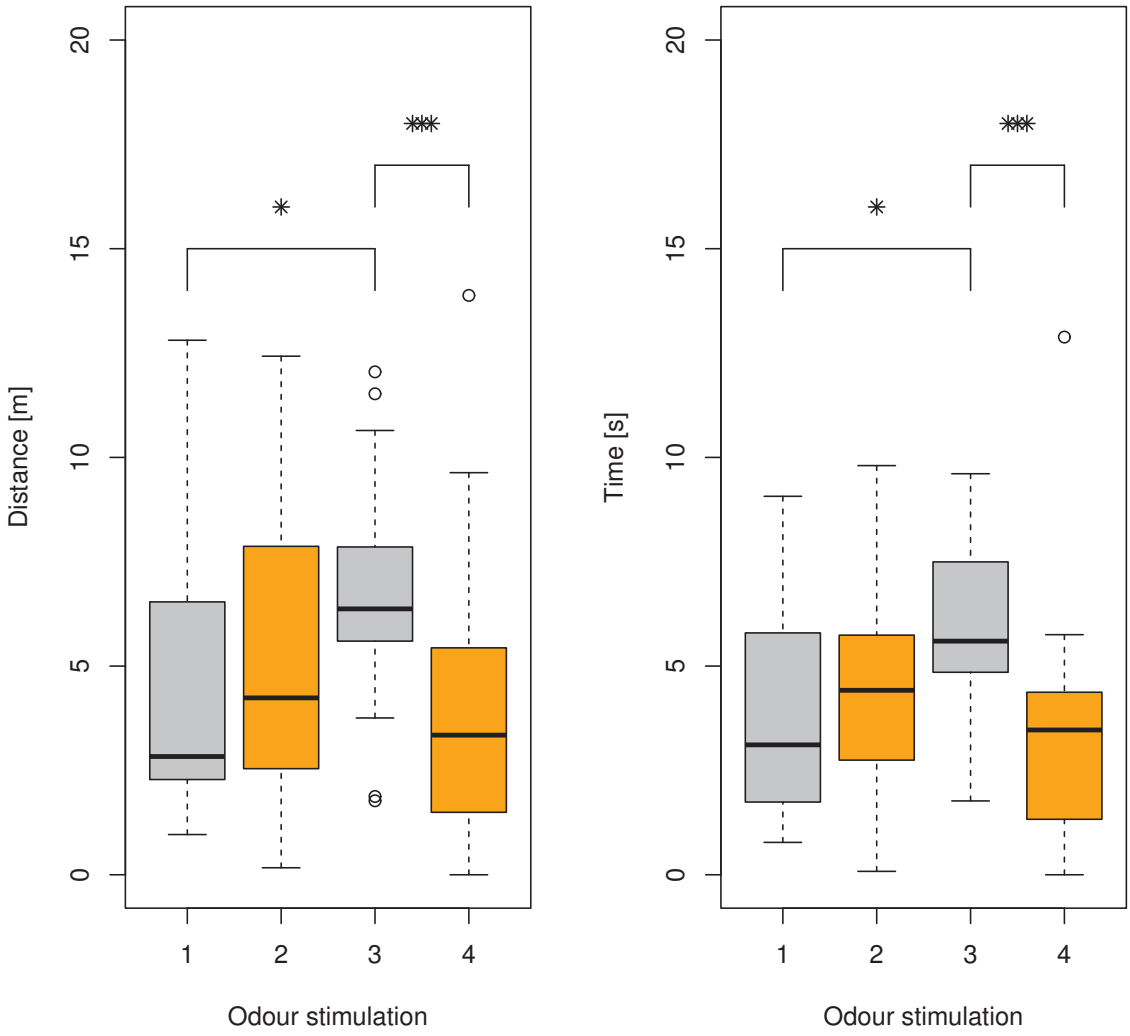

Figure 3.5.: **Distance covered and time spent by *G. pallidipes* stimulated with L-turpentine released at 1 and 10μg/min in the presence of 26 or 80ppm CO_2 and to CO_2 alone in a zone around the target.** N=20 for each treatment. Odour stimulation: 1 L-turpentine released at 10μg/min with 26ppm CO_2; 2 L-turpentine at 10μg/min with 80ppm CO_2; 3 L-turpentine at 1μg/min with 80ppm CO_2; and 4 CO_2 at 80ppm. Treatment 3 was statistically different from treatments 1 and 4 ($p < 0.05$ and $p < 0.01$, respectively; Wilcoxon rank-sum test).

and CO_2 at 80ppm alone (kernel density estimates of 3608.9, 2835.4, 2499.0 and 2198.8, respectively). Fitted multitype point pattern models revealed that those spatial point patterns were significantly different according to treatment ($p < 0.01$; inhomogenous point pattern model (Poisson)).

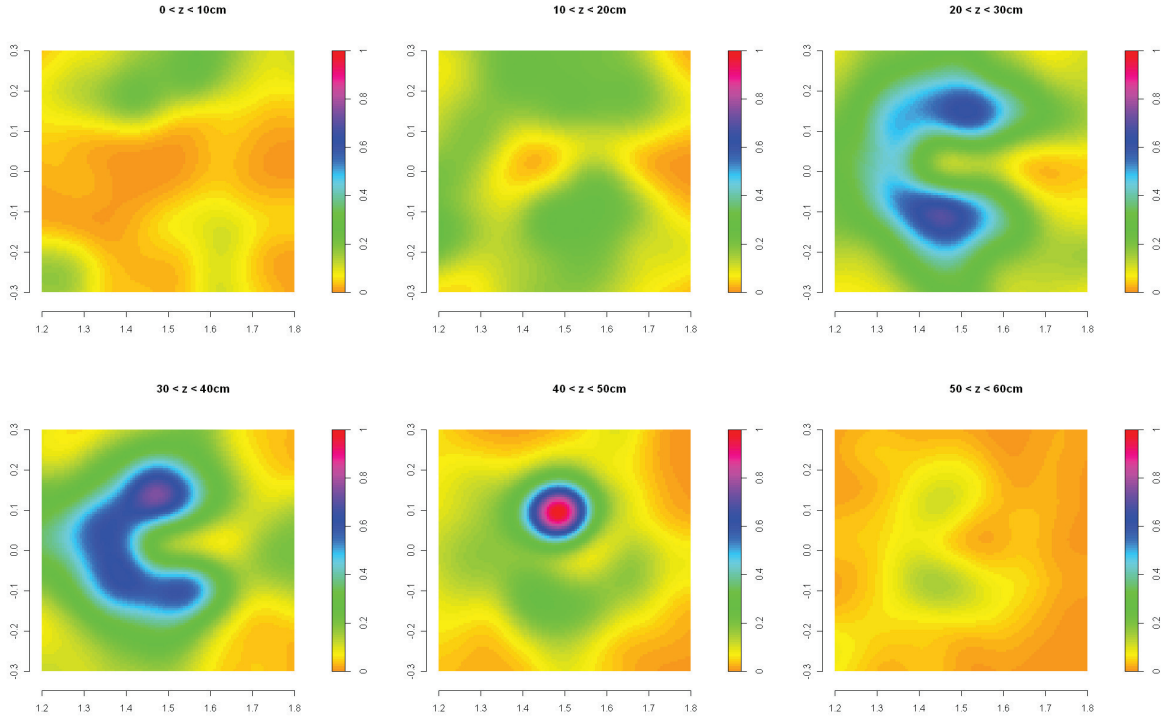

Figure 3.6.: **Estimated smoothed kernel intensities of flights by *G. pallidipes* around a blue sphere in presence of L-turpentine released at 10μg/min with 26 ppm CO_2;** N=20 for each treatment. The area depicted in each intensity plot represents a plane within a column 60cm wide, 60cm long and 10cm high around the sphere, starting from the floor of the wind tunnel. Plots represented for successive 10cm disks from bottom (top left) to top (lower right). Intensities are scaled 0-1 to facilitate comparison. The highest flight intensities were recorded between 20 and 40cm from the floor in the z direction and at a spot between 40 and 50cm from the floor, corresponding to flies landing and walking on the sphere.

The spatial point pattern of the upwind flights was also analysed in a zone downwind of the target (x: 0.45–1.05m, y=-0.3–0.3m, z=0-1m, target at x=1.5m, y=0m, z=0.5;

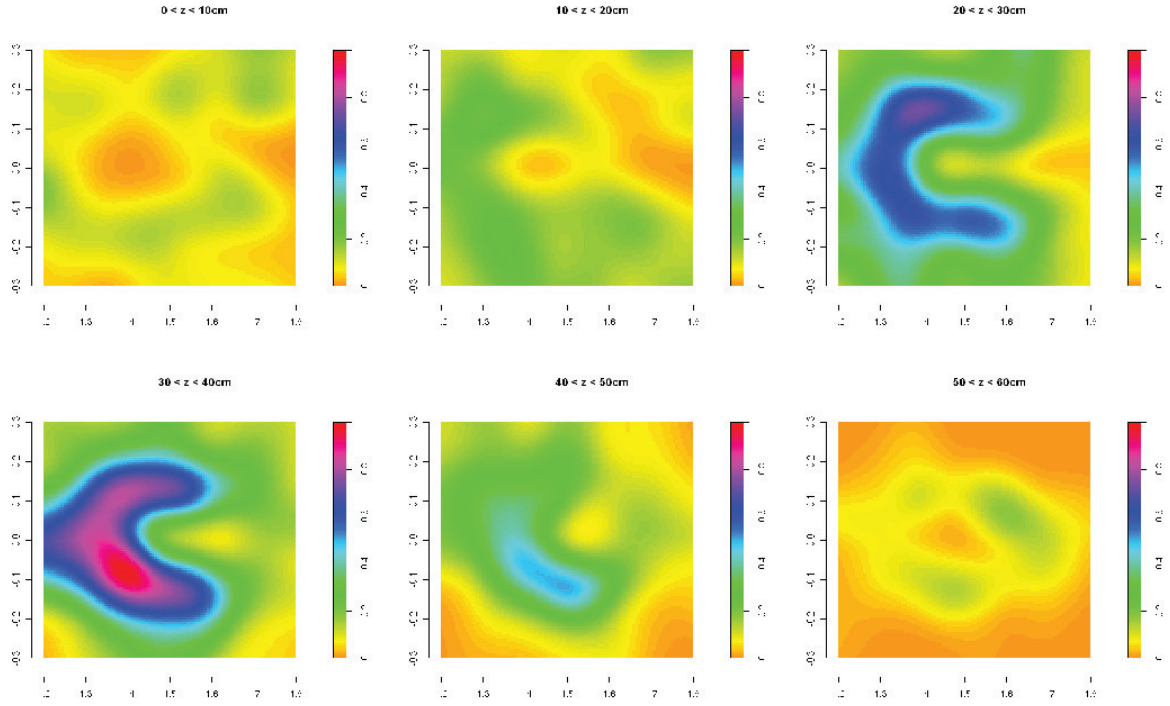

Figure 3.7.: **Estimated smoothed kernel intensities of flights by *G. pallidipes* around a blue sphere in presence of L-turpentine released at 10μg/min with 80 ppm CO_2;** N=20 for each treatment. For details see legend to Figure 3.6. The highest flight intensities were recorded between 20 and 40cm from the floor in the z direction.

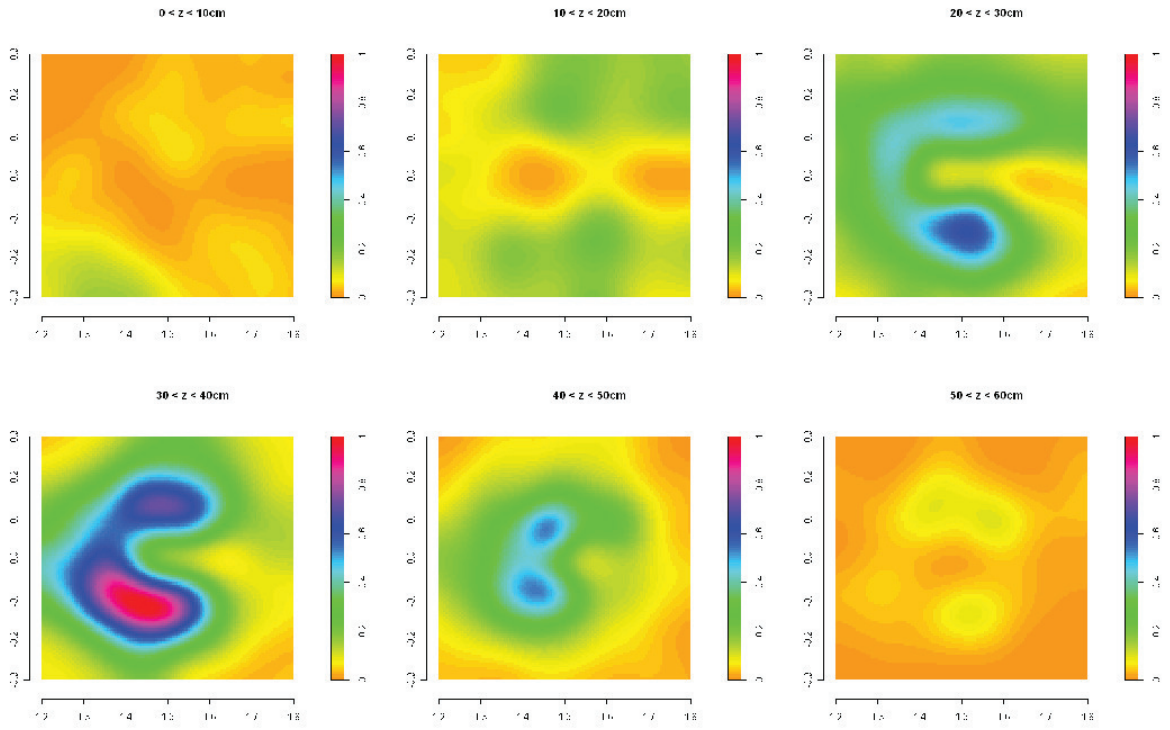

Figure 3.8.: **Estimated smoothed kernel intensities of flights by *G. pallidipes* around a blue sphere in presence of L-turpentine released at 1μg/min with 80 ppm CO_2;** N=20 for each treatment. For details see legend to Figure 3.6. The highest flight intensities were recorded between 20 and 40cm from the floor in the z direction.

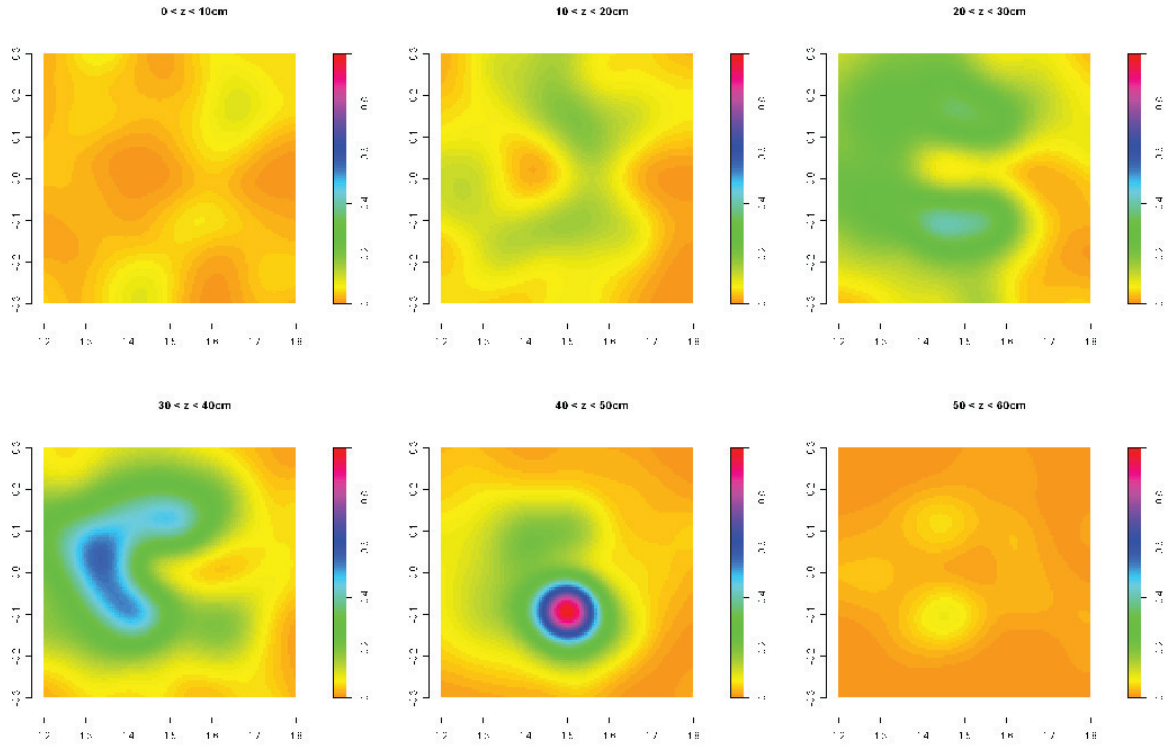

Figure 3.9.: **Estimated smoothed kernel intensities of flights by *G. pallidipes* around a blue sphere in presence of CO_2 at 80ppm;** N=20 for each treatment. For details see legend to Figure 3.6. The highest flight intensities were recorded between 20 and 40cm from the floor in the z direction and on a spot between 40 and 50cm from the floor, corresponding to flies landing and walking on the sphere.

Figure 3.10). With L-turpentine released at either 1 and 10μg/min in the presence of 80ppm CO_2 the highest intensities were recorded between 30 and 40cm in height (Figure 3.10, treatments 2 and 3) while with CO_2 alone or L-turpentine at 10μg/min with 26ppm CO_2 the estimated intensities were more distributed between 20 and 40cm in height (Figure 3.10, treatments 1 and 4). Fitted multitype point pattern models revealed that the total spatial point patterns (all disks combined) are significantly different between L-turpentine released at 1μg/min in the presence of 80ppm CO_2, L-turpentine at 10μg/min with 26ppm CO_2 and CO_2 alone but not between L-turpentine at 1 or 10μg/min in the presence of 80ppm CO_2 ($p < 0.01$; inhomogenous point pattern model (Poisson)).

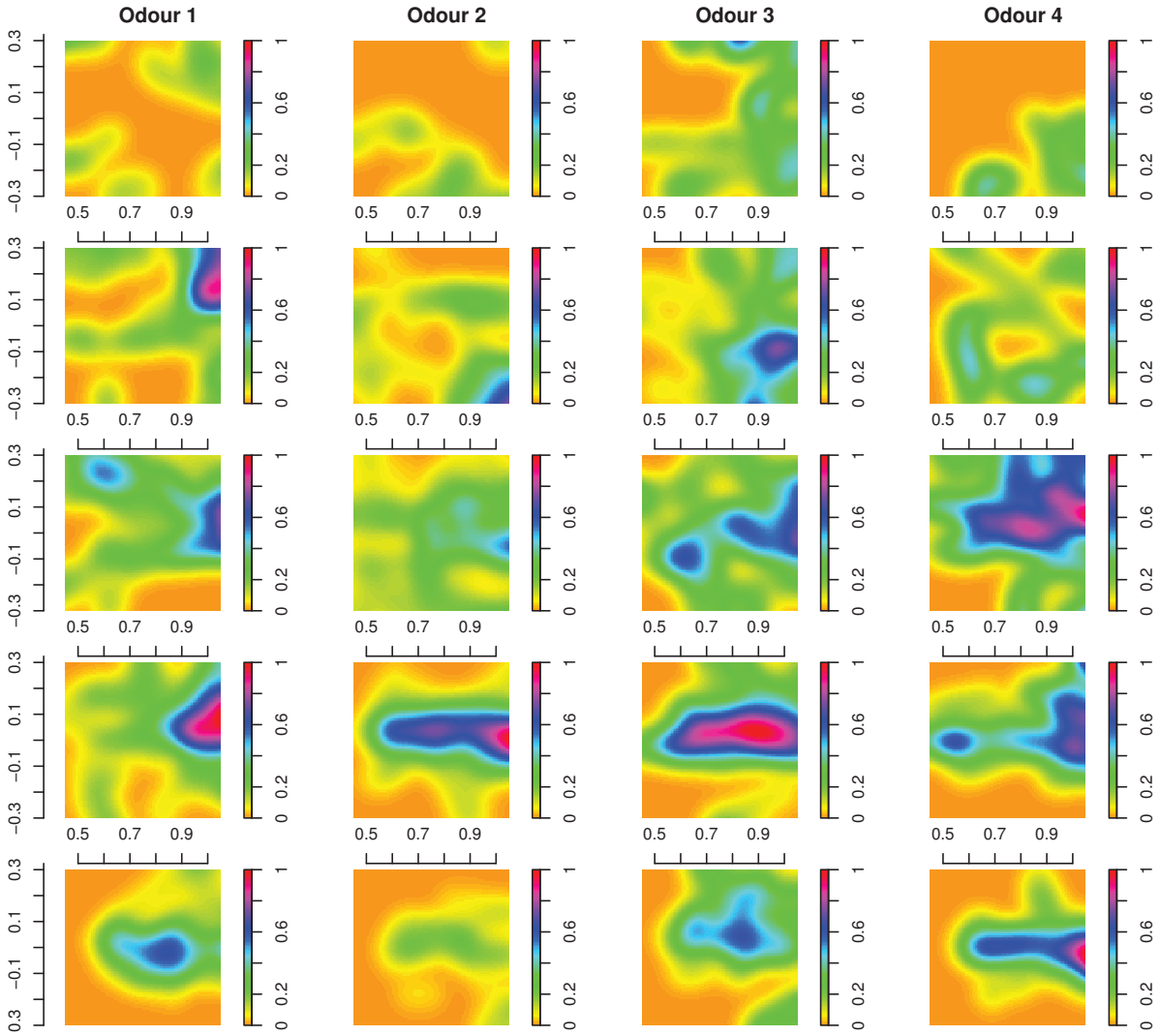

Figure 3.10.: **Estimated smoothed kernel intensities of upwind flights by *G. pallidipes* to a blue sphere in presence of CO_2 at 80ppm alone (Odour 1), L-turpentine released at 1μg/min in the presence of 80ppm CO_2 (Odour 2), L-turpentine at 10μg/min in the presence of 80ppm CO_2 (Odour 3) and L-turpentine at 10μg/min in the presence of 26ppm CO_2 (Odour 4);** N=20 for each treatment. The area depicted in each intensity plot represents a plane downwind of the sphere within a column 60cm wide, starting at 45cm from the downwind end of the wind tunnel, 60cm long, starting at 20cm from the side of the wind tunnel, and 10cm high, starting from the floor of the wind tunnel. Top row 0-10cm, 2^{nd} row 10-20cm, 3^{rd} row 20-30cm, 4^{th} row 30-40cm, bottom row 40-50cm in height. Intensities are scaled to 0-1 to facilitate comparison. The highest flight intensities were recorded between 20 and 40cm from the floor in the z direction.

3.4. Discussion

Our results show that tsetse flies respond to a series of single plant compounds and their mixtures. So far only β-caryophyllene and 1-octen-3-ol have been tested in a wind tunnel study [157]. A problem with tsetse flies in wind tunnel studies is the low rate of activation of flies that makes it very difficult to measure differences in behavioural responses to treatments. The flies need to be in a certain physiological state (hungry, but still able to fly) to be activated in an artificial environment. The identification of this time frame is not easy, depending on the reserves of the fly derived from the puparial state and the amount of blood taken during the last blood meal [28]. Most studies could only differ between activation (e.g. turning in the wind) and attraction [131, 157, 180]. A second problem in wind tunnel studies with tsetse flies is their high flight speeds that excess 4m/s in laboratory and field studies [25, 55]. The flight trajectory of the flies is not discernable by the eye alone, but filming the trajectory poses other problems regarding lighting and floor cover to increase the contrast between fly and surroundings. By combining the sensitising effect of CO_2 [37, 49, 161] with the test compounds we were able to increase the number of activated flies by tenfold and were therefore able to detect differences in the behavioural responses of tsetse flies to treatments. This effect of CO_2 has already been shown for mosquitoes [37] and for tsetse flies in combination with humidity [49]. A low dose of CO_2 close to the detection limit [161] was chosen in order not to interfere with any effects of the volatile plant products tested, as high amounts of CO_2 have been shown to elicit upwind anemotaxis in tsetse flies in the laboratory and the field [32, 49, 70, 132, 139].

The single plant compounds tested with the best activation potential were S-(-)-limonene and *p*-cymene, activating 10 and 20% more flies than either α-pinene or 1-octen-3-ol, respectively, at their best doses, but most of those flies only crossed through to the middle of the wind tunnel. By contrast, although both α-pinene and 1-octen-3-ol activated fewer flies they had a greater capacity to evoke local search behaviour in the odour source zone at the upwind end of the wind tunnel by *G. pallidipes* than S-(-)-limonene and *p*-cymene. Limonene and *p*-cymene are structurally similar. While *p*-cymene is an aromatic related to monoterpenes ($C_{10}H_{14}$) with a propyl group, limonene is a cyclic monoterpene with

a closed ring with 1 double bond ($C_{10}H_{16}$) and a propenyl group. It seems that some compounds have the capacity to activate *G. pallidipes* while others induce local search behaviour at the upwind end of the wind tunnel. The green-leaf aldehyde E2-hexenal did not have any effect on the behavioural responses of *G. pallidipes* activating only as many flies as CO_2 alone or in combination with the solvent. It is possible that either the wrong dose was tested, either under- or overdosed, or that E2-hexenal is of no interest to tsetse flies as it can be found in nearly all green plants and is therefore present in tsetse habitats at rather high doses. Considering the strong EAG responses from *G. pallidipes* to E2-hexenal, the second hypothesis seems less probable, because the maintenance of the sensory system is costly and sensory responses to products without any information content should therefore be less likely.

The monoterpene mixture comprising L-turpentine showed a very good activating effect on *G. pallidipes*, *G. swynnertoni* and *G. brevipalpis* and also induced many flies to perform local search behaviours at the upwind end of the wind tunnel. At the optimal dose tested (10μg/min), L-turpentine was comparable to human breath in terms of activating and inducing local search behaviour by *G. pallidipes* and *G. swynnertoni* at the upwind end of the wind tunnel. The amounts of CO_2 present in human breath in our wind tunnel studies (~60–80ppm) was 2-3 times higher than the amount of CO_2 used in combination with the plant compounds (26ppm). Combining L-turpentine with an amount equal to CO_2 present in breath eliminated every difference in the efficacy of L-turpentine as an attractant compared to human breath. This was not only measured under the behavioural criteria 'activation', 'exit', 'passing 1/4 WT', 'passing the midline', 'passing 3/4 WT' and arriving at the 'source', but also in the speed to activation of flies. Two main components of turpentines are α-pinene and limonene, both eliciting EAG responses in tsetse sp. from all three habitat groups (*G. pallidipes, G. swynnertoni, G. brevipalpis, G. f. fuscipes* and *G. p. gambiensis*). The question arose if a combination of those two products, already active on their own, can account for the effect of L-turpentine. To test this hypothesis two mixtures of α-pinene and limonene at different ratios (5:1 as in L-turpentine and at a 9:1 ratio, both at 10μg/min total amount of products released, i. e. the same total amount as the optimal dose of L-turpentine

(10μg/min)) were tested. Neither of these mixtures was as effective as L-turpentine, but the 9:1 mixture approached the level of biological activity as L-turpentine in terms of flies crossing three quarters of the wind tunnel length but not in terms of activation time. It can therefore be assumed that at least some of the other compounds in L-turpentine add to its effect even though they did not evoke strong EAG-responses from *G. pallidipes* antennae. At the highest dose tested (100μg/min), L-turpentine was less active than at the weaker doses (1 and 10μg/min), possibly due to 3-carene, present in L-turpentine at a small percentage. 3-Carene on its own at 1μg/min activated only 4% of the tsetse flies and in combination with S-(-)-limonene at a 4 times lower dose completely inhibited the response to S-(-)-limonene, suggesting that this compound is either repellent or inhibitory (see Appendix C). It is therefore possible, that the amount of 3-carene in L-turpentine at 100μg/min is sufficiently high to render L-turpentine less attractive. Inhibitory effects of high release rates of 3-carene have been reported for bark beetles, where 3-carene is most attractive at ~100mg/day in field-traps [48].

A further question was if one could increase the efficacy of L-turpentine by adding either a E2-hexenal, a green-leaf volatile found in all green plants, or the known tsetse attractant 1-octen-3-ol to L-turpentine. Even though E2-hexenal on its own did not have any effect, the combination with terpenes could change the behavioural responses of tsetse flies. At the dose and ratio tested this was not the case: E2-hexenal did not alter the behavioural responses of *G. pallidipes* to L-turpentine in terms of activation or flight behaviour responses when added at 1μg/min to L-turpentine at 10μg/min. Visser [174, 175] showed that not only the amount of the GLVs is important but also the ratio of the compounds. Therefore it is possible that the ratio of L-turpentine and E2-hexenal tested was not appropriate to augment the attractiveness of *G. pallidipes* to L-turpentine alone.

1-octen-3-ol is an interesting compound. On its own it very quickly becomes overdosed (0.1μg/min), activating less flies than at its optimal dose of 0.01μg/min, but added to L-turpentine at 10μg/min the higher amount of 1-octen-3-ol (0.1μg/min) activated and induced flight in more flies than the lower amount (0.01μg/min). But neither of these L-turpentine plus 1-octen-3-ol treatments resulted in a better performance than

L-turpentine on its own. Adding an even higher amount of 1-octen-3-ol (1μg/min) to L-turpentine at 10μg/min resulted in a complete loss of the effect of L-turpentine, as did this dose of 1-octen-3-ol on its own.

The behavioural effects of the volatile plant compounds on *G. pallidipes* are supported by tests on *G. swynnertoni* and *G. brevipalpis* with both species showing similar trends in the behavioural responses to the volatile plant compounds tested. *G. swynnertoni* even showed a slightly better performance to L-turpentine than human breath, while *G. brevipalpis* responded best to human breath but was readily activated by L-turpentine at all doses tested.

Feeding and subsequently starving affected the behavioural responses of *G. pallidipes*. Generally, fed and starved *G. pallidipes* were more selective in their responses, only weakly responding to CO_2 on its own, but showing similar responses to single plant compounds and a better performance to L-turpentine. Fed flies were more prone to undertake longer flights (upwind and downwind) with more pronounced local search flights than teneral flies. This certainly is affected by the fact that tsetse flies need a first blood meal to fully develop their flight muscles [103], and having had a blood meal, probably have higher energy reserves [28, 77, 103]. Interestingly though, teneral *G. pallidipes* and *G. brevipalpis* showed a better response to human breath than fed flies (25% and 19% local search compared to 11% and 12% local search, respectively). This was rather unexpected as we activated flies with human breath in order to induce them to feed on the artificial silicon membrane, and tsetse flies are known to prefer the same host for subsequent blood meals as in their first meal [6, 61, 178]. It might be interesting to see whether the 'human' odour source is confounded by the odour source of 'bovine blood' on which they fed. The effects of the dose of L-turpentine was clearer for fed and subsequently starved than in teneral *G. pallidipes* in that teneral flies responded to all three doses similarly with a slight preference for the middle concentration (10μg/min), whereas fed and subsequently starved flies showed a clear preference for the 10μg/min dose. This is consistent with the fact that fed flies seem to be more selective in their responses than teneral flies.

No differences were detected in behavioural responses of female and male tsetse flies in

wind tunnel studies [26, 32, 78, 79], and in field studies [18, 103, 129, 130, 138, 168, 172] effects are either not clear or conflicting. In our study we also found no differences in behavioural responses of male and female *G. pallidipes* to human breath, CO_2 alone and 1-octen-3-ol, products usually associated with hosts, but we could detect differences in behavioural responses of males and females to volatile plant compounds (limonene, *p*-cymene and L-turpentine). The effects were more pronounced in fed flies than in teneral flies but they could already be detected for limonene and L-turpentine in that fed and subsequently starved females had a significantly higher chance (up to 8 times higher) to respond to limonene and L-turpentine than males. Females also responded better to the other single plant compounds tested than males, with the exception of teneral females responding to α-pinene. Interestingly, pregnant *G. brevipalpis* responded only to L-turpentine (N=8) but not human breath (N=5), although pregnant *G. brevipalpis* would take a blood meal every day if offered. One could speculate that volatile plant compounds not only carry information about hosts, but also indicate larviposition or resting sites, both of which are of greater importance due to the higher energy demands of flying in pregnant females than males.

To assess whether volatile plant compounds could be of use in the field, i.e. increasing the trap or target encounters, we needed to test if plant compounds could serve to attract tsetse flies to a target. In a first attempt to combine visual and olfactory stimuli we used a blue sphere as a target and the best treatment from previous experiments, L-turpentine, as an olfactory stimulus. We showed that L-turpentine in the presence of CO_2 increases the time spent and distance flown around a target and also affects the relative height of flight to the target and of the local search behaviour at the target. With L-turpentine, flight to the target was more directed than with CO_2 alone and the local search at the target was lower in elevation. This is particularly interesting as tsetse flies attack their hosts from below. The visual target alone did not activate tsetse flies, nor did it alter the proportion of flies activated or the time to activation by the respective odour. If a fly was activated without odour it did either not fly to the sphere or undertook a very short flight around the sphere. This indicates, that odours clearly modulate the behaviour of tsetse flies around a target [24, 26, 171]. This new method of

testing tsetse fly semiochemicals is promising as it allows to analyse the flight of flies in more detail, minimizing spatio-temporal constraints of wind tunnel studies due to the high flight velocities of tsetse flies and their long flying range. A flight of a tsetse fly stimulated by an odour in a 2.5m long wind tunnel without a sphere lasts about 2-3sec. This flight duration is on the mean doubled by the introduction of the sphere into the system.

To fully assess the potential of volatile plant products in tsetse fly control we need to include compounds with a lower vapour pressure in future wind tunnel studies. The combination of highly volatile monoterpenes with sesquiterpenes of low volatility might enhance the attractiveness of the mixture not only in the vicinity of the target but also from further away. β-caryophyllene could be an interesting candidate as it has a low vapour pressure, is present in both host emanations [87, 124, 154] and plants [80] and has already been shown to activate tsetse flies in a wind tunnel study [157].

4. General discussion and conclusion

Our findings show that tsetse flies possess receptor cells on their antennae for volatile end products of major biosynthetic and catabolic pathways of plants, and that these sensory responses permit the flies to respond to volatile plant compounds from a distance.
Comparison of turpentines and mixtures of green-leaf volatiles (GLVs) by GC-EAG, as well as comparison of EAG responses to volatile plant compounds, and the determination of the EAG response thresholds to some of these volatiles, permitted the identification of chemostimuli that may be implicated in the sensory ecology of tsetse flies (Chapter 2). Tsetse flies showed a low response threshold to L-turpentine and therebenthine, limonene, *p*-cymene, ocimene and myrcene. Of these, the L-turpentine and limonene chemostimulants approached the response threshold of 1-octen-3-ol. The low response threshold to 1-octen-3-ol corresponds to the high number of receptor cells (~50%) on a tsetse fly antennae responding to 1-octen-3-ol as shown by single-cell recording [39, 40, 177]. But the measured EAG-response thresholds of these products, even of 1-octen-3-ol, is still rather high compared to response thresholds of moths to their pheromone product that are detected in the pg range [7, 95, 96]. Following 1-octen-3-ol, the lowest response threshold was measured for L-turpentine, a simple monoterpene mixture only consisting of different $C_{10}H_{16}$–molecules. This product as well as its main components α-pinene and limonene, likewise with low EAG detection thresholds, were of main interest for further analysis, especially since limonene is found in the breath of humans as well as bovids, the main hosts of tsetse flies [79, 87, 133, 154].
Characterizing new chemostimulants is a first step in the direction of the developing new attractants for tsetse flies. The next, more important step, is to prove that these stimulants are not only detected by antennal sensory cells but can act as attractants

in controlled behavioural assays. The wind tunnel studies presented here (Chapter 3) present such a behavioural assays that permit to measure the attractiveness of a product and to estimate the amount of product needed to activate and attract tsetse flies. The use of a piezo-driven aerosol sprayer in combination with a static gas mixer, creating a defined turbulent odour plume, allowed us to determine with good precision the amount of product reaching the antenna. The known tsetse fly attractant, 1-octen-3-ol, was behaviourally active to as low as 50ppt. This amount is approximately a million times lower than the threshold for CO_2 (~30ppm). Interestingly, 1-octen-ol in the presence of CO_2 became behaviourally inactive or repellent already at amounts 10 times higher. Contrary to this, when combined with L-turpentine this higher amount did not alter the behaviour of tsetse flies to L-turpentine, but loss of activity was apparent at a dose 100 times higher of 1-octen-3-ol. This effect of test products reflects the importance not only of amounts tested but also of proportions of products in mixtures [174, 175]. Apart from 1-octen-3-ol and L-turpentine, none of the other tested products were able to produce a behavioural response at such low levels, but these products were more active in terms of activation and inducing flight behaviours at higher doses than 1-octen-3-ol.
Of the volatile plant compounds tested L-turpentine was especially interesting. This product not only activated flies nearly as fast as human breath, considered in this study as a positive control, but activated both teneral and fed and starved tsetse flies quickly and to a high degree, and also induced local search behaviour to the same extent as human breath. The difference in the activation time of human breath and L-turpentine is probably due to the lower amount of CO_2 in the tests with L-turpentine (26ppm) than present in breath (60-80ppm). Adding CO_2 at 80ppm to L-turpentine resulted in equal activation times for breath and L-turpentine, while CO_2 at 80ppm on its own was not different from CO_2 presented at 26ppm. This phenomenon can be explained by the sensitising effect of CO_2 on the perception of chemostimulants [37, 161]. It is possible that the amount of CO_2 used (26ppm) is close to the detection limit of CO_2 for tsetse flies and that some of the variability in the responses of the flies to the plant products at low amounts was due to the amount of CO_2 used and not due to the plant compounds as such. For future studies one should consider the use of CO_2 at ~60-80ppm, thus pre-

venting some variability, making it easier to compare treatments. Turpentine is already used successfully in traps for bark beetles (*Dendroctonus valens*) and known as 'Red Turpentine Beetle Lure' (RTB-lure) consisting of equal amounts of α-pinene, β-pinene and limonene [48].

Regrettably none of the mixtures of L-turpentine plus either 1-octen-3-ol or E2-hexenal performed significantly better than L-turpentine on its own. Possible explanations could be that the proportion of the products was not optimal or that products in the mixtures were overdosed.

For the first time a clear difference between the responses of male and female *G. pallidipes* to volatiles was demonstrated. While host derived stimuli like CO_2 or breath activated and attracted equal numbers of male and female tsetse flies, plant derived volatiles like limonene, *p*-cymene and L-turpentine attracted significantly more female flies. This effect can be explained by the higher importance of plants in the life of female compared to male tsetse flies, since female flies not only use thickets and bushes as resting sites, but also as larviposition sites [28, 166]. On the contrary, it has been argued that hosts not only serve as a food-resource but are also used as place of encounter by tsetse flies for mating, as population densities of the flies are generally low [106].

Adding a visual stimulus to the behavioural set-up produced some interesting results. Using visual stimuli was important as chemical cues predominate in long-range attraction of tsetse flies whereas within a range of less than 20m host visual cues predominate [54, 169]. We could prove that L-turpentine not only activates tsetse flies, but that it induces flight to and around an object. This is important with respect to the development of new attractants as in the field an attractant not only serves to attract flies to the vicinity of the trap or target, but also enhances trap entrance or landing responses of the flies [112, 138]. These two behavioural steps, namely attraction and trap entrance or landing response, respectively, must be regarded separately, as they might be triggered by different chemo or physical stimuli [108, 127, 138, 160, 171]. An interesting observation was that compared to the assays without a target, 1μg/min of L-turpentine showed a better performance with the target than 10μg/min L-turpentine, the best treatment in the assays without a target. This could indicate that products

were slightly overdosed in our behavioural tests with volatile plant compounds tested without the target, or that the amount of CO_2 added to sensitise the tsetse flies was too low. To assess whether terpenes and especially turpentines – that could easily be produced locally in Africa at low cost – could replace the so far used phenol – 1-octen-3-ol mixture, field tests are necessary. For this, appropriate odour release devices must be developed. The polyethylene sachets used so far release about 25 times the amount of L-turpentine compared to 1-octen-3-ol (Annex E). Further we have to consider the role of CO_2. In our wind tunnel assays we used CO_2 as a sensitiser to increase the number of responding tsetse flies and we were not able to activate a significant number of flies without CO_2, showing the importance of that compound. In the field, CO_2 is highly impractical and expensive to use. Nevertheless as trap studies run on a longer time scale (normally 24 hours) our products could still improve trap catches. As the preliminary behavioural experiments with *G. swynnertoni* and *G. brevipalpis* have shown, volatile plant compounds not only attract *G. pallidipes* but there is a potential for attraction of tsetse flies occupying different habitats in Africa.

The results presented in this thesis show that tsetse flies not only perceive volatile plant compounds, but that these vectors of disease, especially females, are attracted by terpenes and simple terpene mixtures presented without and with a visual target at amounts 1000 to 10'000 times lower than background CO_2 levels. Overall this work suggests that volatile plant compounds hold promise for integration into semiochemical-based control techniques for tsetse flies.

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Appendices

A. Wind tunnel specifications

A.1. Air flow conditions in the wind tunnel

To investigate the air flow condition in the wind tunnel two methods were used. First a visible plume was produced by mixing acetic acid and ammonia and released from a Pasteur pipette placed vertically at the upwind end of the wind tunnel. The plume was released at a height of 50cm and at either 10cm and 50cm from the side wall of the wind tunnel. At 10cm from the side wall, a narrow ribbon plume was formed and maintained throughout the length of the wind tunnel, suggesting a quasi laminar flow (Figure A.1, left). In contrast to this, in the center of the wind tunnel, i.e. in front of the mixer, a filamentous plume that became increasingly disrupted towards the downwind end of the wind tunnel was formed, suggesting a turbulent air flow (Figure A.1, right).

Second, wind speed measurements were made at 27 points at three planes in the wind tunnel: 10cm off the upwind, at mid way in the wind tunnel and at 15cm from the downwind end of the wind tunnel. The pressure of the wind tunnel set to 25Pa and a thermoelectric portable measuring anemometer (ThermoAir2, Schiltknecht, Switzerland) mounted on a tripod was used. Wind speed measurements were fairly constant with 0.54m/s outside the mixer region and 0.6m/s inside the mixer region (FigureA.2). Inside the mixer region the wind speeds varied slightly from 0.53 to 0.72m/s with less variability at the downwind end of the wind tunnel. The constant wind speed measurements outside the mixer region support the observation of a quasi laminar flow, while the variability inside the mixer region supports the observation of a turbulent flow in the center of the wind tunnel. This setup permits the production of an odour plume of a defined volume (see also Annex A.2).

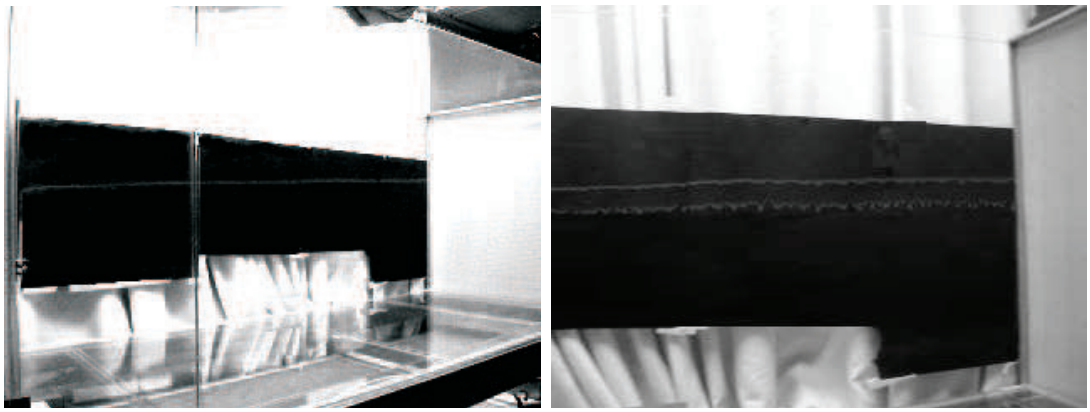

Figure A.1.: **Smoke visualisation of the flow conditions within the wind tunnel.** On the left a narrow ribbon plume outside the mixer region and on the right a disrupted filamentous plume in the center of the wind tunnel, passing downwind parallel to the floor.

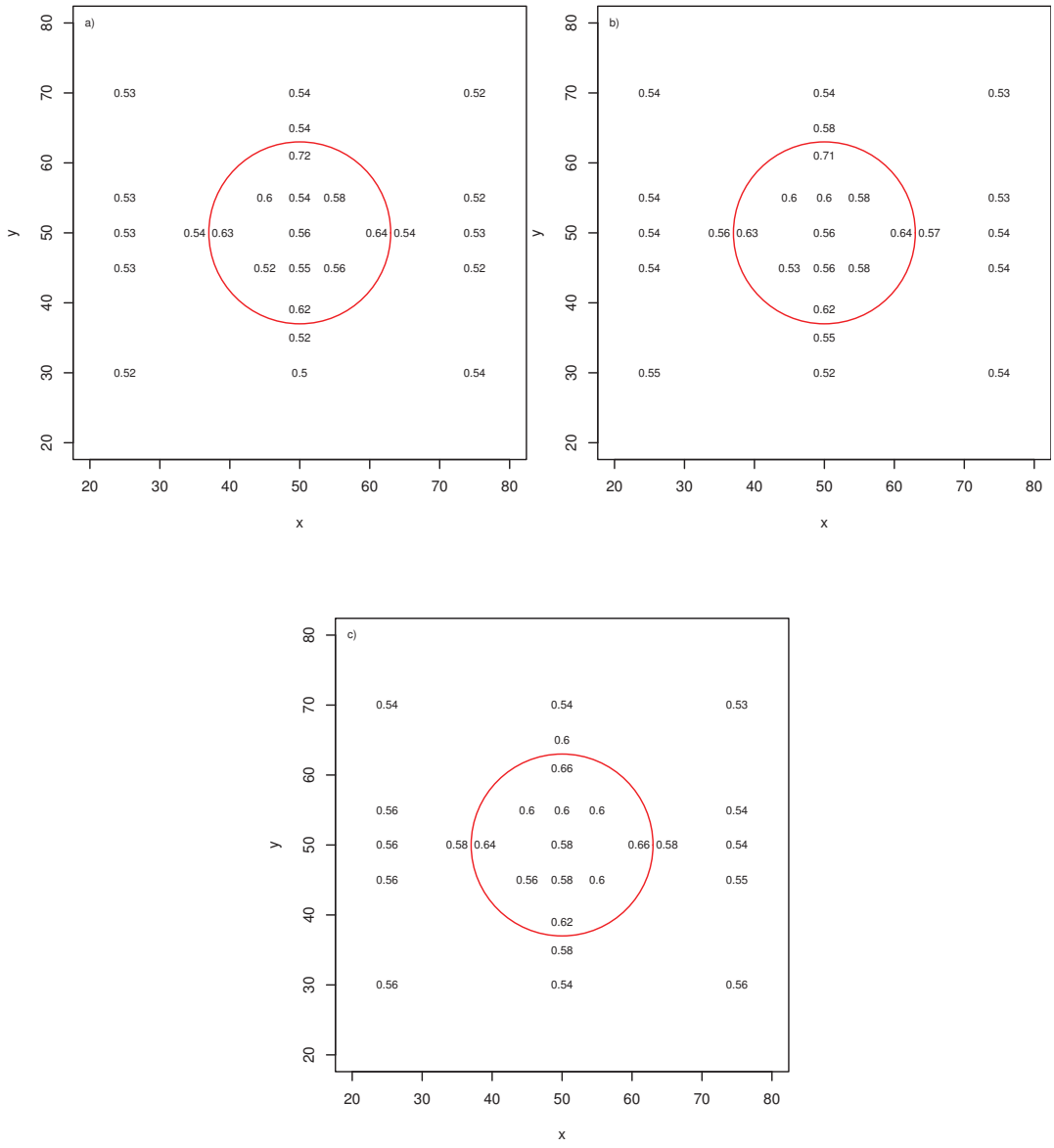

Figure A.2.: **Wind speed measurements at 27 points at three planes of the wind tunnel.** a) 10cm from the upwind end, b) at mid way in the wind tunnel and c) 15cm from the downwind end of the wind tunnel.

A.2. Plume structure

CO_2 measurements (in ppm) in the wind tunnel were performed to define the plume structure using a IR CO_2 analyzer (Li-820, 0-2000ppm range; LI-COR Inc, Nebraska, USA). Pure CO_2 at 50ml/min was released from a gas tank for one minute (test) behind the mixer (see Figure 3.1) and the abrupt CO_2 increase was measured compared to the background (measured for 1 min beforehand). Measurements were taken at two cross-sectional planes in the wind tunnel, the first plane at 10cm from the upwind end and the second plane at the release cage (15cm from the downwind end). The structure of the plume of CO_2 was maintained throughout the wind tunnel, with only a slight dilation at the downwind end of ~2-3cm (Figure A.3). No abrupt CO_2 increase was noted outside the plume but an overall steady increase of 1 to 2 ppm CO_2 per minute in the wind tunnel was recorded. In contrast an abrupt increase of the CO_2 concentration was noted within the plume where the CO_2 concentration varied between 13 and 86 ppm at the upwind end and 13 and 55 ppm at the downwind end of the wind tunnel due to the turbulent nature of the plume. The CO_2 measurements around and within the plume and the maintained structure of the plume throughout the length of the wind tunnel support the assumption of a quasi-laminar flow around the turbulent plume in the center of the wind tunnel as hypothesised after investigation of the flow conditions (see Appendix A.1).

Figure A.4 provides a visualisation of the plume achieved by mixing acetic acid and ammonia. The plume is formed of discrete bursts of ammonium acetate and maintains its form the length of the wind tunnel.

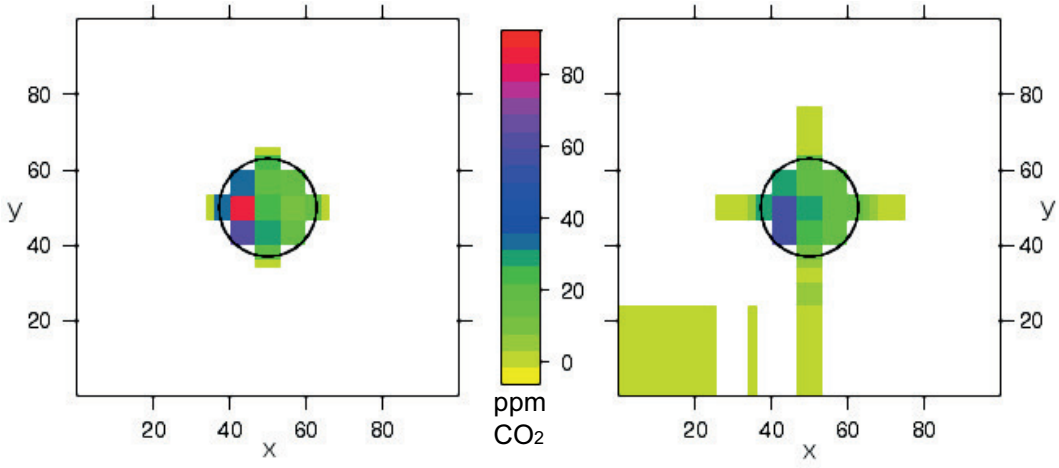

Figure A.3.: **CO_2 Measurements of pure CO_2 released into the wind tunnel to define the plume structure.** CO_2 was measured in two cross-section planes in the wind tunnel: left 10cm from the upwind end and right at the release cage. Colours represent increments of CO_2 in ppm above the background level (~350-400ppm); light green cross and light green box in the lower dorner of the wind tunnel showed no CO_2 increment; the circle represents the mixer zone; the outer square represents the wind tunnel walls; figure according to scale.

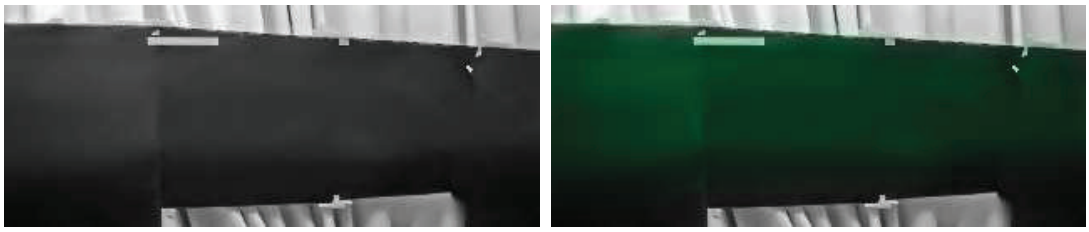

Figure A.4.: **Visualisation of plume with ammonium acetate.** The plume structure was maintained from the upwind end to the downwind end of the wind tunnel. On the left a snapshot of the plume, on the right false colour (green) was introduced to the snapshot to enhance the visability of the plume.

A.3. Colour spectrum of the sphere used as a visual target

The colour spectrum of the glass sphere painted blue (colour specification see paragraph 3.2) used as a visual target was compared to the standard blue cloth used for traps and targets in the field (100% cotton, 180g/m^2, Azur 623, TDV Industries, France). The spectral reflectance was measured using a portable spectrophotometer (Datacolor Check^{PRO}, Datacolor Inc. USA) under standard daylight conditions (D65/10°). The spectral reflectances curves for the glass sphere and the standard blue cloth are comparable with both having a maximum reflectance at 460nm and a shoulder between 410 and 420nm (Figure A.5). This suggests use of the same pigment for both materials. The tissue did have a higher intensity than the glass sphere, but that should not effect the perception by tsetse flies. The vertical light blue bands in the wind tunnel were also measured and showed a different spectral reflectance with a plateau between ~440nm to ~460nm (maximum at 450nm) and a high proportion of red (>620nm).

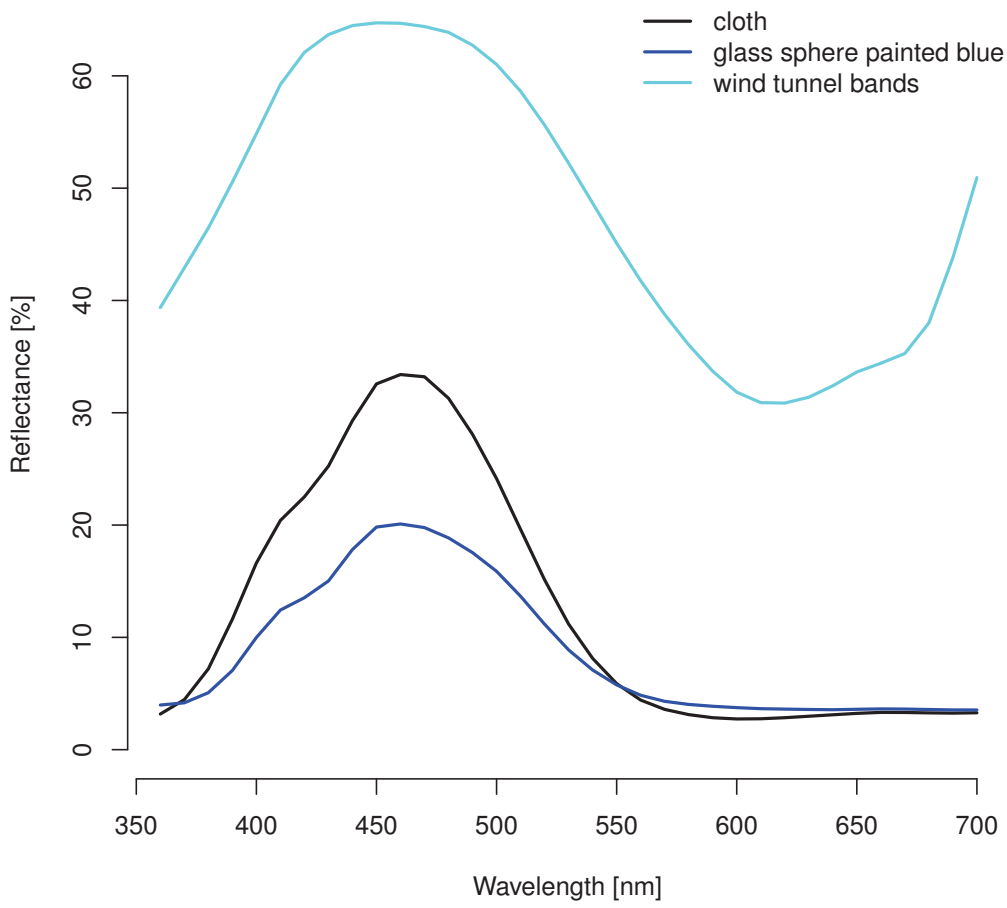

Figure A.5.: **Spectral reflectance curves for the visual target (glass sphere painted blue), the vertical bands on the wind tunnel walls (wind tunnel bands) and the standard cloth used for traps and targets (cloth).** The spectral reflectance curves for the glass sphere and the standard cloth are comparable with maximum reflectance at 460nm and a shoulder between 410 and 420nm, while the wind tunnel bands have a broader spectral reflectance range with a maximum at 450nm and a high proportion of red(>620nm).

B. Additional sensory responses to volatile plant compounds

B.1. GC-linked EAG analysis of L-turpentine (Sigma) with the antennae of *G. f. fuscipes* and *G. p. gambiensis* as biological detector

A GC-EAG analysis of L-turpentine constituents was also made using antennae of *G. f. fuscipes* and *G. p. gambiensis* . On this shorter and thinner phase (see Section 2.2) camphene and 3-carene were hardly visible, and α- pinene already started to elute in the solvent peak. The only response recorded from the *G. f. fuscipes* and *G. p. gambiensis* antennae were to limonene (Figure B.1).

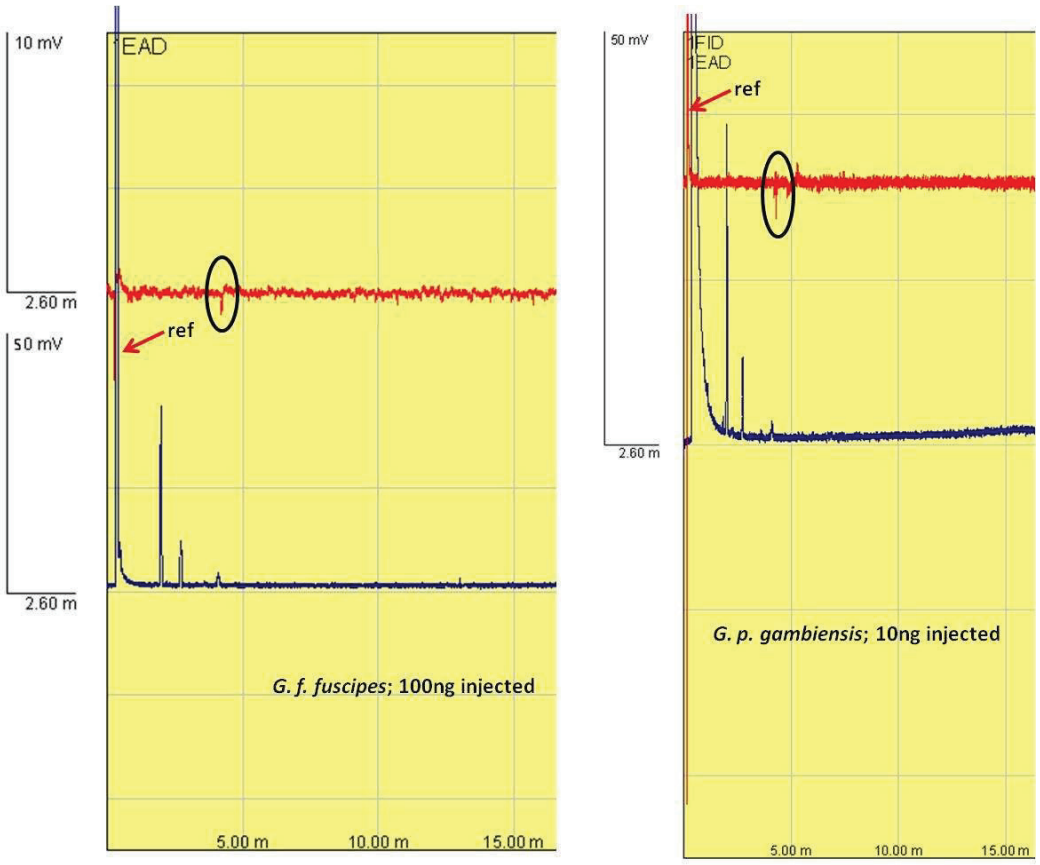

Figure B.1.: **GC-EAG analysis of L-turpentine with the antennae of *G. f. fuscipes* and *G. p. gambiensis* as biological detectors.** The upper trace (red) is the EAG response and the lower one the FID trace of the gas chromatograph. Reference (ref) is the EAG response to an air puff over 1μg of 1-octen-3-ol on a filter paper strip in a stimulus syringe recorded at the beginning of the GC-EAG recording. Peaks identified are 1) α-pinene, 2) β-pinene, 3) limonene. The EAG response to limonene is circled.

B.2. Dose-response curves of *G. pallidipes* to generally occurring leaf volatiles

Relative EAG responses of *G. pallidipes* to 9 synthetic plant volatiles, β-caryophyllene, terpinolene, humulene, 1-heptanol, myrcene, 1-hexanol, E2-hexenol, Z3-hexen-yl-acetate, and hexanal, established by GC-EAG. The results were not included into the main analysis (Figure 2.11, Table 2.5) due to $N < 4$ or a regression fit with $R^2 < 0.1$.

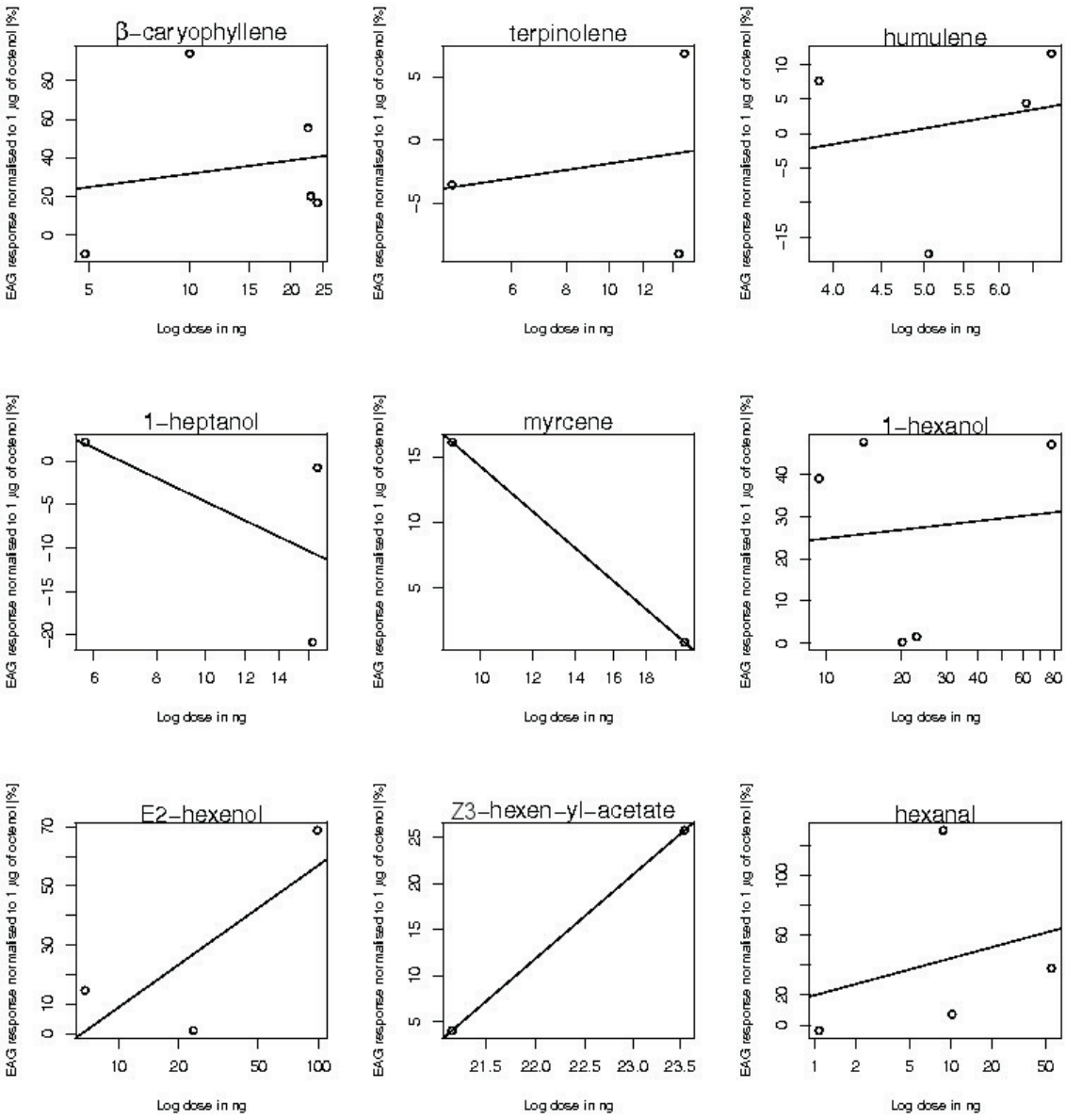

Figure B.2.: **Relative EAG responses of *G. pallidipes* to 9 synthetic plant volatiles as a function of dose injected into the chromatograph (see methods, chapter 2).** Responses are graphed relative to the response to 1μg of 1-octen-3-ol as released from a stimulus syringe (see methods, chapter 2).

B.3. EAG-responses of *G. pallidipes* and *G. brevipalpis* to alcanes

Relative EAG responses of *G. brevipalpis* and *G. pallidipes* to C_6 to C_9 alcanes and acetone in preliminary tests. Alcanes, acetone and the positive control, 1-octen-3-ol, were dissolved in C_{16}. The results are comparable to previous studies [78] with the best responses recorded to C_8 and C_9.

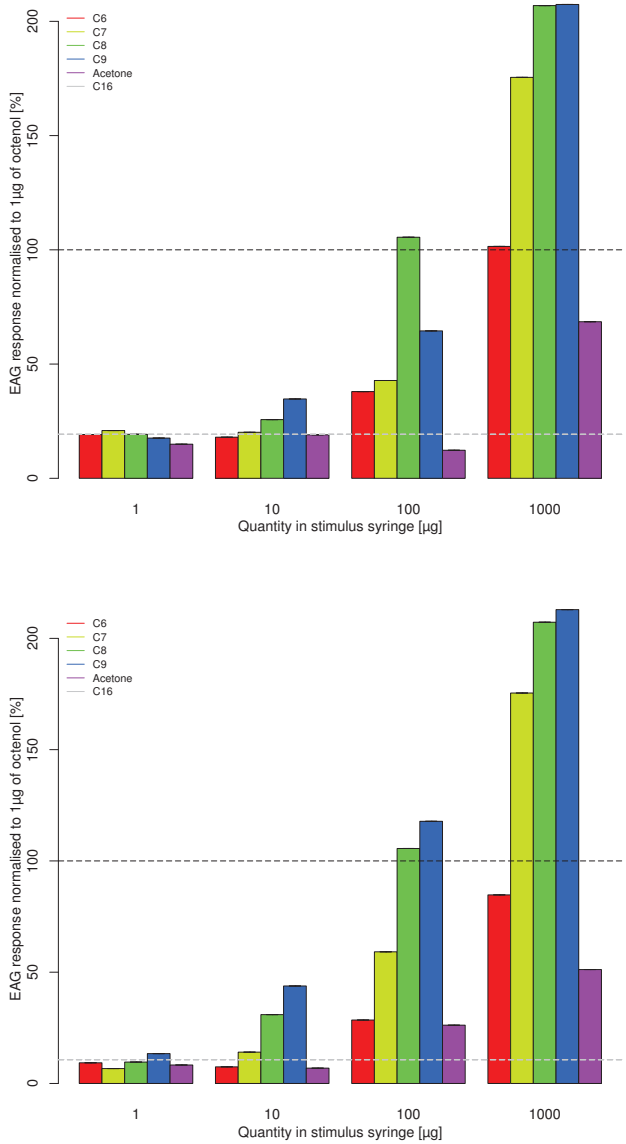

Figure B.3.: **EAG responses of *G. brevipalpis* and *G. pallidipes* to alcanes (C_6 to C_9) and acetone.** Top EAG responses of *G. brevipalpis*, bottom EAG responses of *G. pallidipes*. Hexadecane was used as a solvent. EAG responses are normalised to 1μg of 1-octen-3-ol delivered from a stimulus syringe represented here by the dotted black line. N = 1 each case.

C. Behavioural responses of teneral *G. pallidipes* to overdosed treatments

The responses of teneral *G. pallidipes* were recorded to 1-octen-3-ol at 0.1 and 1μg/min, to 3-carene at 1μg/min, to a mixture of L-turpentine at 10μg/min and 1-octen-3-ol at 1μg/min and to a mixture of S-(-)-limonene at 1μg/min and 3-carene at 0.25μg/min, all in the presence of CO_2 at ~26ppm (Table C.1). 3-carene and 1-octen-3-ol at 1μg/min only activated ~4% of the flies and induced no fly to pass through the midline of the windtunnel, while 1-octen-3-ol at 0.1 μg/min activated 29% of the flies and induced ~5% to pass through the third quarter of the windtunnel without reaching the source zone. Adding 1-octen-3-ol at 1μg/min to L-turpentine strongly diminished the responses to L-turpentine on its own (compare also to Table 3.3) and also compared to L-turpentine plus 1-octen-3-ol released at 0.01 and 0.1μg/min, respectively (Tables 3.3 and 3.5). This reduced behavioural response at higher doses of 1-octen-3-ol compared to those obtained at lower doses might suggest either a repellency of 1-octen-3-ol at the higher levels or the wrong ratio of the mixture. Adding 3-carene released at 0.25μg/min to S-(-)-limonene at 1μg/min had the same inhibitory effect on the response to S-(-)-limonene on its own (Tables 3.3 and 3.5). This suggests that at these doses 3-carene is repellent or inhibitory. Inhibitory effects of high release rates of 3-carene have been suggested for bark beetles, where 3-carene at ~100mg/day is the most attractive monoterpene used in field-traps [48].

Table C.1.: **Behavioural responses of teneral *G. pallidipes* to 3-carene, to 1-octen-3-ol, to a mixture of L-turpentine plus 1-octen-3-ol and to a mixture of S-(-)-limonene plus 3-carene at overdosed levels, all in the presence of 26ppm CO_2.**

Substance	**Activation** [%]	**Exit** [%]	**Passing** 1/4 **WT** [%]	**Passing Midline** [%]	**Passing** 3/4 **WT** [%]	**Source** [%]	**N**
3-carene 1μg/min	5.6	5.6	5.6	0	0	0	18
1-octen-3-ol 0.1μg/min	28.6	9.5	4.8	4.8	4.8	0	21
1-octen-3-ol 1μg/min	3.7	3.7	0	0	0	0	27
L-Turpentine 10μg/min + 1-octen-3-ol 1μg/min	13.8	10.3	6.9	6.9	3.4	3.4	29
S-(-)-Limonene 1μg/min + 3-carene 0.25μg/min	26.7	13.3	10	0	0	0	30

D. The activation time of *G. pallidipes* to terpene mixtures

The activation time of *G. pallidipes* to mixtures of monoterpenes (α-pinene released at 5 and 9μg/min plus S-(-)-limonene at 1μg/min), and mixtures of L-turpentine released at 10μg/min plus 1-octen-3-ol at 10 and 100ng/min and L-turpentine at 10μg/min plus E2-hexenal at 1μg/min was measured and analysed as in chapter 3. As a reference human breath delivered from a Tedlar® bag and L-turpentine released at 10μg/min were included into the analysis. Human breath delivered from a Tedlar® bag activated flies significantly faster than any other treatment (Cox proportional hazard model, $p<0.05$). No mixture of L-turpentine plus 1-octen-3-ol or E2-hexenal approached the activation time of human breath. Instead, there were no differences between L-turpentine released at 10μg/min on its own and mixtures containing L-turpentine at the same release dose plus either 1-octen-3-ol at 10 and 100ng/min or E2-hexenal at 1μg/min. The added substances did not influence the time between onset of stimulation and activation of the fly. There were also no differences between the activation time of *G. pallidipes* to the mixture of α-pinene released at 9μg/min plus S-(-)-limonene at 1μg/min and L-turpentine at μg/min. On the contrary, the mixture of α-pinene released at 5μg/min plus S-(-)-limonene at 1μg/min activated flies significantly slower than L-turpentine at 10μg/min and L-turpentine at 10μg/min plus 1-octen-3-ol at 100ng/min (Cox proportional hazard model, $p<0.05$), but not different from α-pinene released at 9μg/min plus S-(-)-limonene at 1μg/min and L-turpentine at 10μg/min plus 1-octen-3-ol at 10ng/min (Cox proportional hazard model, $p>0.05$). The conclusion is that α-pinene and S-(-)-limonene are not the only components in L-turpentine accounting for its attraction, but

that there must be components in L-turpentine that act synergistically with α-pinene and S-(-)-limonene, although we were not able to record strong EAG-responses to the other components in L-turpentine from *G. pallidipes* antennae (see Figure 2.1).

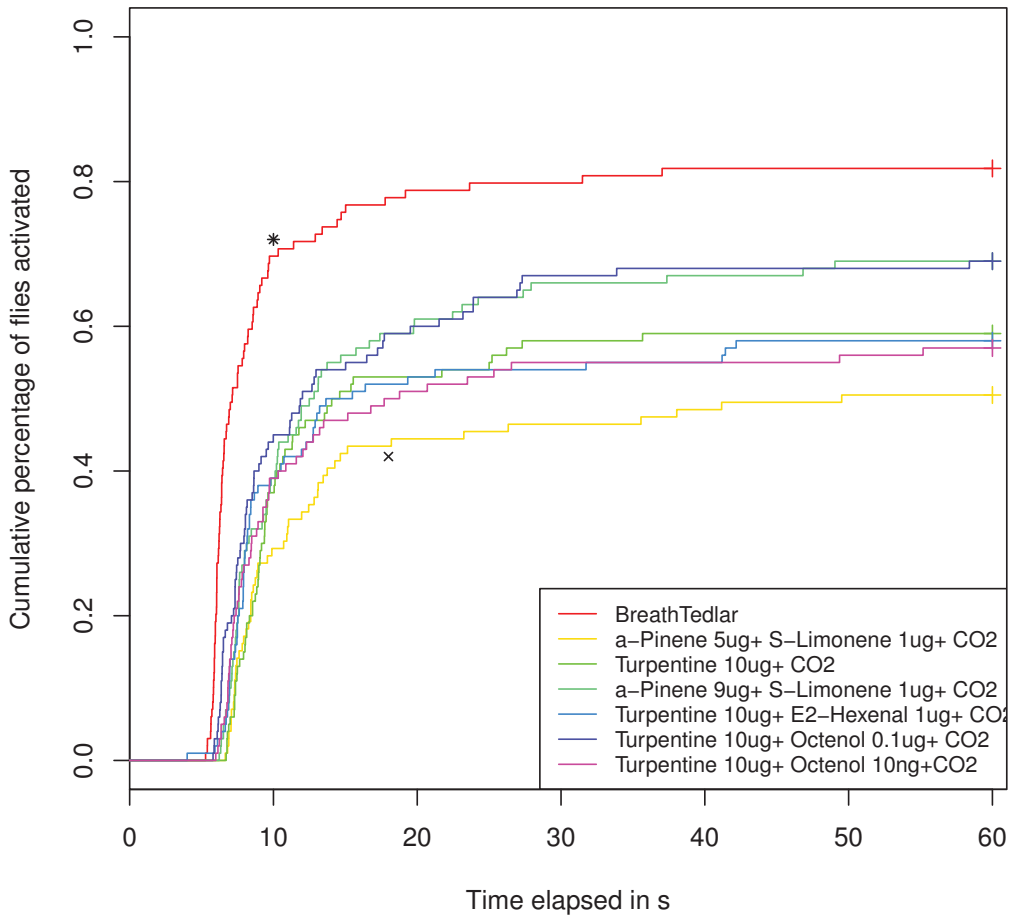

Figure D.1.: **Cumulated percentage of teneral *G. pallidipes* activated over a time period of 60s to mixtures of L-turpentine plus 1-octen-3-ol, L-turpentine plus E-2-hexenal and α-pinene plus S-(-)-limonene each in the presence of 26ppm CO_2 and to human breath delivered from a Tedlar® bag (positive control).** The positive control activated flies significantly faster than any other treatments, while the mixture of α-pinene released at 5μg/min plus S-(-)-limonene at 1μg/min activated flies significantly slower than L-turpentine released at 10μg/min and the mixture of L-turpentine at 10μg/min plus 1-octen-3-ol at 0.1μg/min (Cox proportional hazard model, $p<0.05$). L-turpentine released at 10μg/min and the mixture of L-turpentine at 10μg/min plus 1-octen-3-ol at 0.1μg/min and E2-hexenal at 1μg/min are not different; N=100 flies for each treatment.

E. Release rates of L-turpentine, 1-octen-3-ol and anhydrous terpineol from polyethylene sachets with different wall thicknesses

To test terpene mixtures in field trials in Africa appropriate field dispensers for terpenes need to be developed as has been done for the phenol – 1-octen-3-ol mixtures [159]. A first step into this direction has been undertaken by comparing the cumulated release rate of L-turpentine (Sigma-Aldrich), 1-octen-3-ol (Fluka) and anhydrous terpineol (Fluka) from freely suspended single- and double-walled low density polyethylene (LDPE) sachets of different wall thickness (150μm (Medewo,Switzerland), 200μm (Medewo, Switzerland) and 250μm (Tegum, Switzerland)). Increased wall thickness, using double-walled sachets, and decreasing the amount of product decreased the release rate of all products (Figures E.1 and E.2). Mean release rates for L-turpentine were 36–250mg/24hrs (Figure E.1), for 1-octen-3-ol 5–8mg/24hrs (Figure E.2) and for terpineol 3–5mg/24hrs (Figure E.2).

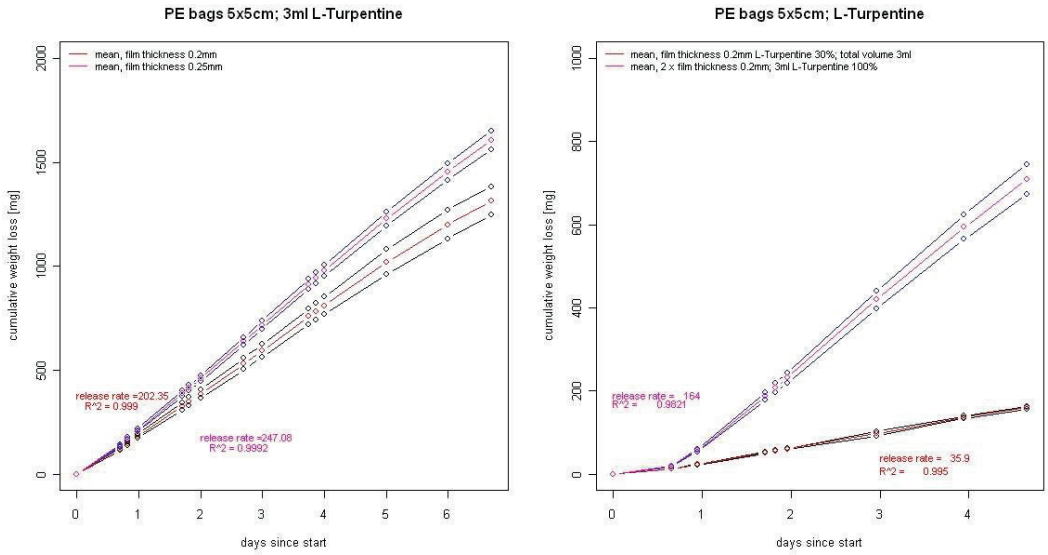

Figure E.1.: **Comparison of cumulated release rates of L-turpentine (Sigma) from freely suspended single- and double-walled PE-sachets of different wall thickness over a time period of 5 to 7 days.** The sachets were made of low density polyethylene film (LDPE) of 200μm and 250μm wall thickness, respectively. The 5x5cm^2 sachets were filled with either 3ml of L-turpentine or 1ml of L-turpentine and 2ml of di-*n*-octyl phthalate (Fluka) to attain 3ml per sachet. For single-walled sachets with 3ml L-turpentine the mean release rates were 202 and 247mg/24hrs, respectively, for 200μm and 250μm sachets wall thickness. For double-walled 200μm wall thickness sachets with 3ml of L-turpentine and for 1ml L-turpentine in a single-walled sachets the mean release rates were 164 and 35.9mg/24hrs, respectively.

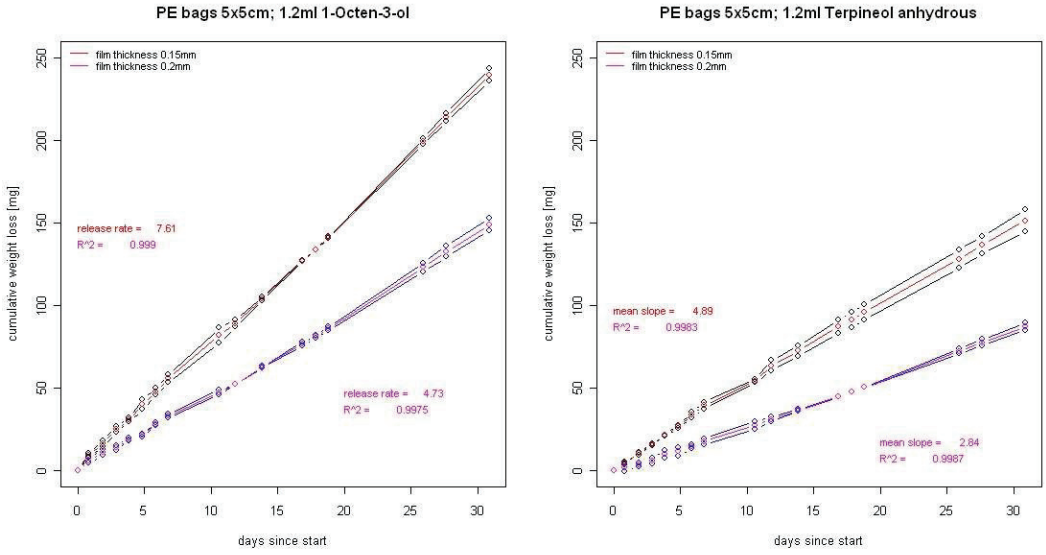

Figure E.2.: **Comparison of cumulated release rates of 1-octen-3-ol (Fluka)and anhydrous terpineol (Fluka) from freely suspended single-walled PE-sachets of different wall thickness over a time period of 30 days.** The sachets were made of low density polyethylene film (LDPE) of 150μm and 200μm wall thickness, respectively. The 5x5cm^2 sachets were filled with 1.2ml of 1-octen-3-ol and anhydrous terpineol respectively and 2.7ml of di-*n*-octyl phthalate (Fluka) to attain 3.9ml per sachet. For 1-octen-3-ol the mean release rates were 7.59 and 4.71mg/24hrs, respectively, for 150μm and 200μm sachets wall thickness. For anhydrous terpineol the mean release rates were 4.88 and 2.83mg/24hrs, respectively.