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The behaviour of the tsetse fly *Glossina pallidipes* (Diptera, Glossinidae): from host seeking to biting

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by

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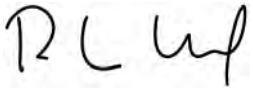

Content

Summary

Key words: tsetse, *Glossina pallidipes*, ectoparasite, insect behaviour, semiochemicals, carbon dioxide, volatile organic compounds, insect olfaction, wind tunnel, 3D flight tracking, fly landing response, visual target, fly biting response, haematophagy, hygroreceptor

Tsetse flies are major vectors of African trypanosomes causing sleeping sickness in humans and nagana in cattle. These diseases are substantial burdens on human health and prosperity. One way to break the trypanosomes life-cycle is the control of tsetse populations using visual odour-baited trapping devices. A better understanding of how tsetse use visual and chemical cues to locate hosts could help to design even more efficient trapping devices. It is in this context that critical host seeking behaviours of *G. pallidipes* were investigated in a wind tunnel. These behaviours include fly activation, optomotor anemotaxis, approach flights to a visual target, local search flights around a visual stimulus, landing responses of the fly and its biting response.

I was interested in how a host odour emanation such as human breath intervenes in the host-seeking behaviour of *G. pallidipes*. Breath is a complex blend of more than 200 volatile organic compounds (VOCs) originating in part, from the exchange between air and blood in lungs. Quantifying critical behaviours such as activation, optomotor anemotaxis and local search flights around a blue sphere with a 3D tracking system in a wind tunnel allows us to understand how *G. pallidipes* responds to breath as an olfactory stimulus. Furthermore, I demonstrate how CO_2, a constituent of breath known to attract tsetse and other blood-sucking insects, is not responsible on its own for the behavioural responses recorded for *G. pallidipes* to breath in the wind tunnel. In fact, CO_2 combines with VOCs present in breath to elicit host-seeking behaviours in tsetse. Electroantennogram (EAG) recordings and the wind tunnel behavioural experiments reveal that breath constituents such as acetone, C_5-C_{10} alkanes, isoprene and geranylacetone play a role in the host-seeking behaviour of *G. pallidipes*.

I demonstrate in the wind tunnel that an object such as a black column supporting a blue sphere can serve as a landing stimulus for *G. pallidipes* and that the fly's landing

behaviour can be modulated by changing the width of the column and the size or shape of the object it supports.

The manner in which tsetse exploit the cooling system of mammals to elicit their biting response was studied in a specially designed experimental set up. In this manner it could be demonstrated that simultaneous increases in the humidity and temperature of air act synergistically to induce biting in *G. pallidipes*. These combined physical stimuli influence the biting response latency, biting persistence and the dynamics of this fundamental behaviour. In addition, I report on neurones with hygroreceptive properties present in wall-pore sensilla on the maxillary palps of *G. pallidipes* that are probably implicated in biting behaviours.

Résumé

Mots clés: tsé-tsé, *Glossina pallidipes*, ectoparasites, comportement chez les insectes, semiochimiques, dioxide de carbone, composés organiques volatiles, olfaction chez les insectes, chambre de vol, 3D flight tracking, réponse d'atterrissage, cible visuelle, réponse de piqûre, haematophagie, hygrorécepteur

Les tsé-tsé sont les principaux vecteurs des trypanosomes africains qui causent la maladie du sommeil chez l'humain et le nagana chez les bovins. Ces maladies représentent un lourd fardeau en ce qui concerne la santé et la prospérité humaine. Une façon de lutter contre ces trypanosomes est de s'attaquer au vecteur en contrôlant sa population à l'aide de pièges qui le trompent par des moyens visuels et olfactifs. Par conséquent, une meilleure compréhension de la manière dont une mouche tsé-tsé utilise les indices olfactifs et visuels laissés par l'hôte peut aider à développer des méthodes de captures plus efficaces encore. Et c'est dans ce contexte que nous avons étudié les phases critiques du comportement de recherche d'hôte de *G. pallidipes*, telles que l'activation, l'anémotaxie optomotrice, l'approche d'une stimulation visuelle forte, la recherche locale autour d'elle, l'atterrissage et finalement, la piqûre.

Nous nous sommes intéressés plus particulièrement à savoir comment l'haleine intervient dans le comportement de *G. pallidipes*. En effet, l'haleine est un mélange complexe de plus de 200 composés organiques volatiles (COV) qui proviennent en partie des échanges dans les poumons entre l'air inspiré et le sang. En quantifiant des phases comportementales aussi critiques que l'activation, l'anémotaxie optomotrice, la recherche locale autour d'une stimulation visuelle forte, nous montrons comment *G. pallidipes* répond à l'haleine dans une chambre de vol. De plus, nous démontrons que le CO_2, une molécule constituante de l'haleine et connue pour attirer les mouches tsé-tsé ainsi que d'autres insectes haematophages, n'est pas entièrement responsable des réponses comportementales obtenues avec l'haleine. En effet, c'est la combinaison du CO_2 avec les COVs de l'haleine qui induit le comportement de recherche d'hôte chez les tsé-tsé. Nos enregistrements d'électroantennogramme (EAG) et nos données comportementales révèlent que les constituants de l'haleine tels que l'acétone, les

alcanes de C_5 à C_{10}, l'isoprène et le geranylacétone, jouent un rôle dans le comportement de recherche de l'hôte chez *G. pallidipes*.

Nous démontrons en chambre de vol qu'une barre noire coiffée d'une sphère bleue est un stimulus déclenchant l'atterrissage chez *G. pallidipes*. De plus, le comportement d'atterrissage peut être modulé en changeant la taille de la barre et la taille ou la forme de l'objet qui coiffe la barre.

Finalement, nous démontrons que le système de refroidissement des mammifères induit la piqûre chez les tsé-tsé. En effet, l'humidité et la chaleur agissent d'une façon synergique pour induire la piqûre et influencent le temps de latence, la persistance à piquer et la dynamique d'un comportement aussi essentiel que la piqûre. En plus, nous avons pour la première fois identifié chez les tsé-tsé des neurones démontrant des propriétés hygroréceptives dans les sensilles basiconiques des palpes maxillaires.

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1. General introduction

Glossina pallidipes Austen is a dipteran belonging to the tsetse fly family (Glossinidae), a group of obligatory blood-sucking flies that transmit sleeping sickness in humans and nagana in cattle. Sleeping sickness, also known as human African trypanosomiasis (HAT), is a debilitating disease whose outcome is death when no treatment is administered. The etiological agents are two parasitic trypanosomes. Most of the cases reported (97%) are due to *Trypanosoma brucei gambiense* causing chronic infection spread across 24 countries of Western and Central Africa. The remaining 3% is due to *T. b. rhodesiense* that causes an acute infection in 13 countries of Eastern and Southern Africa (Simarro et al., 2008). These trypanosomes develop inside the human body in two distinct phases. In the first phase trypanosomes mainly invade blood and lymphatic tissue causing various symptoms such as a chancre at the biting site, cardiovascular disturbance, adenopathy, fever, general malaise, and disturbance of the vegetative system, sexual disturbance and headache (Büscher and Lejon, 2004). In the second phase, trypanosomes cross the blood-brain barrier and invade the nervous system resulting in neurological disorders with symptoms such as mental changes, poor coordination, drowsiness, disturbance of the sleep cycle and finally coma (Büscher and Lejon, 2004). This dramatic disease had almost disappeared in the middle of the past century but the incidence has increased again and reached almost 40'000 new cases in 1998. In the same year, WHO estimated that 300’000 infected people were left undiagnosed because only 3 to 4 million of the 70 million threatened people are covered by surveillance (WHO, 2013). Thanks to sustainable control efforts, the incidence decreased to 7’197 reported cases in 2012. Although the number of HAT cases is less than those of AIDS or malaria, such a disease is a major concern in public health management. In fact, the fatality rate is very high. It is estimated that between 50’000 and 100’000 people die every year due to HAT resulting in about 2 million disability-adjusted life years (DALYs) (Cattand et al., 2001). Furthermore, relaxation of the surveillance leads to a rapid increase in the number of cases reaching epidemic levels (Shaw, 2004).

The burden of African trypanosomes is not entirely due to HAT. In fact, African trypanosomes do not affect only humans but also animals. Both trypanosome species mentioned above can also infect wild animals and domestic livestock resulting in reservoirs for HAT. *Trypanosoma brucei rhodesiense* can infect cattle, pigs, goats and a variety of wild animals such as antelopes whereas *T. b. gambiense* was only found in pigs and sheep (Stevens and Brisse, 2004). Although the Gambian form is found in animals, it was thought that the implication of wild animals as reservoirs was negligible. A recent study is at odds with this view and demonstrates that wild animals play an important role as reservoirs of the Gambian form (Funk et al., 2013). However, animal African trypanosomiasis (AAT) can be caused by many other trypanosomes such are *T.b. brucei*, *T. congolense* and *T. vivax*. *T. congolense* and *T. vivax* have dramatic effects on their hosts and cause anemia, weight lost, fever, abortion, reduced milk yield and infertility. *T. vivax* can cause a hyperacute form of AAT which leads to a haemorrhagic syndrome. Both infections are lethal if no treatment is administrated. *T. b. brucei* is less pathogenic, especially for African cattle, but imported breeds can be more susceptible (Eisler et al., 2004). The economic burden of AAT is very heavy. According to the FAO, 3 million cattle die every year from AAT and 1-1.2 billion US dollars are lost yearly in cattle production.

To complete their life cycle, African trypanosomes pass from one host to another conveyed by the tsetse vector. A primary way to break this life cycle is to kill the parasite in hosts such as humans and domestic livestock by active case detection followed by drug treatment. A second way is the control of tsetse populations. For example, since the prevalence of both mature *T. b. gambiense* and *T. b. rhodesiense* is less than 1% in tsetse populations (Auty et al., 2012; Jamonneau et al., 2004), a reduction of tsetse density can drastically reduce the transmission rate (Solano et al., 2013).

Furthermore, the control of tsetse populations is facilitated by their low reproduction rate. Indeed, in closed tsetse populations, a constant 4-8% reduction in female numbers is sufficient to result in a significant decline in population density until complete eradication (Hargrove, 2004). This low reproduction rate, making tsetse k strategists, is

due to the adenotrophic vivparity mode of reproduction. After copulation, only one egg is fertilized at a time, and the development of the 3 larval stages occurs in the uterus. The larva is fed with a form of milk produced by specialized glands inside the uterus. The third instar is deposited on the ground and the larva burrows into the soil to pupate. The pupal duration varies with temperature and is about 30 days at 25°C. A female can produce a larva every 6-12 days (Hargrove, 2004), giving an average of 6 offspring within a life span of 2 months (Turner, 1987).

Several methods to control tsetse populations are available, exploiting different stages of the tsetse life cycle. In the past, the life cycle of tsetse was disrupted by eliminating bush and wild animals that constituted the habitat and reservoir hosts for tsetse (Steverding, 2008). In addition, adults and pupae were killed with massive insecticide spraying campaigns. This method was similar to that used after the Second World War to eradicate malaria in Europe: kill adult mosquitoes and destroy their breeding sites. Despite the success of such a method to eliminate vectors, it results in ecological disasters and more specific ways of reducing tsetse populations are currently preferred. The reproductive cycle can be targeted in a very specific manner using the sterile insect technique (SIT). The idea is to supplant the natural male population with sterilized males that transfer sterile sperm to females. In fact, the sterile sperm is kept inside the spermatheca and renders the female infertile for the rest of her life. SIT was used successfully to eradicate tsetse and African trypanosomes on the island of Zanzibar. Attempts were also made on the African continent, but tsetse-free zones thus created were re-infested from nearby populations. SIT is a very efficient method of tsetse control but reaches its highest efficiency at low population densities (Feldmann, 2004). In Zanzibar, the tsetse population was reduced at first by treating cattle with insecticides, thus disrupting the tsetse life cycle during interaction with the host.

Feeding on the host's blood is essential for tsetse as they are obligate hematophagous insects, meaning that they procure nutrients and water only from their blood meal. But obtaining a blood meal is not without risk. Upon landing on the host, flies are exposed to predators and to the host's defence responses such as grooming and tail flicking. The trade-off between feeding and risk avoidance could explain the adaptation where flies

visit hosts infrequently but feed to repletion. For example, tsetse such as *G. pallidipes* feed every 3 to 4 days (Randolph et al., 1992; Turner, 1987). The method of insecticide-treated cattle would be efficient in controlling tsetse populations where the high density of cattle is able to compete with wild hosts (Van den Bossche and de Deken, 2004). In fact, tsetse can exploit a wide range of hosts such as bovinae, suidae, caprinae, equidae, humans and reptiles (Moloo, 1993; Moloo et al., 1993).

The life cycle of tsetse can also be disrupted during their host and refuge seeking behaviour by contact of flies with insecticide-impregnated targets and traps (Figure 1.1). Both targets and traps exploit the visual responses of tsetse, a very important sense for these ectoparasites to find a host or a refuge. When the host is within visual range, vision becomes the most important sense used by tsetse to reach the resource (Gibson and Torr, 1999). In practice, tsetse are attracted by devices coloured in blue, white or black whereas devices in green and yellow are unattractive (Green, 1986; Green, 1993; Green and Cosens, 1983; Lindh et al., 2012). Tsetse can perceive colours from UV to the red part of the light spectrum with a maximal sensitivity in the UV and blue parts (Green and Cosens, 1983). The strong response of tsetse to blue and black remains unexplained but could be linked to the fact that tsetse spend most of the time in shaded areas. In fact, short-wavelength light (blue) is more scattered by air than longer wavelength light (red), rendering shadows more bluish (Steverding and Troscianko, 2004).

In addition to vision, olfaction plays an important role in the host seeking behaviour of tsetse. Tsetse flies can perceive a wide range of odours emanating from plants and hosts (Harraca, 2008; Joris, 2013) using olfactory neurones situated inside fine structures called sensilla situated mainly on the funiculus of the antennae (Figure 1.2). The number of tsetse caught by traps or on targets can be significantly increased by adding odour baits to these devices releasing volatile organic compounds (VOCs) isolated from the odour emanations of hosts. The host emits odours that are carried by the wind and so form a plume that is used by tsetse as cues to locate a host situated even out of sight.

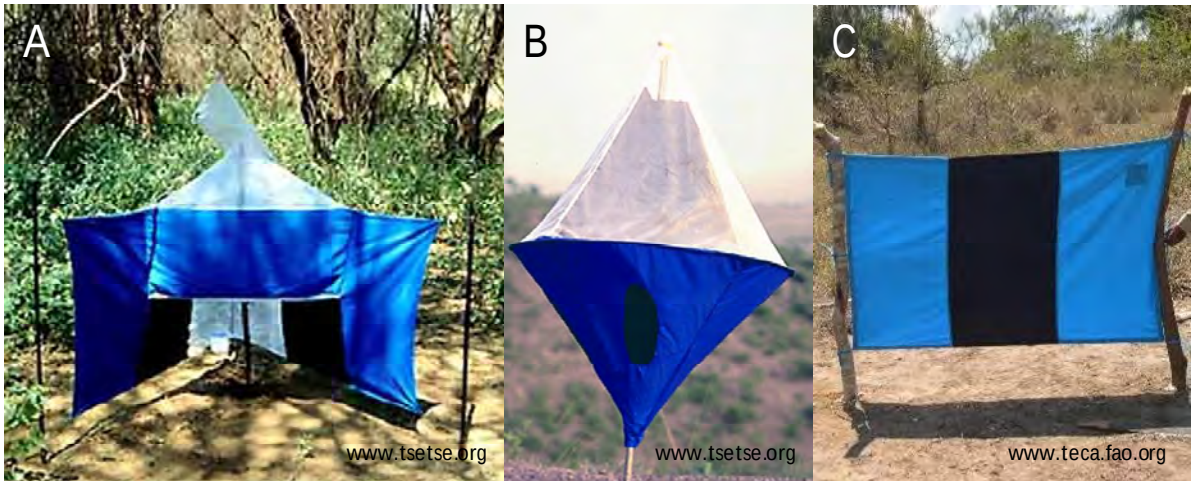

Figure 1.1: Examples of devices used in the field to control tsetse. A nzi trap, B biconical trap, C target.

Tsetse respond to a host odour plume by optomotor anemotaxis meaning that tsetse stimulated by host odours assess the wind direction to fly fast upwind regulated by visual stimuli. When tsetse lose the odour plume during upwind flight, they turn downwind bringing them back into the plume and continue again upwind (Vale and Torr, 2004). This process draws tsetse to within the visual attraction range of the host. When tsetse arrive within the visual attraction range of a trapping device they modify their flight trajectories (Torr, 1989), aiming for the device in directed flights (Gurba et al., 2012) and following this, investigate the device by circling it (Gibson et al., 1991). Olfactory baits attract tsetse and increase the recruitment of tsetse to trapping devices and consequently decrease the number of trapping devices needed to control tsetse populations.

The most frequently used olfactory bait in the field is the POCA mixture made of two phenols, namely 3-n-propylphenol (P) and p-cresol (C), and 1-octen-3-ol (O) released from polyethylene sachets and acetone (A) released from a bottle. These chemicals present several drawbacks. Bottles with acetone must be refilled frequently due to its high volatility and the two phenols are highly toxic. Moreover, these compounds do not entirely explain the attractivity of hosts (Torr et al., 2006). Further research is thus needed to identify other chemicals that could enhance the POCA mixture, replace it, or at least some of its constituents.

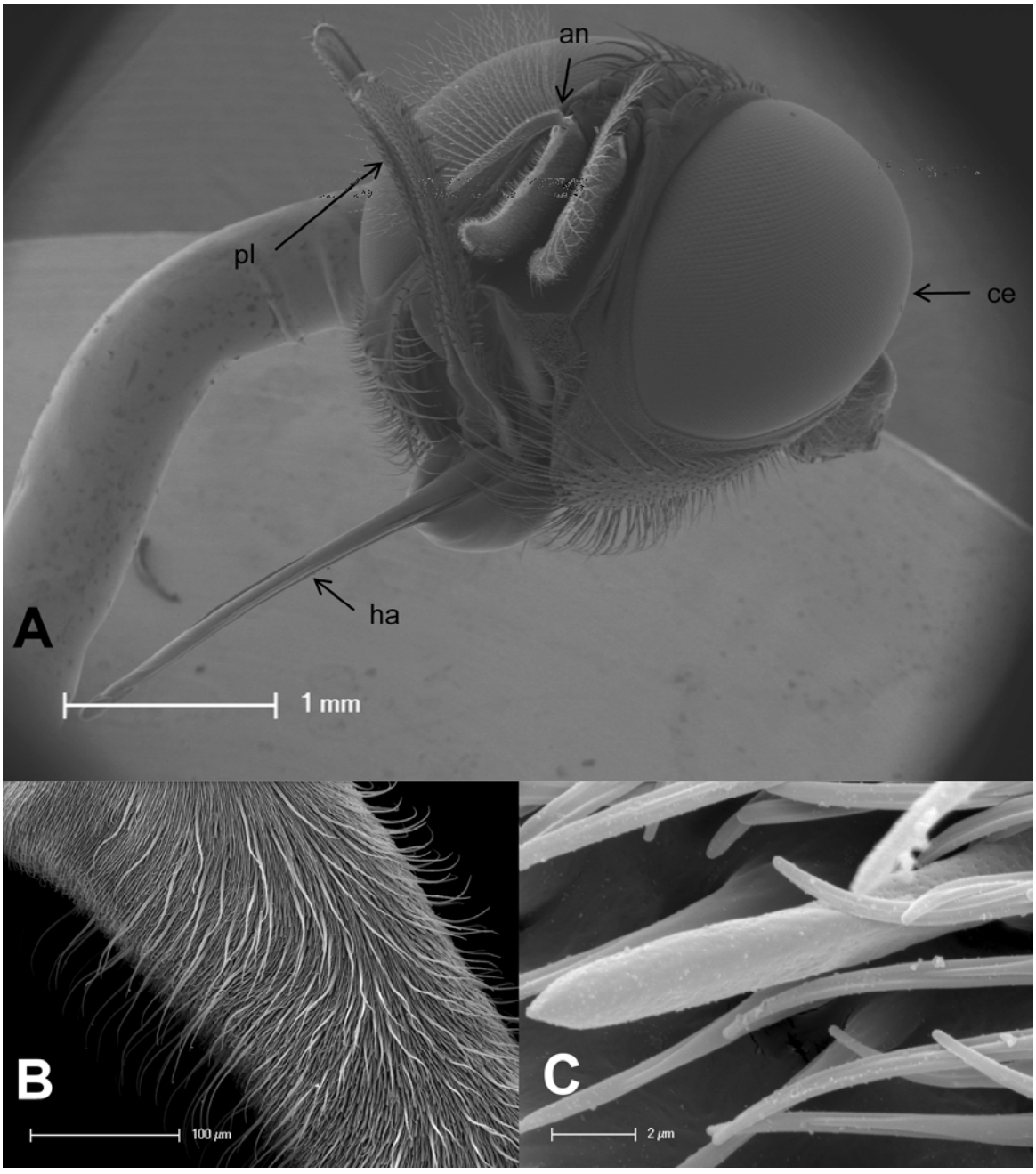

Figure 1.2: A Overview of the head of *G. pallidipes* head with palp (pl), haustellum (ha), antenna (an) with the assiociated arista and compound eye (ce). B Image of the proximal half of the antennal funiculus bearing a dense population of sensilla. C Magnified wall-pore sensillum on the antenna.

In order to identify new semiochemicals, I focused on one host effluent, namely breath that serves as a window linking tsetse to host metabolism. With this paradigm I developed experimental setups that allowed us to quantify the behavioural and

electrophysiological responses of tsetse to breath and its constituents. The response of tsetse to breath and its constituents were quantified in the absence of a strong visual stimulus, simulating the distant host situation (Chapter 2). As visual cues play an important role in the behaviour of tsetse, a second experimental series was made to investigate the interaction between olfactory and visual cues in tsetse host finding responses. Breath and some of its constituents were tested in the presence of a visual target allowing us to quantify tsetse behavioural responses close to a target (Chapter 3). I was also interested in the final step of the host seeking behaviour, namely the biting response, as any knowledge on steps drawing tsetse to blood-meals is a key means to disrupt the life cycle of tsetse and consequently that of African trypanosomes. Keeping in mind the notion that tsetse exploit the physiology of their hosts, I investigated how heat and water loss, constituting the heat regulation system of mammals, can induce the biting response in tsetse (Chapter 4).

2. Responses of *G. pallidipes* to alkanes, end-products of lipid peroxidation

2.1. Introduction

Tsetse flies are obligatory hematophageous insects, able to take bloodmeals from a wide range of mammals and reptiles depending on their ecology (Jordan et al., 1958; MohamedAhmed and Odulaja, 1997; Weitz and Jackson, 1955). Finding a host is critical for individual survival and reproduction, and interestingly, host choice is influenced by the host selected for the first bloodmeal (Bouyer et al., 2007). Different sensory modalities such as olfaction and vision intervene depending on the distance between tsetse and host animals. Since chemical cues are carried by wind, Vale (1977) estimated that olfactory perception is extended to 60-120m downwind from the host whereas within a range of less than 20m host visual cues predominate (Gibson and Torr, 1999). Since the beginning of the 20th century when researchers revealed the critical importance of olfaction in host seeking behaviour by tsetse (Glover, 1967) efforts were made to establish the nature of the attractive chemical cues emanating from hosts with a view to improving the efficiency of visual traps (Vale, 1974a). This led to the development of baits such as the POCA mixture made up of two phenols, namely 3-n-propylphenol (P) and p-cresol (C), and 1-octen-3-ol (O) released from polyethylene sachets and acetone (A) released from a bottle (Vale and Torr, 2004). These chemicals present several drawbacks. Bottles containing acetone must be refilled frequently due to its high volatility and the two phenols are highly toxic. Moreover, these compounds do not entirely explain the chemical attractivity of hosts (Torr et al., 2006) and several species of tsetse, especially riverine, hardly respond to these compounds. Further research is thus needed to identify other chemicals from host odour that are involved in the host seeking behaviour of tsetse.

Host odours originate in 3 different ways: in breath, sweat and excretions (urine and faeces; Table 2.1). Tsetse respond positively to old bovine urine (Owaga, 1985).

Indeed, a mixture of p-cresol and 3-n-propylphenol, isolated from urine, act synergistally to attract tsetse flies (Bursell et al., 1988; Owaga et al., 1988a). In contrast to urine, the role of ox sebum in the sensory ecology of tsetse 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 (Warnes, 1990b; Warnes, 1995).

Table 2.1: Metabolites mediating host seeking behaviour by tsetse flies and *Anopheles gambiae*.

Host effluent	***Main metabolic origin in the host***	***Metabolite***	***Vectors***		***References***	
			A. gambiae	***Tsetse flies***	***A. gambiae***	***Tsetse flies***
Breath	Glycolysis, Krebs cycle	Carbon dioxide	+	+	a	b
Breath, waste products, bacterial decomposition	Amino acid deamination	Ammnonia	+	?	c	NA
Breath	Ketogenesis	Acetone	+	+	d	e
Breath	Lipid oxidation	1-octen-3-ol	+	+	f	g
Breath and Sweat	Triglyceride degradation, Carboxylic acid biosynthesis	Carboxylic acids	+	+	h	i
Waste products, bacterial decomposition	Aromatic amino acid degradation	Phenols	+	+	j	k
Breath	Isoprene metabolism	Terpenes	?	+	NA	l

"+": positive behavioural response, "?": not investigated yet and NA: not available. Letters in the two last columns refer to: a) (Dekker et al., 2005; Gillies, 1980; Mboera and Takken, 1997) b) (Colvin et al., 1989; Paynter and Brady, 1993; Rennison and Robertson, 1959) c) (Meijerink et al., 2001; Smallegange et al., 2005) d) (Takken et al., 1997) e) (Vale and Hall, 1985b) f) (Takken et al., 1997) g) (Hall et al., 1984) h) (Acree et al., 1968; Dekker et al., 2002; Smallegange et al., 2005) i) (Harraca et al., 2009b) j) (Cork and Park, 1996) k) (Hassanali et al., 1986; Owaga et al., 1988a) l) (Harraca et al., 2009b; Syed and Guerin, 2004).

Breath is an interesting host effluent that attracts tsetse (Gurba et al., 2012; Joris, 2013; Vale, 1979) because it is a quasi-direct window into host metabolism. In fact, Its principal component is the end-product of the glycolysis and Krebs cycle, CO_2, known to attract *G. pallidipes* on its own (Rennison and Robertson, 1959). Further studies show

that CO_2 elicits upwind anemotaxis in *G. morsitans morsitans* (Colvin et al., 1989; Paynter and Brady, 1996). In addition to CO_2, breath is a conduit for end-products of lipid metabolism that has been implicated in the host seeking behaviour of tsetse since acetone and 1-octen-3-ol are known to attract tsetse in the field (Bursell, 1984b; Hall et al., 1984) and to elicit upwind turns in wind tunnel experiments (Paynter and Brady, 1993). Acetone is produced by the decarboxylation of excess acetyl-CoA derived from lipolysis and 1-octen-3-ol is produced by autoxidation of unsaturated fatty acids and by enzymatic oxidation of linoleic acid in mushrooms (Amman and Smith, 2005; Frankel et al., 1981; Wurzenberger and Grosch, 1982). Acetone and 1-octen-3-ol are constituents of a blend of host animal products that enhance catches of tsetse in visual traps and on targets (Kappmeier and Nevill, 1999; Torr et al., 2011; Vale et al., 1988).

In this context, end-products of a supplementary pathway of the lipid metabolism, namely lipid peroxidation have retained my attention. Lipid peroxidation produces short chained alkanes that are released in human and bovine breath (Phillips et al., 2000b; Spinhirne et al., 2004). So I investigated here the power of short chain alkanes as chemical stimulants for *G. pallidipes*. My electroantennogramm (EAG) recordings reveal the perception of short-chain alkanes by antennal receptor cells and my wind tunnel studies provided evidence that the hydrocarbons play a role in the host seeking behavior of *G. pallidipes*.

2.2. Materials and methods

2.2.1. Insects

G.pallidipes Austen pupae, supplied by the IAEA (Siebersdorf Laboratories, Austria), and imagos were kept in 2 climate chambers offset by 2 hours and programmed as follows: 8h light at 26°C, 85%RH with a 1h ramp for light and temperature at dawn and dusk and 14h dark at 22°C 85% RH. Females and males were separated at emergence to prevent mating and placed in cotton netting cages (25 cm * 15 cm * 15 cm). Flies were tested on day 2 after emergence (day 0) and were fed on day 3 with defibrinated bovine blood through a silicon membrane. The behaviour of tsetse flies was observed during the 2 daily activity peaks during the first 1h 30 min and the last 2 hours of the photophase.

2.2.2. Electroantennograms

Electroantennograms (EAG) from *G. pallidipes* antennae were accomplished as described in Harraca et al. (2009a). Schneider (1957) introduced an electrophysiological method permitting measurements of the response of an insect antenna to volatile chemical stimuli. This method was adapted for tsetse flies from that described in Guerin and Visser (1980). Briefly described, the tsetse head was removed from its body and placed on a glass capillary reference electrode (2mm o.d., with filament) filled with 0.1M KCl with the tip of the electrode placed at the base of the antenna (Figure 2.1). The tip of the glass capillary measuring electrode (2mm o.d. with filament) was also filled with 0.1M KCl and placed on the tip of the antenna (Figure 2.1). The measuring electrode was connected to a computer via a high impedance preamplifier (Syntech, Kirchzarten, Baden-Württemberg, Germany), a DC amplifier (UN-03, Syntech) and an analog-digital converter (USB-IDAC box, Syntech). EAG responses were recorded with EAG pro from Syntech. The mounted antenna was placed in a humidified charcoal-filtered air stream (1m/s) conveyed in a water-jacketed tube 7mm i.d. diameter. The temperature of the air stream was always set at 2°C higher than the ambient temperature.

The antenna was stimulated with 1mL charcoal-filtered air passing through a 5mL polypropylene syringe (BD Plastipack(TM), Spain) containing a filter paper strip soaked with the products listed in Table 2.2. During recordings, stimulations with a positive control (10µL of a 100ng/µL solution of 1-octen-3-ol in dichloromethane (DCM)) were regularly made and data were normalized to compensate for a drop in the antennal response with time as described in (Guerenstein and Guerin, 2001). In a first recording series, acetone and *n*-alkanes from pentane to decane were injected directly onto the filter paper into syringes using a 1µL glass syringe (Microliter#7001, Hamilton, Reno, Nevada, USA) in a range of volumes from 20nL to 1000nL and a 10µL syringe (SGE Analytical Science Pty Ltd, Victoria, Bayswater, Australia) for 10000nL. Puffs were made immediately after disappearance of the halo created by the compound on the filter paper. In the case where the halo did not disappear, puffs were made 2min after the injection of the product into the syringe. It appeared that the threshold for heptane, octane, nonane and decane was lower than 20nL. A second recording series was made where these 4 alkanes were diluted in hexadecane from 100µg/µL to 10ng/µL. One µL of these solutions was directly injected onto the filter paper strips in stimulating syringes as for the previous experiment. Hexadecane was used as solvent as the EAG responses of *G. pallidipes* antenna to 1µL or 10µL of hexadecane were not significantly different to those of an empty syringe (T-test, df=9.303, P=0.06277; T-test, df=11.826, P=0.1851).

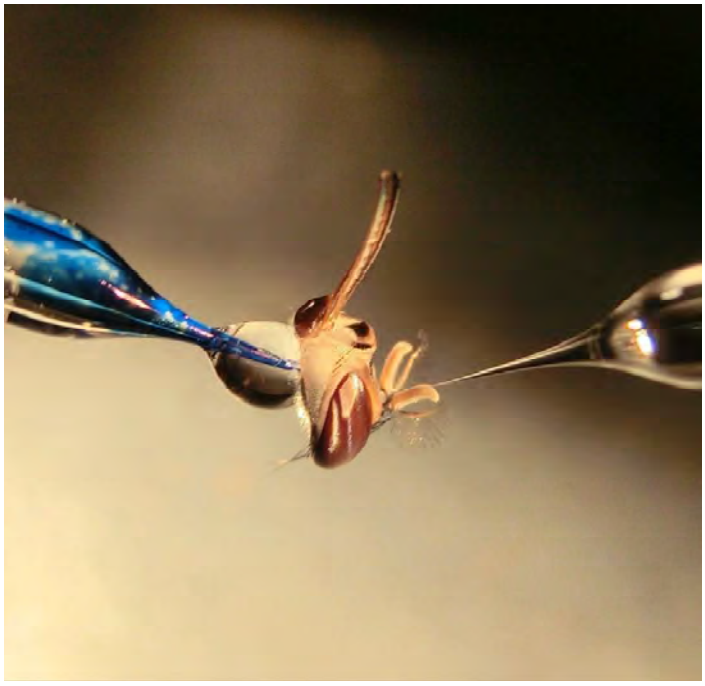

Figure 2.1: Picture of a *G. pallidipes* head prepared for EAG recordings. The head is removed from the body and mounted between the reference electrode painted in blue and the measuring electrode placed at the tip of the funiculus. The reference electrode crosses the occipital condyle and arrives at the base of the antenna.

Table 2.2: List of products tested on *G. pallidipes* by electroantennogram recording (EAG) and in the wind tunnel (WT).

Chemicals	Suppliers	Purity [%]	Use
Pentane	Flucka	99	EAG, WT
Hexane	Merck	97	EAG, WT
Heptane	Flucka	99.5	EAG, WT
Octane	Flucka	99.5	EAG, WT
Nonane	Flucka	95	EAG, WT
Decane	Flucka	98	EAG
Hexadecane	Flucka	98	EAG
Acetone	Acros	99.8	EAG,WT
Dichloromthane	Merck	99.9	EAG
1-octen-3-ol	Flucka	98	EAG
CO_2	Carbagas	99.99	WT

2.2.3.Wind tunnel

Based on the wind tunnel used by Gurba et al. (2012), several modifications were made as follows. The closed circuit wind tunnel (working area: 250cm long, 100 x 100cm) made of non reflecting glass had a centrifugal ventilator to move humid air (85 ± 1% R.H., 26 ± 0.1°C) in a laminar flow of 0.54m/s. Active charcoal filters cleaned the air at either end. On both side walls of the wind-tunnel light blue vertical bands 7 cm wide spaced 7 cm apart provided visual cues to enable tsetse to orient. To ensure that *G. pallidipes* could perceive the blue bands, band width was calculated as 2.5 times the interommatidial angle in the peripheral eye zone at 1m as measured by Gibbson & Young (1991). High frequency lighting neon tubes (1 kHz, Luniscontrol Gmbh, Lanzenhäusern, Bern, Switzerland) simulated daylight with 700Lux on the floor of the wind-tunnel. The floor was covered with brown panels.

Tracking system

The flight of tsetse flies was filmed and recorded at 24 frame/s by two video cameras (eXcite exA640-120m, Basler, Ahrensburg, Schleswig-Holstein, Germany) with wide-angle objectives (SV M0614, C mount, f=6 mm, VS Technology, Tokyo, Japan) linked to 3D software (v7.1, MVTec, München, Bayern, Germany) placed 87cm above the wind-tunnel center (distance between cameras 160 cm) and linked to a computer as in Gurba et al. (2012). The program (CrowCameras, University of Neuchâtel, Neuchâtel, Neuchâtel, Switzerland) calculates the x, y, and z coordinates of each detected point to determine the location of a fly in flight within the wind tunnel at a given time (Gurba et al., 2012). In some cases, when 3D-tracking system was in maintenance, flies were filmed with 1 camera (The IMAGING SOURCE DMK236618, 120 frame/s, Bremen, Frei Hansestadt Bremen, Germany) placed in the middle of the wind tunnel 76cm above the roof of the wind tunnel. 2D-tracking analysis was done with Matlab (The MathWorks, Inc, Natick, Massachusetts, United States) in a post-treatment manner.

The plume generator

I build an odor release system composed of two parts: first the odor release system which contained piezo nebulizers to quantitatively release volatiles and with tubes for the release of gases (Figure 2.2) and secondly, the plume generator that mixed the

vapours with the wind tunnel air (Figure 2.3). The principle of the piezo nebulizer has been described by El-Sayed et al. (1999) and by Schmidt-Busser et al. (2009). A piezo disc (Philips PXE5 25/2.0, Megatron AG, Kaltbrunn, St. Gallen, Switzerland) connected to a frequency generator (FG-5000A Wavetek, Germany) vibrated at an amplitude of 40 V and a frequency of 70 kHz and transmitted the vibration to a glass capillary (8-9 cm long, o.d. 1 mm, i.d. 0.46 mm) with a drawn-out tip (1-3 cm, tip opening of 10-50 µm) via an aluminium clip. The transmitted vibration induced the capillary tip to oscillate and nebulized the liquid solution passing through it. Solutions were pumped from a syringe (5 ml, 60 mm long, Hamilton, Bonaduz, Graubünden, Switzerland) with a syringe pump (CMA 400, Microdialysis, Solna, Stockholms län, Sweden) into a PTFE microtube (1.5 m long, 1.02 mm o.d., 0.56 mm i.d., Hamilton, Switzerland) connected to the oscillating glass capillary. Two piezo nebulizers were mounted on the head of the odor release system to be able to simultaneously nebulize substances that were not miscible. Gases were released from 6 mm in diameter glass tubes (4.25 mm i.d., Figure 2.2). The production of the aerosol could not be interrupted and quickly re-launched without a delay in the stable production of the aerosol. Instead, the aerosol was continuously produced and the interruption of the release was done by sucking the aerosol with an aspirator (T 12/1, Kärcher, Harsewinkel, Nordrhein-Westfalen, Germany) connected via a 12 mm diam. glass tube to a solenoid valve (6 mm i.d., Lucifer, Fluid control Division Europe, Geneva, Geneva, Switzerland). The aspirator was connected to a variac (Philips, Amsterdam, Nordholland, Netherland) to adjust the flow rate to about 100 L/min (corresponding to 0.3% of the total wind tunnel flow). To stimulate flies with the aerosol, a switch enabled the solenoid valve to close and shutdown the aspirator to allow the aerosol to enter the plume generator. Switching off the aspirator did not immediately interrupt the flow as the motor continued to turn, but the valve stopped the aspiration flow immediately. The aerosol was illuminated from below with a red LED and was monitored with a web cam (QuickCam Vision Pro 9000, Logitech S.A., Morges, Vaud, Switzerland) to ensure that correct production of the aerosol occurred. All tested substances were accompanied with the release of CO_2 that was also continuously released or removed by aspiration in the same way as the aerosol to avoid any lag between the release time of CO_2 and the test chemical (Figure 2.2).

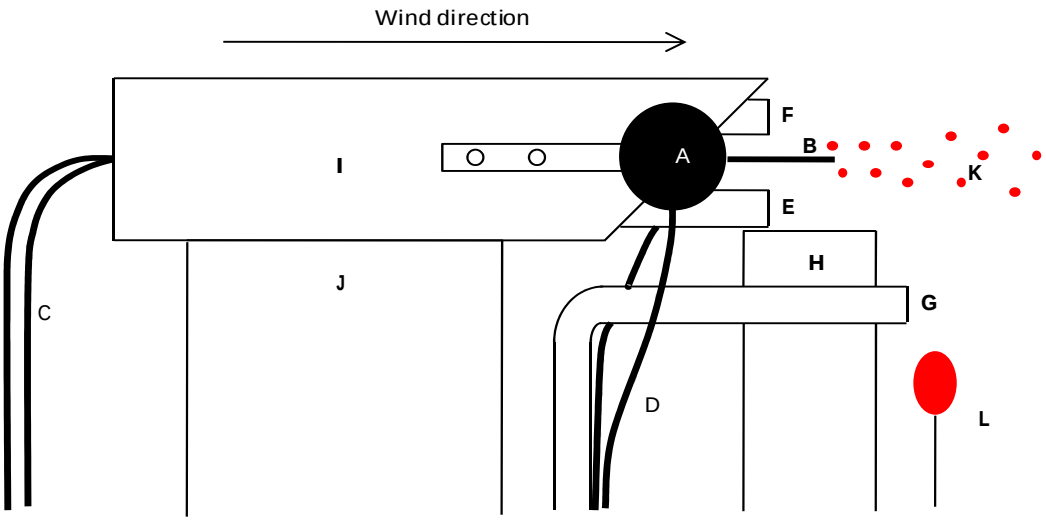

Figure 2.2: Magnified sideview of the odour release system. Two piezo nebulizer discs (**A**) were mounted on each side of the head (**I**) that held a glass capillary with a drawn-out tip (**B**), PTFE microtubes (**C**) and electric wire (**D**). Gases such as CO_2 or breath were released from tube **E**. **H** aspiration flow. The aerosol (**K**) and CO_2 were sucked through tube **H** out of the wind tunnel when not required. The production of the aerosol was monitored with a web cam filming the diffraction of red light produced by a LED (**L**) in the aerosol (**K**). Other tubes (**F**, **G**) were added in prevision for other experiments but were not used.

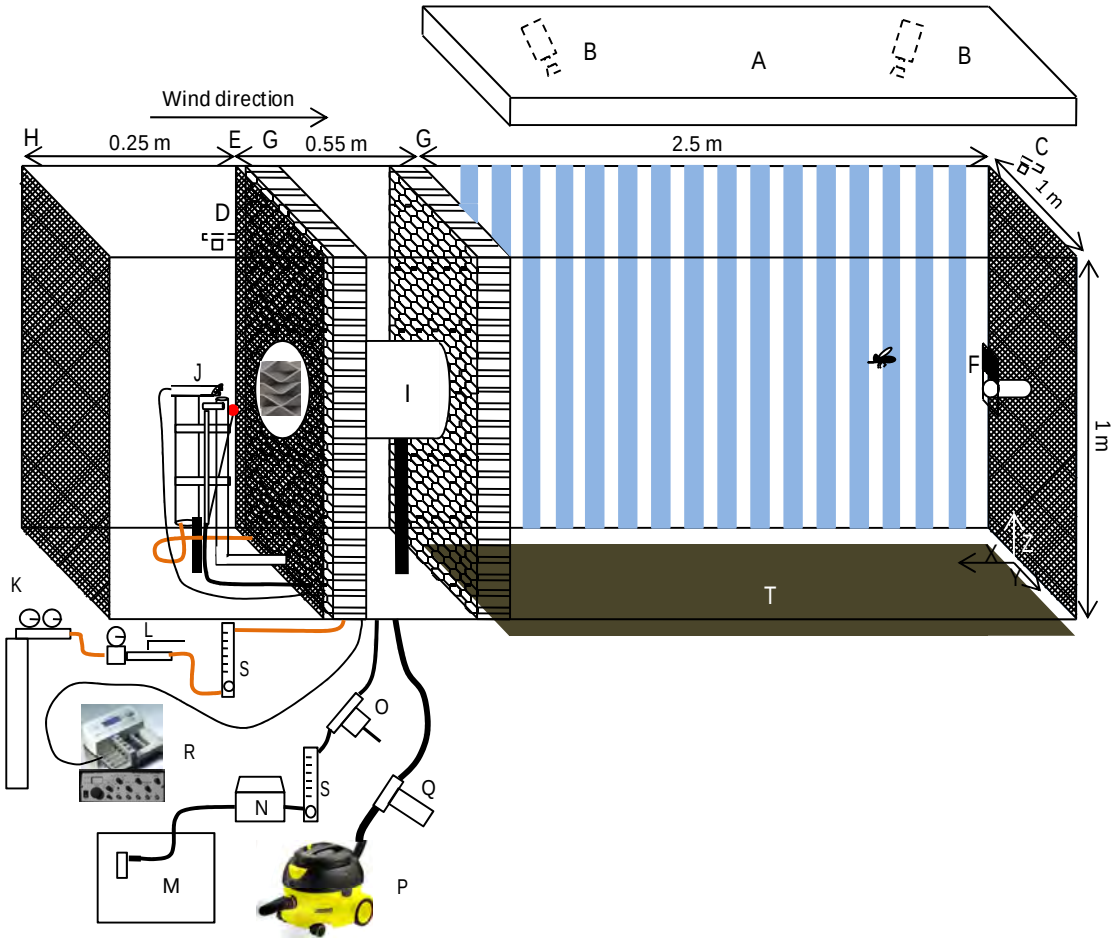

Figure 2.3: Scheme of the wind tunnel, not to scale. **A** High frequency neon lighting; **B** Cameras of the tracking system in the middle of the overhead light field A; **C** Web cam to observe fly activation behavior; **D** Web cam to observe the plume generated by the piezo nebulizer as highlighted by a red LED (red dot); **E** 0,5 mm mesh mosquito netting; **F** Fly release cage; **G** Honeycomb sheets; **H** Nylon laminar flow screen; **I** Static gas mixers housed in a steel tube; **J** Odour delivery system (for details see Figure 2.2); **K** CO_2 pressurized cylinder; **L** Manometer followed by a hand valve; **M** Gas sampling Tedlar bag; **N** Pump to push breath into the wind-tunnel; **O** Three-way solenoïd valve controlling entrance of breath into the wind tunnel; **P** Aspirator; **Q** Valve to control the aspiration flow; **R** Syringe pump with frequency generator; **S** Flow meter; **T** brown panel.

The odor in aerosol form entered the plume generator that was composed of two 26 cm diameter static gas mixers (Sulzer Chemtech Ltd, Winterthur, Zürich, Switzerland) housed in a 27.2 cm diameter steel tube (50 cm in length, 1 mm thick) that mixed the released product with the air of the wind tunnel. The housing of the gas mixers was set in the centre of the wind tunnel cross-section on 2 steel rods (30 mm diameter) on 2 steel holders (50 mm high, 4 mm thick) mounted on a sliding rack allowing rapid mounting and re-mounting of the plume generator and to clean it as necessary. The 26 cm diameter cylindrical shape of the plume was maintained as a laminar flow generated

first by a nylon laminar flow screen (40 µm mesh, Sefar AG, Heiden, St-Gallen, Switzerland) to equalize all velocity vectors upwind of the plume generator and secondly by mosquito netting (nylon, 0.5 mm mesh) placed at the upwind end of the static gas mixers to equalize pressure loss across the static gas mixers (but not covering them) and 2 downwind aluminium honeycomb sheets (57 mm thick, 9.525 mm cell size, Plascore GmbH & Co KG, Waldlaubersheim, Rheinland-Pfalz, Germany; Figure 2.3). Mosquito netting (nylon, 1 mm mesh) was placed 1cm downwind of the last honeycomb sheet and also at the downwind end of the wind tunnel on a perforated steel screen (1 mm thick, 3 mm round holes, 51% of air passage) to avoid tsetse escape. The laminarity of the flow was controlled by measuring wind speeds and was visualized with a plume of ammonium acetate. This confirmed that the plume maintained its shape and integrity along the wind tunnel length as measured by the carbon dioxide concentration with an infrared detector (LI 820, LI COR Inc, Lincoln, Nebraska, USA) at 3cm from the upwind end and at 46 cm from the downwind end of the wind tunnel (Figure 2.4).

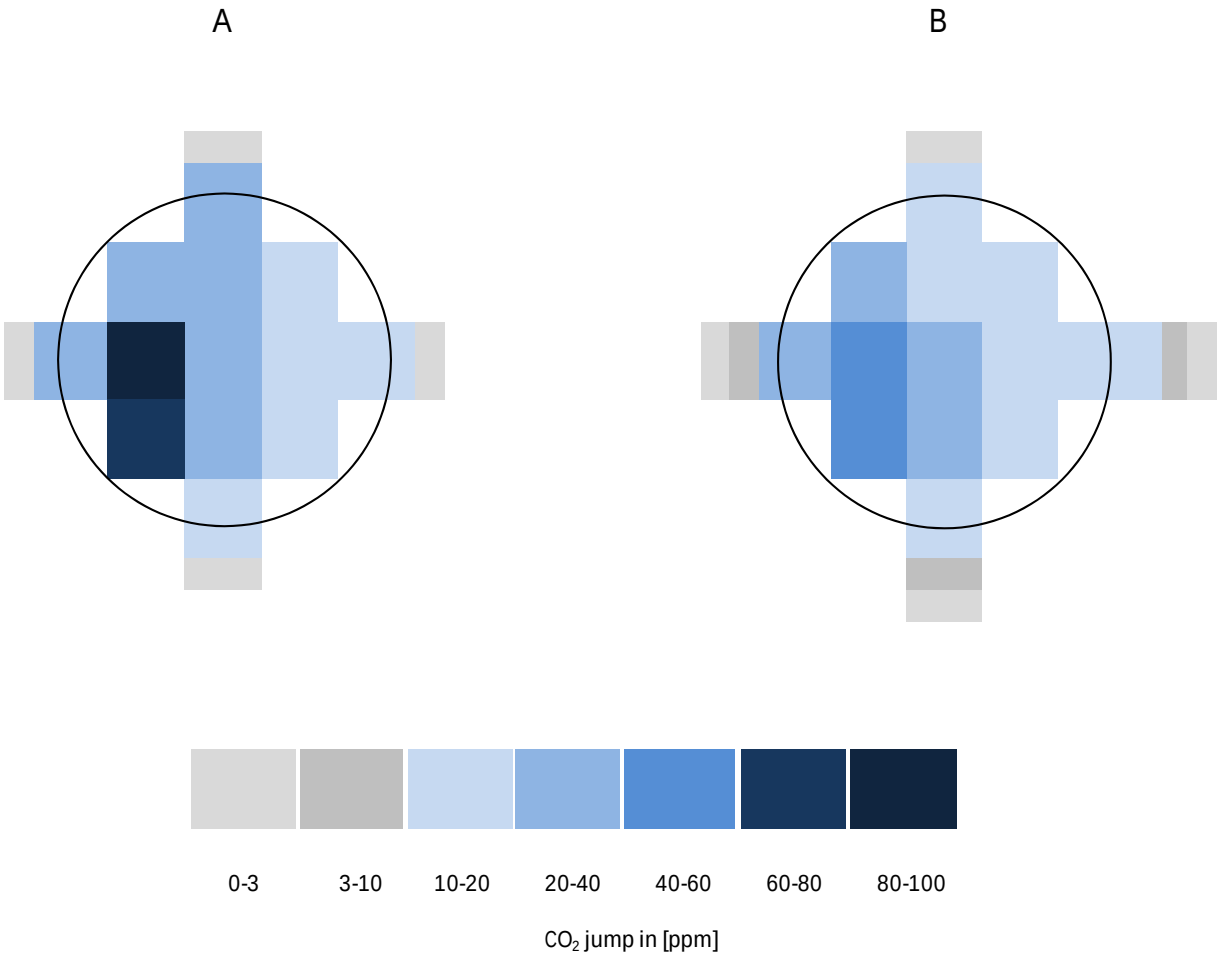

Figure 2.4: CO_2 measurements within 3cm of the upwind end (**A**) and 46 cm of the downwind end of the wind tunnel (**B**). Air samples were taken in the middle of each square (6.75 cm*6.75 cm). Samples collected outside the plume were spaced at 2 cm steps from its circumference. CO_2 was released at 60-80ml/min. The circle is the 26 cm diameter of the plume generator. Each square shows the CO_2 level difference between 1min baseline level sampling followed by 1min of CO_2 delivery. No jump in CO_2 was observed 4-6 cm outside the circumference of the plume. The plume enlarged by a factor of 2cm per meter along the wind tunnel length. The inner structure of the plume is inhomogeneous with CO_2 levels not reaching the same maxima.

Human breath was collected from only one individual (a male caucasian experimenter, 30 years old) and no attention was paid to his diet. The experimenter expired via a Teflon® tube into 25L Tedlar gas sampling bags (CELscientific corp, Santa Fe Springs, California, USA) that were held at room temperature for 15min before experiments. Breath was then pumped (MPC 100 E, Saskia, Germany) into the wind tunnel at a rate of about 5L/min (Table 2.3). CO_2 was used as baseline and was released from a pressurized cylinder at a rate of 60 to 80ml/min (Table 2.3). Breath and CO_2 were released from the same tube (Figure 2.2 & Figure 2.3). Acetone, *n*-alkanes from hexane

to nonane were released with the piezo nebulizer described above (release rate shown in Table 2.3).

Table 2.3: Release rates and concentrations measured for CO_2 alone, for CO_2 in breath and for substances tested in the wind tunnel. Concentrations for acetone and for hexane to nonane were estimated assuming the plume to be homogenous.

Substances	Release rate	Concentration [ppm]
Breath	5L/min	59.37 ± 1.38
CO_2	60-80ml/min	22.10 ± 0.76
Acetone	10µl/min	1.81
Hexane	10µl/min	1.03
	100µl/min	10.32
Heptane	1µl/min	0.091
	10µl/min	0.91
	100µl/min	9.15
Octane	10µl/min	0.83
	100µl/min	8.30
Nonane	0.01µl/min	0.00074
	0.1µl/min	0.0074
	1µl/min	0.074
	10µl/min	0.74

Test Procedure

Six flies (a batch) were transferred into 6 plastic release cages (transparent PVC cylinders 15 cm × 10 cm). During the transfer to the release cages, flies with wing damage were discarded. The cages were placed on the floor at the downwind end of the wind tunnel. After 15 min for fly acclimatization to the wind-tunnel conditions the first cage was placed horizontally on a stand at 50 cm from the floor and at 5 cm from the downwind end of the wind-tunnel. The tracking system and a stopwatch were launched (time 0) and the door of the cage was lifted within 15 s. The fly was successively exposed for an equal period of time to odor-free air (negative control) and then to the test odor. Stimulation began at time 75 s and lasted 60 s. Each fly was removed from the wind tunnel at the end of the experiment and the empty cage was placed on the

floor of the wind tunnel. The same procedure was followed for the following five flies. Flies were used only once. Flies that sat in cages on the floor of the wind tunnel were not exposed to odors delivered from the plume generator (Figure 2.4). As the downwind end of the wind tunnel was not visible to the cameras of the tracking system the time to activation of a fly was measured by observing flies moving in the cage with a web cam (SPCI 1300NC, Philipps Electronics, Amsterdam, Nordholland, Netherland).

2.2.4.Data analysis

EAG

To compare the normalized responses to products tested and to verify if the normalized responses of the antenna increased with the dose for hexane, heptane, octane, nonane and decane, a 3 parameter logistic regression was applied to the data using lns function with a selfstart function SSlogis in R. An exponential model fitted with the lns function with R (R Development Cor Team, 2012) was used for acetone, pentane and for the second experiment where heptane, octane, nonane and decane were tested at very low doses. In the first recording series the dose to obtain 50% of the response to 1 µg of 1-octen-3-ol was calculated from the models and was used to compare the response of the different product tested. In the second series, the threshold was calculated from the models where y was equal to the response to the solvent.

Behavioural analysis

The behavioral criteria used were: “activation”, the fly moved in the cage; “activation time”, time between the onset of stimulation and the first movement of the fly; “exit”, the fly exited the cage; “1/3”, the fly passed through the first third of the wind tunnel (0.8m); “1/2”, the fly passed through the midline of the wind tunnel (1.25m); “2/3”, the fly passed through the top third of the wind tunnel (1.7m) near the odor source. The effective volume of the 2D-tracking system was reduced compared to the 3D-tracking system. This meant that the first and the last quarter of the wind tunnel were not visible with the 2D tracking system, so one third sections of the wind tunnel were used to compare data obtained with the two tracking systems. The criteria such as “1/3”,”1/2”,”2/3” were determined as the furthest point that a fly reached before it touched any wall of the wind tunnel. For a particular behavioural criterion the response of flies was measured as the

proportion of responding flies of the total flies tested for a treatment. Proportions for each behavioral criterion were analyzed separately using a generalized linear model (GLM) with a logit link function (R Development Cor Team, 2012). Odd ratios were used to compare the effect of a treatment over the control. Each step in reducing GLMs was controlled by a deviance analysis following a X^2 distribution (ANOVA). Flies that were activated during the negative control were not taken in account. To determine if a treatment increased the probability of activating a fly and reduced the time to activation, Cox hazard regression was used to take into account censored data (flies that were not activated during the 1min test interval). The level of significance was set at 0.05. As I tested males and females, the variable sex was always integrated in the models to check possible interactions or significant changes in the intercept. No significant interaction between the variables sex and substance was found for all behavioural parameters. Only the intercept of sex was significant for “activation” and “exit”. However, data obtained with males and females were pooled in the interest of clarity as the differences between the sexes did not exceed 10%.

2.3. Results

2.3.1. EAG recordings

The EAG responses of *G. pallidipes* to acetone and to alkanes form pentane to decane were, in a first experiment, recorded to four doses (20µg, 120µg, 1120µg, 11120µg). Stimulation began with the lowest dose and ended with the highest. As the syringes were not completely flushed between successive doses, the amount for each compound added previously was taken into account. The results of this experiment are summarized in Figure 2.5. EAG responses increased with increasing dose of all products tested following exponential curves for acetone and pentane and logistic curves for the other alkanes in the range of doses tested. In fact, doses tested for acetone and pentane may not have been high enough to show logistic curves as for the other alkanes. The dose at which the response reached the 50% level to 1µg of octenol (positive control) was used to compare the effect of each alkane. The dose needed to obtain 50% of the positive control follows a hyperbolic curve with the number of alkane carbon (Figure 2.6A). The lowest effective dose was recorded with octane and nonane. As seen in Figure 2.5, the range of doses tested was too high to determine the threshold for heptane, octane, nonane and decane. In a second experiment alkanes from heptane to decane were diluted in hexadecane (Figure 2.7), thresholds were calculated and are represented in Figure 2.6B. A hyperbolic relationship between the threshold and the number of carbons was also found, with the highest threshold for pentane and hexane and the lowest for octane. The dose-response curve for acetone was very similar to pentane (Figure 2.5) but the threshold was 10 times lower than that for pentane. As a dose of 100 µg of alkanes from heptane to decane diluted in hexadecane provided only the half of the responses obtained with these alkanes injected directly into stimulus syringes, threshold for alkanes from heptane to decane may be underestimated.

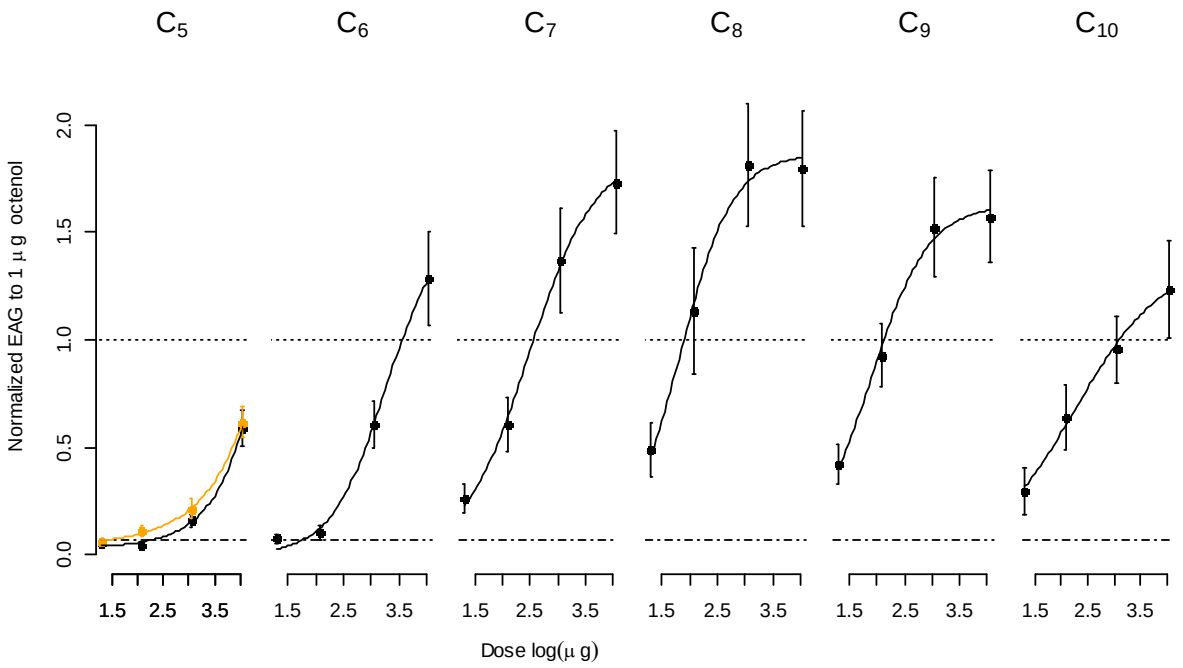

Figure 2.5: EAG responses of *G. pallidipes* to alkanes at different doses in [µg], mean ± SEM (N=4). Results for acetone are shown on the graph for pentane in orange. Trend lines show models used to described the dose-response curve for acetone and alkanes. C_5 pentane, C_6 hexane, C_7 heptane, C_8 octane, C_9 nonane, C_{10} decane.

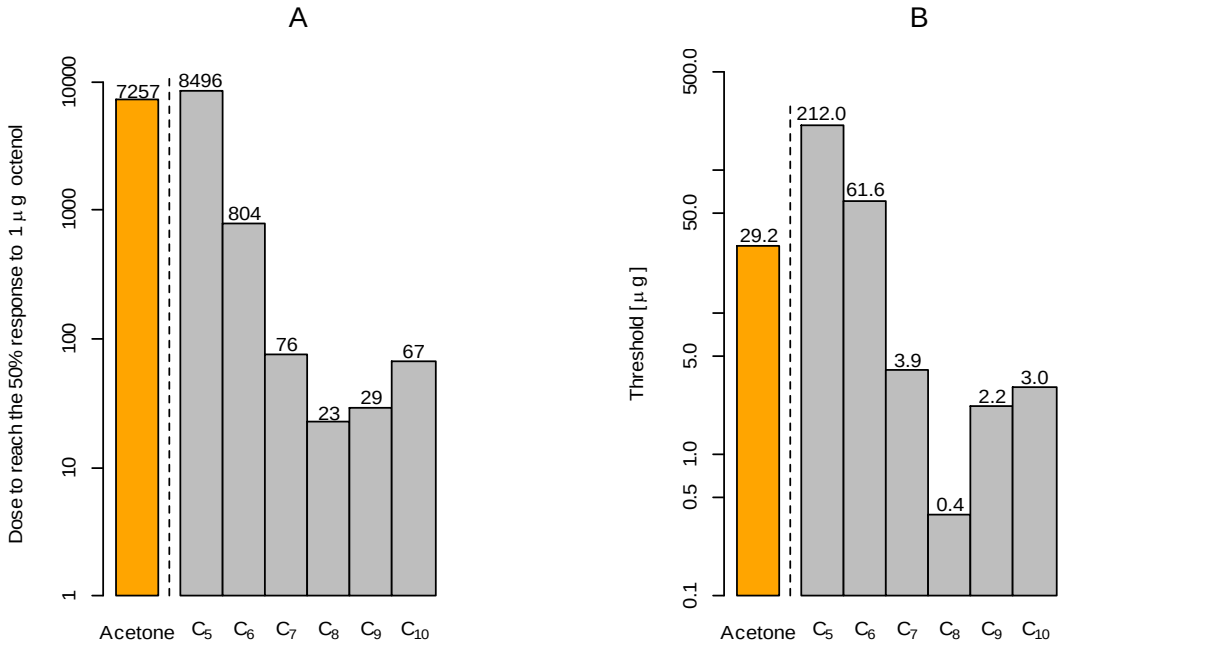

Figure 2.6: comparison of the dose (µg) that was needed to obtain 50% of the response to octenol (**A)** and threshold obtained for each treatment (**B**). Unit of values on bar is expressed in µg. C_5 pentane, C_6 hexane, C_7 heptane, C_8 octane, C_9 nonane, C_{10} decane.

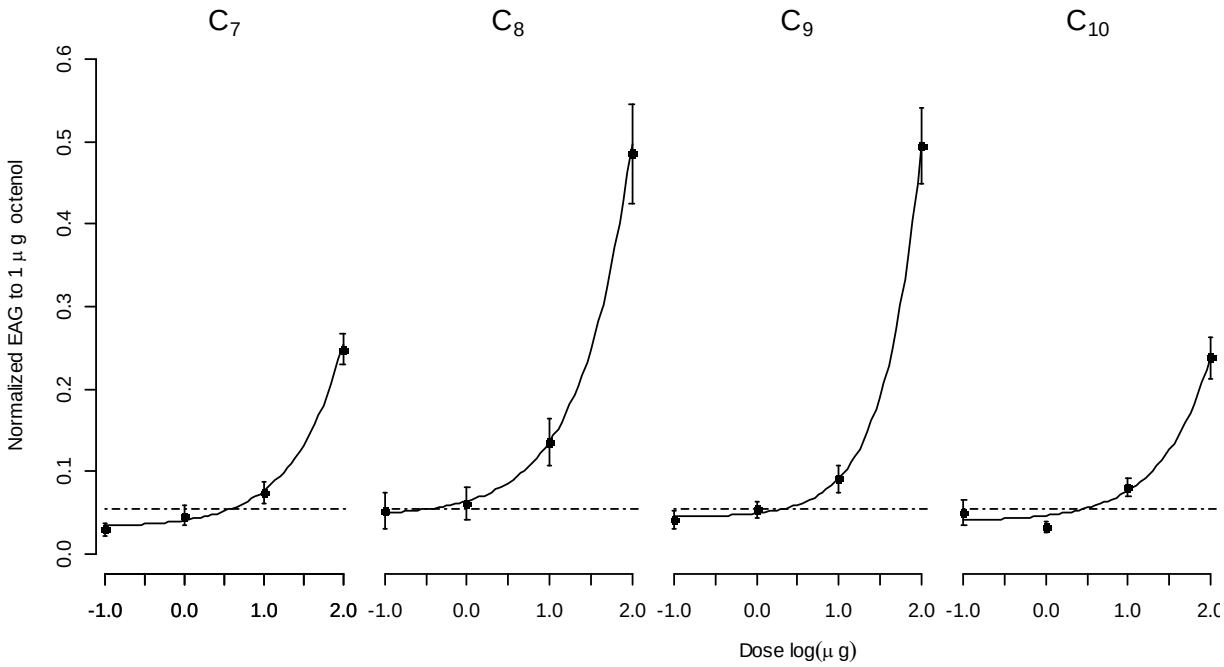

Figure 2.7: EAG responses of *G. pallidipes* to alkanes diluted in C_{16} at different doses in [µg], mean ± SEM (N=4). Trend lines show models used to described the dose-response curve. C_7 heptane, C_8 octane, C_9 nonane, C_{10} decane.

2.3.2.Behavioural responses

G. pallidipes were exposed to acetone and alkanes from hexane to nonane supplemented by CO_2 in the wind tunnel. The first recorded parameters of tsetse responses were the time to activation and the proportion of flies activated. Figure 2.8 and Figure 2.9 summarize the effect of acetone and the alkanes supplemented by CO_2 on the time to activation and the proportion of *G. pallidipes* activated. The addition of 10 µl/min acetone to CO_2 (mean of 36s, censored data were taken into account) significantly decreased the time to activation by CO_2 by a factor of 1.77 (Figure 2.8) and significantly increased the proportion of activated flies to 73.08% from 46.62% for CO_2 alone (Figure 2.9). When the effects of the alkanes at the same release rate (10 µl/min) are compared, heptane significantly reduced the time to activation by a factor of 3.63 compared to CO_2 and significantly increased the proportion of activated flies to 86.32% from 46.62% for CO_2. A same dose of nonane significantly increased the time to activation by a factor of 2.19 compared to CO_2 and significantly reduced the proportion

of activated flies to 25.93%. No significant effect was found for hexane and octane. Breath, used as positive control, significantly decreased the time to activation by a factor of 3.34 compared to CO_2 and significantly increased the proportion of flies activated to 81.72%. Compared to acetone plus CO_2, heptane with CO_2 decreased the time to activation by a factor of 1.975 (Cox hazard regression model, P<0.001, Figure 2.8). A significant difference in activation rate between males and females was found, as indicated by the intercepts, indicating that females were activated 1.28 times faster and in higher number than males.

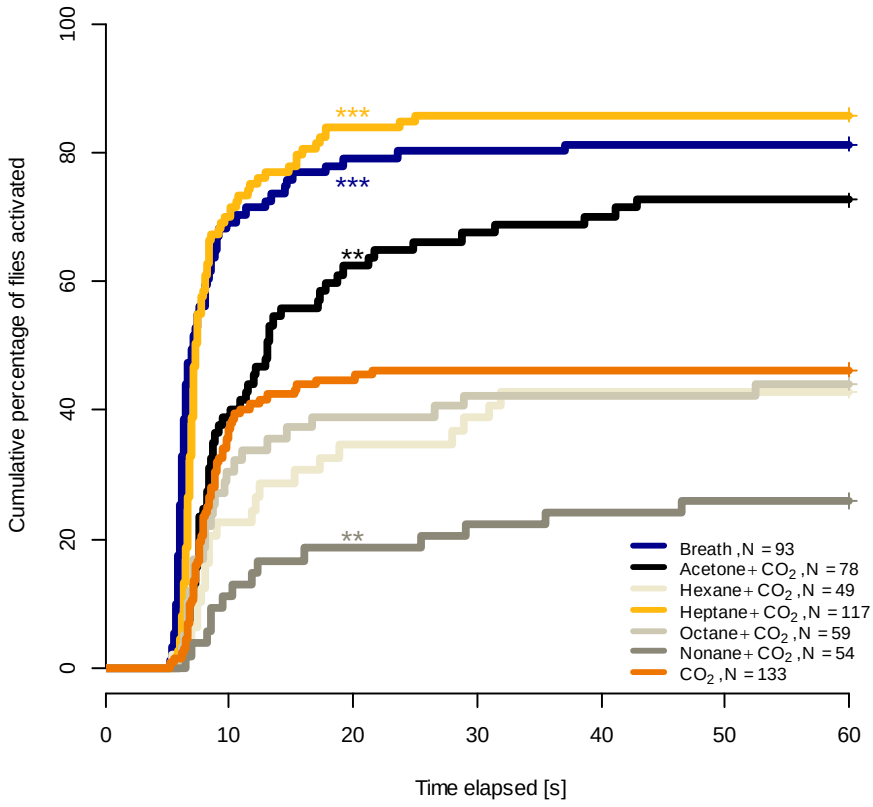

Figure 2.8: Cumulative percentage of activated *G.pallidipes* over a 60s period of time to breath (5l/min), CO_2 (~50ml/min), acetone supplemented with CO_2 and to alkanes supplemented by CO_2. Acetone and alkanes were released at 10µl/min. Zero on the abscissa is the beginning of treatment release that needs about 5s to reach the insect at the downwind end of the wind tunnel. Asterisks indicate levels of probability (Cox proportional hazards model, P<0.01(**) or 0.001 (***)) increased or decreased significantly the time to activation compared to CO_2 alone. Data for males and females were pooled for clarity. No interaction between treatments and sexes was found. Male responses were significantly lower for every treatment compared to females (Cox proportional hazard model, P<0.01).

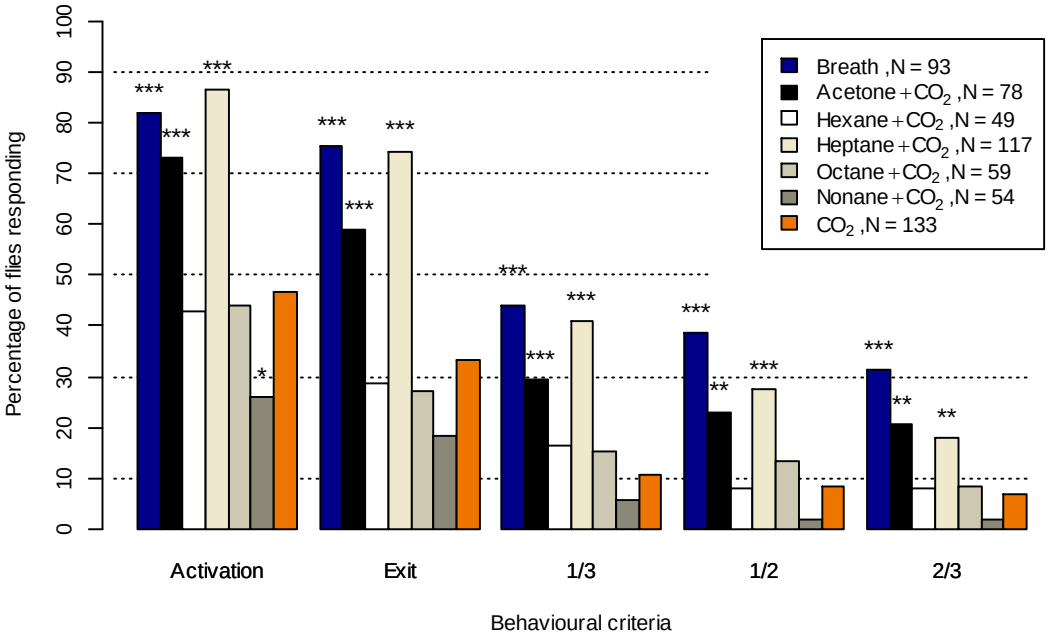

Figure 2.9: Response of *G. pallidipes* in the wind tunnel to breath, CO_2, acetone and to alkanes. Acetone and alkanes were released at the rate of 10µl/min and supplemented by CO_2. The criteria are: "Activation" flies move in the cage, "Exit" flies exit the cage, "1/3" flies pass the first third of the wind tunnel (0.8m), "1/2" flies pass the midline of the wind tunnel (1.25m), "2/3" flies pass the top third of the wind tunnel (1.7m) near the odour source. The percentage of flies responding is the proportion of flies tested that responded under the behavioural criterion. Asterisks indicate that the percentage response was significantly different from that of CO_2 at P< 0.05 (*), 0.01(**) or 0.001 (***) levels of probability following a GLM with a logit link function. Data for males and females were pooled for clarity. No interaction between treatments and sexes was found under any behavioural criterion. For the "activation" and "exit" criteria, male responses were significantly lower for every treatment compared to females (GLM, logit link function, P<0.001).

The addition of either acetone, heptane or nonane to CO_2 changed the activation rate of CO_2, indicating that these products are perceived by *G. pallidipes*, but no information on their potential attractiveness is given by this parameter. So I investigated the distance that *G. pallidipes* flew upwind before touching any wall of the wind tunnel, hypothesing that the more a compound is attractive the more *G. pallidipes* will fly upwind. The wind tunnel was divided into 4 parts beginning with flies exiting the cage at the downwind end, the first third "1/3" (0.8m), midway "1/2" (1.25m) and the top third "2/3" (1.7m) near the odour source. The distance *G. pallidipes* flew upwind was categorized according to these 4 parts. Table 2.4 shows the results in the form of odds ratios with CO_2 as baseline, for breath and for acetone and the alkanes supplemented by CO_2. Breath was the most attractive treatment as its odds ratio for flies reaching the top third section was

the highest. Breath was also more attractive than CO_2 as the probability that a fly exited the cage and flew upwind through the wind tunnel sections was significantly higher than the probability for CO_2 alone. Indeed, 31.18% of flies stimulated with breath reach the top third section of the wind tunnel whereas only 6.77% of flies stimulated with CO_2 reached this section (Figure 2.9). Comparing substances delivered at the same release rate (10 µl/min), the best attractant was acetone when added to CO_2 with odds ratios between 3.18 and 3.56, significantly higher for every behavioural criterion, meaning that the proportion of flies attracted to the mix of acetone plus CO_2 (20.51% of flies that passed to the top third section of the wind tunnel) was significantly higher than CO_2 alone (Figure 2.9). Heptane also proved attractivity when added to CO_2 with a significant odds ratio of 3.014 for flies reaching the top third section of the wind tunnel (17.95% in total). Odds ratios for the heptane-CO_2 mix varied widely from 7.622 to 3.014, significant for all behavioural criteria Table 2.4. The addition of hexane, octane and nonane to CO_2 did not significantly change the responses of *G. pallidipes* to CO_2 alone. As already found for the activation rate parameter, the intercept for males was significantly different from the one for females (GLM with a logit link function, intercept for females 50.17%, intercept for males 40.90%, P<0,01), the intercept of “exit” for males was significantly different to the one for female indicating that more females exited the cage on average than males (according to GLM with a logit link function, intercept for females 37.93%; intercept for males 27.24%; P<0,001). No significant interaction between the variables sex and treatment were found.

Table 2.4: Odds ratio for behavioural responses of *G. pallidipes* to different treatments such as breath, acetone supplemented by CO_2, alkanes supplemented by CO_2 and to CO_2 alone. The behavioural criteria are: "Activation" flies move in the cage, "Exit" flies exit the cage, "1/3" flies pass the first third of the wind tunnel (0.8m), "1/2" flies pass the midline of the wind tunnel (1.25m), "2/3" flies pass the top third of the wind tunnel (1.7m) near the odour source. Values in bold indicate that differences between treatments and CO_2 are significantly different (GLM, logit link function, P<0.05). N is the number of flies tested. Data for males and females were pooled for clarity. No interaction between treatments and sexes was found for any behavioural criteria. For the "activation" and "exit" criteria, male responses were significantly lower for every treatment compared to females (GLM, logit link function, P<0.01 and P<0.001 respectively).

Substances	***Dose***	***N***	***Activation***	***Exit***	***"1/3"***	***"1/2"***	***"2/3"***
CO_2	60-80ml/min	133	1.007	0.589	0.118	0.090	0.073
Breath	5l/min	93	**5.361**	**6.587**	**6.702**	**7.005**	**6.243**
Acetone	10 µl/min	78	**3.326**	**3.181**	**3.555**	**3.327**	**3.556**
Hexane	10 µl/min	49	0.879	0.833	1.659	0.986	1.225
	100 µl/min	57	0.810	0.911	1.190	0.837	0.765
Heptane	1 µl/min	50	1.672	1.708	**2.397**	1.805	0.574
	10 µl/min	117	**7.622**	**6.316**	**5.913**	**4.175**	**3.014**
	100 µl/min	73	**10.196**	**8.867**	**10.891**	**9.150**	**5.943**
Octane	10 µl/min	59	0.939	0.790	1.530	1.740	1.276
	100 µl/min	53	0.602	0.823	1.977	2.269	1.759
Nonane	0.01 µl/min	53	0.939	0.714	1.739	1.972	0.827
	0.1 µl/min	57	1.176	1.183	2.267	1.553	1.621
	1 µl/min	55	1.077	1.230	1.240	0.870	0.795
	10 µl/min	54	**0.413**	0.478	0.500	0.209	0.260

Further investigations of the data were made to establish the dose dependence of the responses of *G. pallidipes* to heptane and nonane. Heptane released at either 100 or 10µl/min with CO_2 significantly decreased and in a similar manner the time to activation by factors 3.51 and 3.63 respectively, whereas release of heptane at 1µl/min with CO_2 did not significantly reduce the time to activation (factor of 1.27; Figure 2.10A). The proportion of flies responding under each behavioural criterion significantly increased with increasing release rates of heptane (Figure 2.11). The best result was obtained with the addition 100µl/min heptane where 30.14% of tested flies reached the top third of wind tunnel, a percentage that was very close to that recorded for breath, the positive control. The addition of nonane released at 10µl/min with CO_2 significantly increased the time to activation by a factor 2.19 whereas responses of *G. pallidipes* to doses

under 10µl/min were not significantly different to the responses recorded for CO_2 alone (Figure 2.10B & Table 2.4).

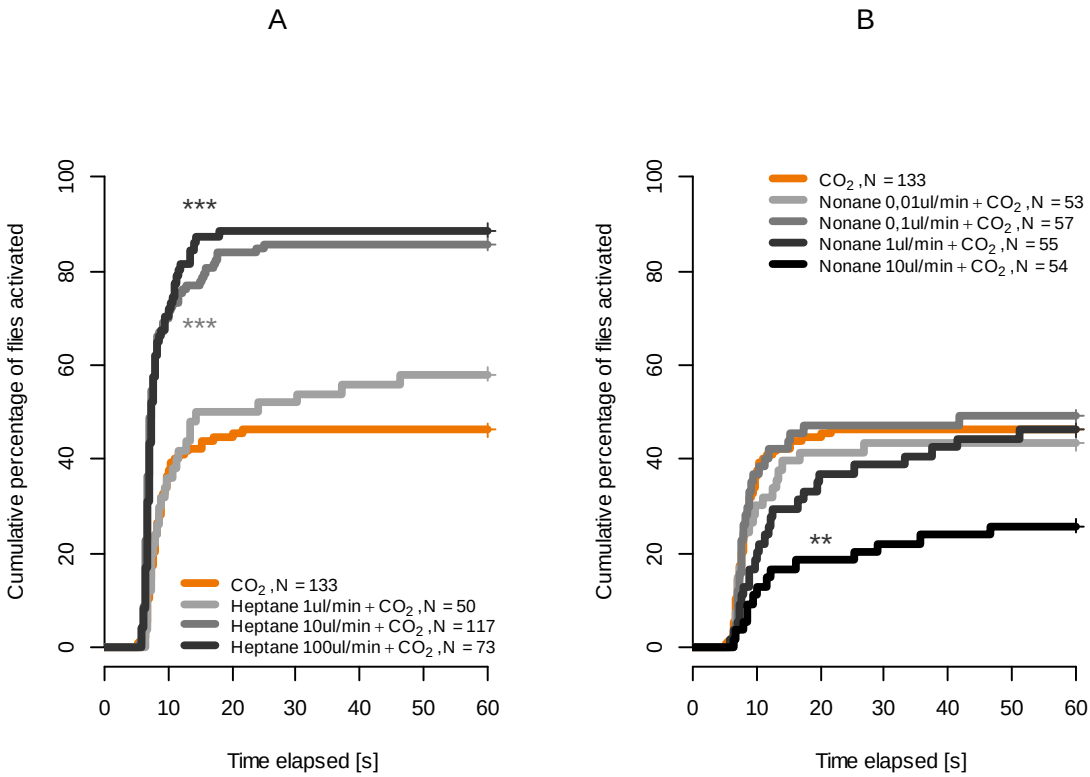

Figure 2.10: Cumulative percentage of activated *G. pallidipes* over a 60s to different doses of heptane (**A**) with CO_2 and to nonane (**B**) supplemented by CO_2. Asterisks indicate levels of probability (Cox proportional hazard model, P<0.01(**) or 0.001 (***)) that a treatment increased or decreased significantly the time to activation compared to CO_2.

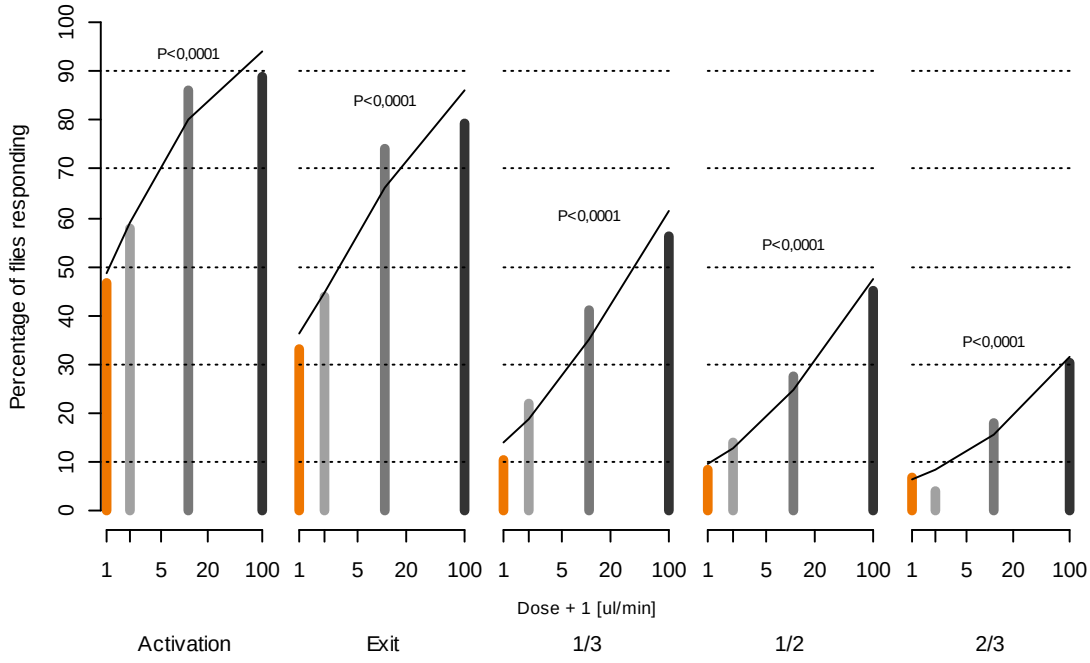

Figure 2.11: Response of *G. pallidipes* in the wind tunnel to different doses of heptane (shades of gray to black) added to CO_2 (in orange). The behavioural criteria are: "activation" flies move in the cage, "exit" flies exit the cage, "1/3" flies pass the first third of the wind tunnel (0.8m), "1/2" flies pass the midline of the wind tunnel (1.25m), "2/3" flies pass to the top third of the wind tunnel (1.7m) near the odour source. The P-value represents the level of probability that the response increases with increasing dose (GLM, logit link function). Trend lines show the predicted values from the GLM model.

As all substances tested were supplemented by CO_2, the possibility of an interaction between heptane and CO_2 was investigated. Heptane was tested without CO_2 at a release rate of 10µl/min. Then the results of this test were combined with the results for CO_2 alone by adding the proportion of responding flies for the two treatments, thus creating a hypothetical additional effect between heptane and CO_2. The results of this experiment were analyzed by GLM with a logit link function, with the hypothetical effect as baseline to test whether the tested combination of heptane and CO_2 significantly exceeded the hypothetical effect (Figure 2.12). *G. pallidipes* responses to heptane without CO_2 were very low (only 2 responding flies over 51 tested or 3.92% of flies activated) and only one fly exited the cage and did not pass the mid line of the wind tunnel. This explains why responses to CO_2 were not significantly different across all behavioural criteria from the hypothetical additional effect. However, responses of *G. pallidipes* to heptane supplemented by CO_2 were significantly different from the

hypothetical additional effect under all behavioural criteria, indicating that heptane presented with CO_2 provides strong synergism. In this analysis, no significant effect of sex was detected except that the proportion of females that exited the release cage was higher than for males (GLM with logit link function: intercept for females 43.74%; intercept for males 26.26%; P<0.01).

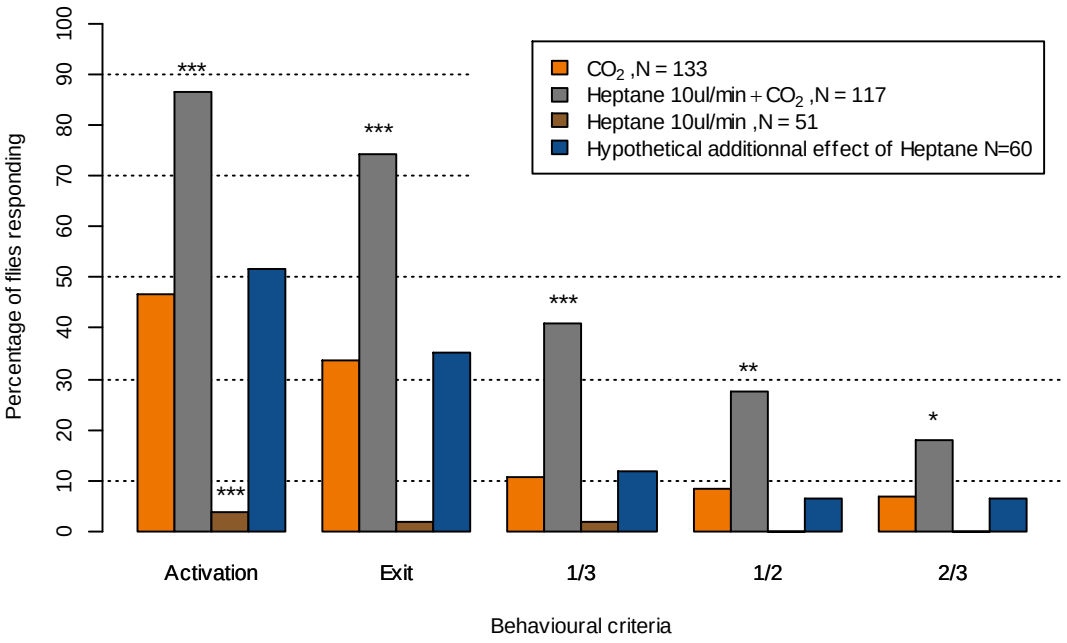

Figure 2.12: Response of *G. pallidipes* to CO_2, to heptane + CO_2 and to heptane. The hypothetical additional effect of heptane and CO_2 was calculated as the proportion of flies responding to CO_2 alone plus the proportion of flies responding to heptane alone (N was arbitrarily fixed at 60 tested flies). Asterisks indicate that the percentage response was significantly different from that of the hypothetical effect at P< 0.05 (*), 0.01(**) or 0.001 (***) levels of probability following a GLM with a logit link function. For the "exit" criterion, responses of tsetse to heptane were removed from the analysis because no male exited the cage. No interaction between sex and treatment was found. Proportions of males exiting the cage was significant lower than the proportion of females (P<0.01) for every treatment.

An attempt to increase the attractiveness of breath was made by adding heptane at release rates of 10µl/min and 100µl/min to breath. Only the highest dose of heptane significantly changed the response to breath. Indeed, adding heptane at 100µl/min to breath decreased the time to activation by a factor of 1.7 compared of breath alone (Figure 2.13A). This treatment provoked an unusual response from *G. pallidipes* in that all flies activated exited the cage. The proportion of flies stimulated with breath plus

heptane at 100µl/min of that exited the cage was significantly higher than the proportion of flies stimulated with breath alone (Figure 2.13B). The other behavioural criteria for attraction were a little higher when heptane was added to breath but were not significant to breath alone.

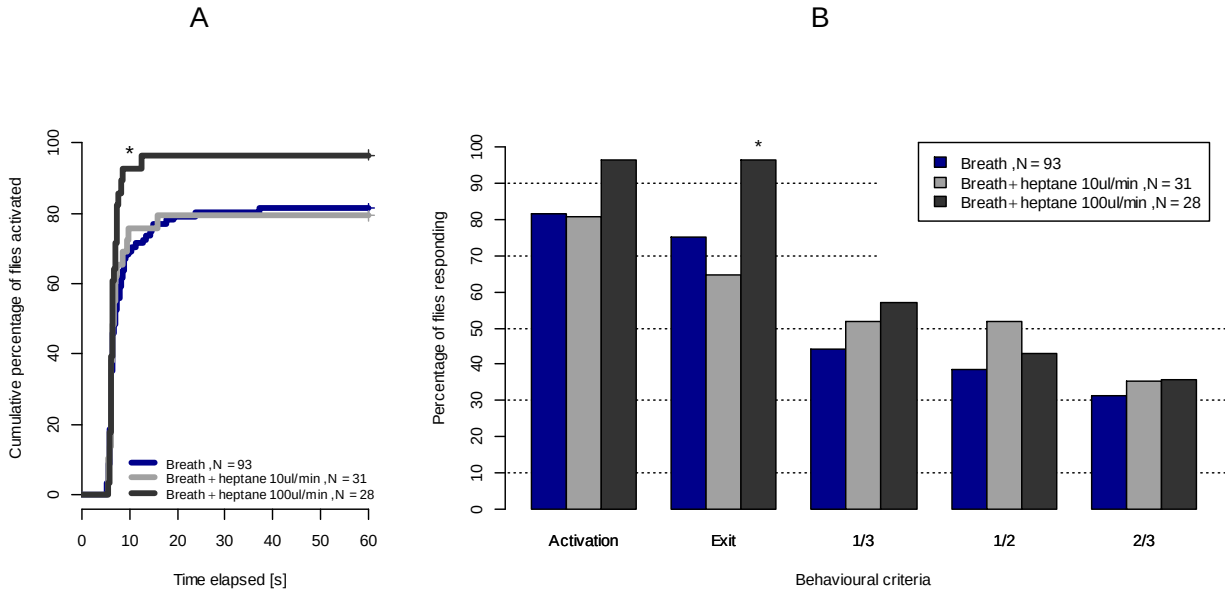

Figure 2.13: Responses of *G. pallidipes* to breath and to breath supplemented by 2 doses of heptane. **A** Cumulative percentage of activated *G. pallidipes* over a 60s. Asterisks indicate levels of probability (Cox proportional hazard model, P< 0.05 (*)) that a treatment decreased significantly the time to activation compared to breath alone. **B** Proportion of flies that completed behavioural criteria “Activation” the fly moves in the cage, “Exit” the fly exits the cage, “1/3” the fly passes the first third of the wind tunnel (0.8m), “1/2” the fly passes the midline of the wind tunnel (1.25m), “2/3” the fly passes the top third of the wind tunnel (1.7m) near the odour source. Asterisks indicate that the percentage response was significantly different from that of breath at P< 0.05 (*) level of probability following a GLM with a logit link function.

2.4. Discussion

My results show that *G. pallidipes* responds to short-chain alkanes in presence of CO_2. The behavioural response is dependent on the length of the carbon chain. Indeed, the mixture of heptane and CO_2 enhanced all behavioural criteria compared with those obtained with CO_2 alone, approaching the score for human breath. On the other hand, nonane tended to weaken the responses to CO_2. Binary mixtures of CO_2 and pentane, hexane and octane did not significantly affect the responses obtained with CO_2 (for pentane see Appendix A). Moreover, I demonstrate that the effect of the heptane and CO_2 mixture on the behavioural criteria was similar if not higher than that of the acetone-CO_2 mixture. The strong effect of the mixture of heptane with CO_2 stems from a clear synergism between the two components as responses to the mixture were higher than the sum of responses obtained with either separately.

Furthermore, my results show that *G. pallidipes* is able to perceive short-chain alkanes with its antenna receptor cells. EAG recordings reveal that alkanes from pentane to decane evoke responses from antennae in a dose dependent manner and that alkanes from heptane to nonane are more effective than acetone at evoking EAG responses. Indeed, 50% of the response amplitude to 1-octen-3-ol was reached with alkanes from heptane to nonane at a dose 100 times lower than that of acetone. Moreover, from heptane to nonane alkanes show the lowest threshold situated between 400ng to 4µg on the filter paper strip, 7 to 70 times lower than the threshold for acetone. EAG responses of several tsetse species to acetone were already described by Den Otter et al. (1988) and their findings corroborate ours in that only high amounts of acetone evoke EAG responses. Within the alkanes tested, lower amounts of heptane to nonane are needed to evoke EAG responses in *G. pallidipes*, consistent with results obtained by Harraca (2008) on *G. brevipalpis* and *G. pallidipes*. However, the amount of alkanes from pentane to decane needed to obtain half of the response to 1µg 1-octen-3-ol ranges from 23 to about 8000 times higher. Assuming that the EAG response represents the response of a population of responding neurones, the amplitude of the responses should be correlated with the number of responding receptor cells and with the strength of their responses (Wibe, 2004). So it is not possible with the EAG

technique to affirm whether short chain alkanes stimulate a smaller population of neurones than 1-octen-3-ol does, whether they stimulate weakly responding receptor neurones or whether they stimulate only weakly a small population of neurones. More information is available for acetone as some studies have investigated responses of single olfactory neurones found on tsetse antenna to this product. In fact, tsetse antennae seem to have less neurones responding specifically to acetone than those responsive to 1-octen-3-ol (Voskamp et al., 1999) and the threshold for acetone would also appear to be higher than for 1-octen-3-ol (Otter and Naters, 1992). Even though more acetone than 1-octen-3-ol is needed to obtain an EAG response, acetone is an important host cue intervening in the host seeking behavior of tsetse. The amount of acetone released by an ox is about 500 times higher than the amount of 1-octen-3-ol (Torr et al., 1995). It is possibly more pertinent for the fly to be equipped with receptor neurones that are tuned to the amount released by the host. Similar to acetone, I could imagine that short chain alkanes stimulate a small population of neurones with a dose response curve shifted to higher doses than the curve for 1-octen-3-ol.

Alkanes play a substantial role in tsetse sensory ecology. Methylated long-chain alkanes such as 15,19,23-trimethylheptatriacontane and 13,23-dimethylpematriacontane are found on the cuticle of *G. morsitans morsitans* and *G. pallidipes* females, respectively, and act as contact pheromones eliciting copulatory behaviours by males (Carlson et al., 1984; Carlson et al., 1978). However, short-chain alkanes have been neglected in the behavioural ecology of tsetse and other hematophageous arthropods but they have already been described as chemostimulants for mosquitoes (Healy and Jepson, 1988; Puri et al., 2006). Vale (1980) already tested a mixture of CO_2 and hexane in field traps, but found no significant effect of this mixture in the catch index relative to CO_2 alone. More recently, Harraca (2008) showed that *G. brevipalpis* hardly responds to short-chain alkanes on their own. When combined with 1-octen-3-ol or with a mixture of 1-octen-3-ol, p-cresol and 3n-propylphenol, heptane showed the noteworthy property of eliciting flight responses in a wind tunnel, comparable to the effect of acetone with these products. Similar results in a context with CO_2 for *G. pallidipes* are described here. Heptane alone elicits poor responses in *G. pallidipes* but acts synergistically with CO_2 as well as for acetone. Acetone act

synergistically with CO_2 in a similar manner as shown by Joris (2013) under the same experimental condition. Tsetse hardly responded to 1.8ppm acetone alone. This type of synergism was already demonstrated by Torr (1990) and Vale and Hall (1985b) who found that the combination of acetone and CO_2 is more attractive to *G. morsitans* and *G. pallidipes* in the field than either separately.

The CO_2 context was used for two main reasons. Firstly, VOCs such as acetone and alkanes are released in breath (Krotoszynski et al., 1977; Spinhirne et al., 2004) and so accompanied by CO_2. Secondly, I wanted to benefit from the sensitizing effect of CO_2 described by Dekker et al. (2005) to detect possible synergism between compounds that occur together in nature. CO_2 alone at 30ppm above ambient level was sufficient to activate about 50% of flies tested and, to a lesser extent, to elicit upwind flight. A low dose of CO_2 near the detection threshold was used to maintain the resolving power of the behavioural tests as high as possible, knowing that tsetse respond in a dose dependent manner to CO_2 (Turner, 1971) and that CO_2 can elicit upwind anemotaxis on its own (Colvin et al., 1989; Evans and Gooding, 2002; Paynter and Brady, 1996). Bursell (1984a) proposed that the threshold for CO_2 is situated near 100ppm but I demonstrate here that it is probably lower than 30ppm. The response to CO_2 was much weaker than the response to human breath, letting us consider that CO_2 is not entirely responsible for the response of tsetse to breath. Admittedly, the amount of CO_2 in the two treatments was not the same (Table 2.3). This adds a qualifier to these remarks. However, human breath proved an attractive host effluent supporting the findings of Gurba et al. (2012), Joris (2013), Vale (1979) and Warnes and Finlayson (1985b). Human breath shares some compounds with bovine breath: CO_2, water, acetone, heptane, nonane and other short-chain alkanes were found in both (Elliott-Martin et al., 1997; Mottram, 1997; Spinhirne et al., 2004).

Alkanes occurring in breath arise from oxidative stress that is typically characterized by higher production of reactive oxygen species (ROS). In turn, ROS attack biomolecules such as DNA, proteins and lipids (Davies, 1995). Of interest here are the effects of ROS on lipids, known as lipid peroxidation. Since lipid peroxidation occurs in blood, polyunsaturated fatty acids (PUFAs) of erythrocytes are attacked by ROS (Phillips et al.,

2000a). Critical end products of this reaction are short chain alkanes, methylated alkanes and other VOCs which diffuse through the blood-lung barrier and are expired in breath. Much of the information available to us on lipid peroxidation stems from breath analysis in clinical medicine where the end-products are used to monitor the degree of oxidative stress that signal various physiological disorders (Amann and Smith, 2005).

The lowest concentration of heptane used in my behavioural assay is 91ppb, 23-758 times higher than the concentration of heptane found in breath of a healthy human that ranges from 0.12 to more than 4ppb (Krotoszynski et al., 1977; Poli et al., 2005). However, my results show that tsetse respond in a dose dependent manner to CO_2 combined with increasing amounts of heptane. Moreover, the mixture of 91ppb heptane and CO_2 did not significantly increase all behavioural criteria except the proportion of flies that pass the first third of the wind tunnel. Thus it is reasonable to propose that the threshold for any behavioural responses to heptane is lower than the dose used in my behavioural tests, approaching the physiological concentrations found in breath. Heptane is also found in bovine breath but unfortunately no quantitative information is available. Another factor has to be taken in account. According to Phillips et al. (1999), the emission of alkanes into the environment is the balance of the production rate and the clearance rate from the body. This balance is termed the alveolar gradient and is estimated by subtracting the amount of alkanes in ambient air (inhaled air) from that in exhaled air. In healthy people the alveolar gradient for short chain hydrocarbons is negative indicating that clearance by the organism is faster than the production rate. Contradictory data have been presented as a positive gradient was found for heptane in earlier papers (Phillips et al., 1994; Poli et al., 2005) but a negative gradient is accounted for other papers (Phillips et al., 2000a; Phillips et al., 2000b; Phillips et al., 1999). This could be explained by the fact that the breath extracts of Phillips were conducted in Staten Island (NJ, USA) an urban site. It is known that urban sites are polluted with high concentrations of alkanes (Fraser et al., 1997). This could influence alveolar gradients, especially those that are close to 0 such as for heptane. In fact, an alveolar gradient close to 0 means that ambient air contains a concentration of alkanes close to that of exhaled air. So a small decrease in the alkane concentration found in ambient air due to the dilution effect of wind for example could flip the alveolar gradient

to a positive value. However, the alveolar gradient of heptane, like that of other short chain alkanes, approaches zero or becomes positive in older people as oxidative stress increases with age (Droge and Schipper, 2007; Phillips et al., 2000a; Phillips et al., 2000b). Furthermore, some evidence of oxidative stress associated with African trypanosomiasis has been highlighted in the past decades. Ameh (1984) discovered that a decrease of glutathione in blood and liver is associated with an increase of erythrocyte susceptibility to oxidative stress after infection with *T. brucei gambiense*. The administration of antioxidant vitamins to rats infected by *T. brucei* decreases anaemia and tissue damage (Umar et al., 2007). Recently, it has reported that infection of rats and camels with *T. brucei* and *T. evansi* is associated with lipid peroxydation (Eze et al., 2008; Saleh et al., 2009; Wolkmer et al., 2009). Since VOCs originating from lipid peroxidation such as short-chain alkanes are exhaled in human and bovine breath (Phillips et al., 2000b; Spinhirne et al., 2004) they could be used as chemical cues by tsetse flies and other vectors of disease to discriminate between individuals within a deme. Indeed, there is some evidence for selective host choice in tsetse flies. Vale (1981) has already suggested that production of specific chemical cues could lead to a preference by tsetse for a particular individual within a species type. Tsetse are known to exploit particular individual animals that differ in age (Torr, 1994; Torr and Mangwiro, 2000; Torr et al., 2006; Torr et al., 2001), size (Hall et al., 1984; Vale, 1974b), nutritional status (Vale, 1981) and infection status by *T. congolense* (Baylis and Mbwabi, 1995). A possible advantage for tsetse to exploit older animals for example is that older oxen defend themselves less than younger ones in term of legs movements, thus increasing the feeding success of tsetse (Torr and Mangwiro, 2000). Exploiting more infected animals than uninfected ones is maybe an advantage for trypanosomes but also for tsetse. In fact, feeding indices showed that the blood meal was facilitated when tsetse fed on *T. congolense* infected cattle compared on uninfected animals resulting in a possible reduction of exposure to host defense (Moloo et al., 2000).

Intriguingly, heptane was the only alkane among those tested in this study that increased all behavioural criteria. No significant effect was obtained with the others except nonane that significantly decreased the activation rate at the highest dose tested. Moreover, behavioural responses of *G. pallidipes* to the linear alkanes of a

specific carbon number showed a higher degree of selectivity than EAG responses. This could indicate that alkanes are more or less efficient at stimulating different receptor cells according to their number of carbons. However, the reasons for the specificity in behavioural responses of tsetse to alkanes of a specific carbon number are unknown.

The results of my behavioural tests showed that mixtures of CO_2 plus acetone and CO_2 plus heptane increased the number of flies that reached the upwind third of the wind tunnel, indicating that these mixtures increased the number of flies that undertook an anemotactic flight compared to CO_2 alone, whereas the mixtures of CO_2 with the other alkanes had no effect. This conclusion should, however, be interpreted with caution as few flies crossed the upwind third of the wind tunnel and as the number of flies that crossed this section seemed to be directly dependent on their activation rate. As *G. pallidipes* reaches the upwind end of the wind tunnel in less than 1s, the spatial and temporal resolution of my wind tunnel assay is not appropriate to analyse flights of such fast flying insects and this could explain why the probability of a fly flying across the midline and final third of the wind tunnel remains constant over different treatments: as flies fly more, the proportion that reaches the upwind end of the wind tunnel increases (Figure 2.9). The pertinent variable to discriminate responses of *G. pallidipes* to different treatments in my wind tunnel tests is the proportion of activated flies, the time to activation and the proportion of flies that fly to cross the first third of the wind tunnel. In Chapter 3, this problem was overcome by using an experimental paradigm where flies are stimulated by a visual target surrounded by olfactory cues (differences in flight tracks obtained with and without sphere are shown in Appendix B.1).

2.5. Conclusions

The results provide evidence that short chain alkanes are perceived by tsetse flies and intervene in the host seeking behaviour of this insect. These end-products of lipid peroxidation may serve to signal to tsetse to the level of oxidative stress of the host. The remarkable response to heptane described here for *G. pallidipes* and by Harraca (2008) for *G. brevipalpis* suggests a possible alternative to acetone in the POCA attractive blend. Heptane presents at least two advantages for field tests. Firstly, heptane evaporates slower than acetone as the vapour pressure of heptane at 20°C is 5.3hPa and that of acetone 24.5hPa, reducing the necessity to refill dispensing bottles. Secondly, I showed that a mixture of heptane with either CO_2 or breath remains attractive even with high dose of heptane. However, taking into account the poor temporal and spatial resolution of the wind tunnel assay, further experiments are needed to assess in more detail the importance of such a molecule in presence of a visual target (see Chapter 3).

3. Responses of *G. pallidipes* to human breath and its constituents in presence of a visual target in a wind tunnel

3.1. Introduction

Breath retained my attention in the context of host seeking by hematophageous arthropods. Due to the enormous alveolar surface of exchange between air and blood, air in lungs picks up metabolites from blood and this results in exhaled breath, a complex mixture of CO_2, water, and more than 200 volatile organic compounds (VOCs) (Phillips et al., 1999). Many hematophageous arthropods such as tsetse (Bursell, 1990; Green, 1993; Warnes, 1989; Warnes, 1990a), mosquitoes (Gillies, 1980; Klun et al., 2013) and ticks (McMahon and Guerin, 2002) are attracted by this host effluent. Past research has revealed that several components of the breath mixture are implicated in this attraction. CO_2, the end-product of aerobic metabolism, plays a major role during host seeking in tsetse as in many other hematophageous arthropods (Guerenstein and Hildebrand, 2008). CO_2 attracts mosquitoes and sensitizes them to body odours (Dekker et al., 2005). In tsetse, CO_2 increases catches in the field (Torr, 1990; Vale and Hall, 1985b) and elicits upwind flights by tsetse in a wind tunnel (Paynter and Brady, 1996). Acetone and 1-octen-3-ol, derived from the lipid metabolism, are also described as attractants in tsetse (Hall et al., 1984; Vale and Hall, 1985b), mosquitoes (Takken et al., 1997) and ticks (McMahon et al., 2001). Furthermore, breath is regularly contaminated through frequent eructation with volatiles emanating from the rumen, known to attract hard ticks and tsetse (Donzé et al., 2004; Harraca et al., 2009a). Breath provides a tight link between the physiology of the ectoparasites and that of the host as it represents a window into the host metabolism for hematophageous arthropods to exploit hosts.

Odour cues carried by the wind play an important role in the host seeking behaviour in tsetse, major vectors of African trypanosomes causing sleeping sickness in humans and nagana in cattle. Indeed, olfactory cues carried by the wind are detected well beyond the visual attraction range of the host, from up to 60-120m downwind according to Vale (1984). Olfactory stimulation activates resting tsetse (Warnes, 1992) and elicits anemotaxis (Colvin et al., 1989; Gibson et al., 1991; Paynter and Brady, 1993; Torr and Mangwiro, 1996), behaviours that draw tsetse into the range of visual attraction to the host. Olfactory baits, such a mixture of 3n-propyl-phenol (P), 1-octen-3-ol (O), p-cresol (C) and acetone (A) (POCA) enhance catches of trapping devices, such as traps and targets used to control tsetse populations (Kappmeier and Nevill, 1999; Torr et al., 2011; Vale et al., 1988). However, tsetse flies show a poor ability to find an odour source in the absence of visual stimulation (Bursell, 1984b; Vale, 1984). Tsetse are attracted by devices coloured in blue, white or black (Green, 1986; Green, 1993; Green and Cosens, 1983; Lindh et al., 2012), that may contrast with the background. When tsetse arrive within the visual attraction range of trapping device they modify their flight trajectories (Torr, 1989), aim for the device in directed flights (Gurba et al., 2012) and following this, investigate the device by circling it (Gibson et al., 1991). A substantial number of flies end up investigating the trapping device without landing on or entering it, thus reducing the efficiency of trapping devices. In addition to attraction, i.e. sufficiently affecting the directed flight behaviours of tsetse to ensure that flies reach the trapping device, a target needs to provide an appropriate landing stimulus to finally lure an approaching fly to land. Consequently, a better understanding on how tsetse flies approach a visual target and how visual and olfactory stimulation work in unison could help to design even more efficient trapping devices.

To investigate this, I presented tsetse in a wind tunnel with strong visual stimulus, a blue sphere and surrounded it with human breath as an olfactory cue. This combined stimulus provides the opportunity to record behavioural steps of the approach to a visual target never recorded before in sequence for tsetse in 3D. The effect of olfactory cues on crucial behavioural steps such as activation, directed flight to the visual target and local search could be quantified in this manner. My results show that the rich content of VOCs in breath play an important role at every behavioural step recorded and reveal

the role of fundamental chemical stimulants stemming from the host physiology that have been ignored to date in host seeking in tsetse. Moreover, the landing behaviour of *G. pallidipes* exposed to different objects surrounded by human breath was quantified. My results show that a blue sphere provides a landing stimulus.

3.2. Materials and methods

3.2.1. Insect

G. pallidipes Austen pupae, supplied by the IAEA (Siebersdorf Laboratories, Austria), and imagos were kept into 2 climate chambers offset by 2 hours and programmed as follows: 8h light at 26°C 85%RH with a 1h ramp for light and temperature at dusk and dawn and 14h dark at 22°C, 85% RH. Females and males were separated at emergence to prevent mating and placed in cotton netting cages (25 cm * 15 cm * 15 cm). Teneral flies were tested on day 2 after emergence (day 0). Fed and starved flies were fed on day 3 with defibrinated bovine blood through a silicon membrane and were tested in the wind tunnel on day 9. The behaviour of tsetse flies was observed during the 2 daily activity peaks in the first 2 hr and the last 2 hr of the photophase.

3.2.2. Wind tunnel

The wind tunnel, the odor release system and the tracking system is described in Chapter 2. However some modification was made. A target made of a round-bottomed 2L flask (8cm in diameter) painted with Gentian Blue (Dupli-color, Migro, Marin, Neuchâtel, Switzerland) was placed in the odour plume on a transparent plexiglas 2,5 cm diameter stem erected on a base (15 cm * 47,5 cm * 1,2 cm). The center of the target was situated at the followed coordinates: X=1.5 m, Y=0 m, Z=0.5 m (Figure 3.1). The reflectance spectrum of the gentian blue paint was analysed with a spectrophotometer (Datacolor, Lawrenceville, New Jersey, USA). The peak of reflectance was at 460nm with a shoulder at 410nm, a spectrum comparable to the standard phthalogene blue used on the field (Green, 1994). To increase the contrast between the floor and the flying insect, a diffuse red light was placed under the floor of the wind tunnel made of glass. This light was generated by a panel of LEDs (Luniscontrol Gmbh, Lanzenhäusern, Bern, Switzerland) covered with sintered glass and with a light filter (Roscolab Ltd., Schloss Holte-Stukenbrock, North Rhine-Westphalia, Germany) with a cut off at 600 nm (Figure 3.1).

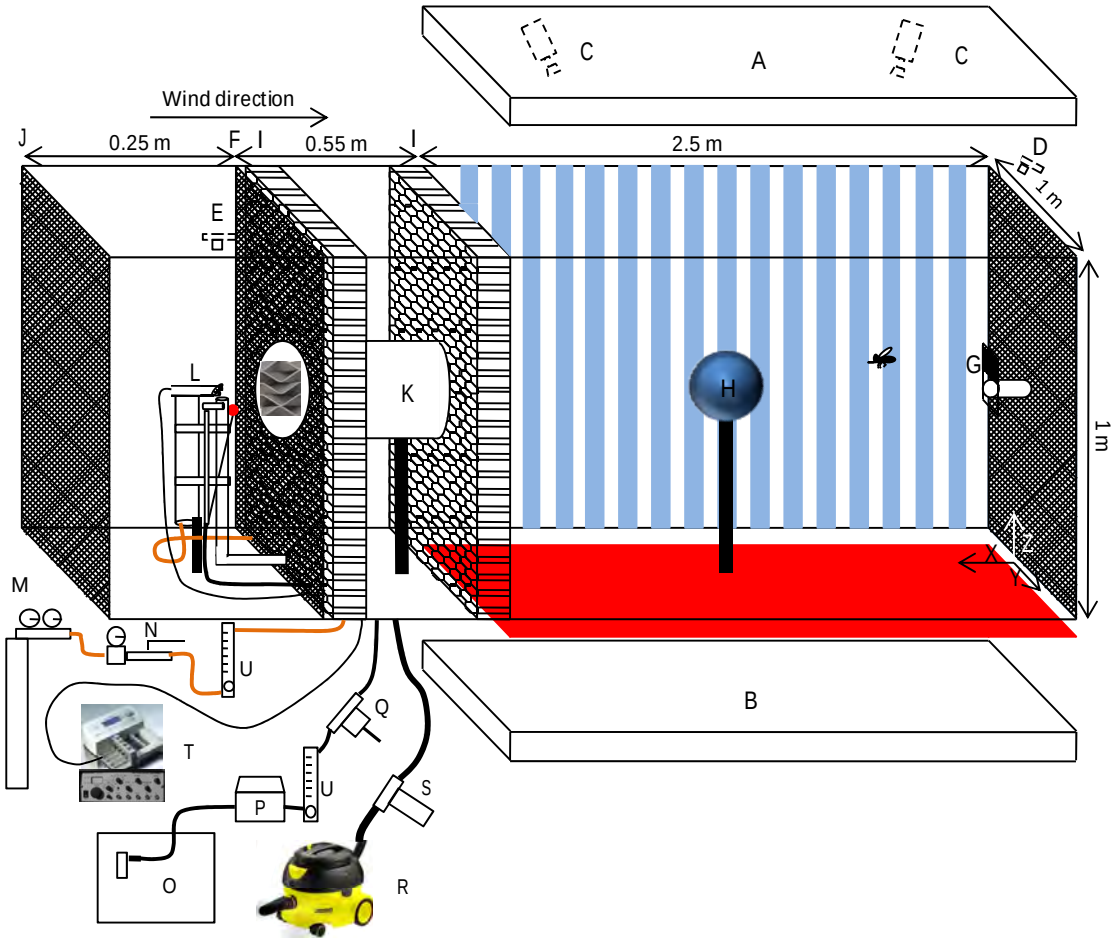

Figure 3.1: Scheme of the wind tunnel, not to scale. **A** High frequency neon lighting; **B** Panel of LEDs covered with a filter cutting out light above 600nm; **C** Cameras of the tracking system were in the middle of the light field A; **D** Web cam to observe fly activation behavior; **E** Web cam to observe the plume generated by the piezo nebulizer and highlighted by a red LED (red dot); **F** 0,5 mm mesh mosquito netting; **G** Fly release cage; **H** Target; **I** Honeycomb sheets; **J** Nylon laminar flow screen; **K** Static gas mixers housed in a steel tube; **L** Odour delivery system (for details see Figure 3.2); **M** CO_2 pressurized cylinder; **N** Manometer followed by a hand valve; **O** Gas sampling Tedlar bag; **P** Pump to push breath into the wind-tunnel; **Q** Three-way solenoïd valve controlling entrance of breath into the wind tunnel; **R** Aspirator; **S** Valve to control the aspiration flow; **T** Syringe pump with frequency generator; **U** Flow meter.

Breath versus CO_2

In this experiment, the effect of human breath and CO_2 was compared in teneral and in fed and starved flies. Human breath was blown into 25L Tedlar gas-sampling bags (CELscientific corp, Santa Fe Springs, California, USA) that were cooled to room temperature for 15min before experiments. Breath was pumped (MPC 100-E, Saskia, Germany) into the wind tunnel at a rate of 5L/min. CO_2 was released from a pressurized cylinder (99,99%, Carbagas, Gümligen, Bern, Switzerland) at a rate of 220 ml/min corresponding to a dose of CO_2 higher than that found in breath (concentration

of CO_2 measured in the release cage: mean ± sd, breath 59,6 ppm ± 1,57; CO_2: 69,58 ppm ± 8,10). Breath and CO_2 were released from the same tube (Figure 3.1 & Figure 3.2). As the content of a Tedlar bag is only 25L, breath was not continuously released and aspirated as for CO_2. To release breath, the pump was switched on at the same time as the aspiration valve was closed and the aspirator switched off. Tedlar bags had the advantage of providing a better control of the release rate of breath compared to a release system where breath was directly blown inside the wind tunnel. However, potential migration of plastic pollutants from the bag walls to the breath sample or transformation of the breath sample quality due to the pump could not be ignored. A control was made where breath was replaced by charcoal-filtered air in Tedlar bags. This air was pumped from bags into the wind tunnel at a rate of 5L/min according to the release system of experiment "The pulse of breath", accompanied by CO_2 released at 220ml/min.

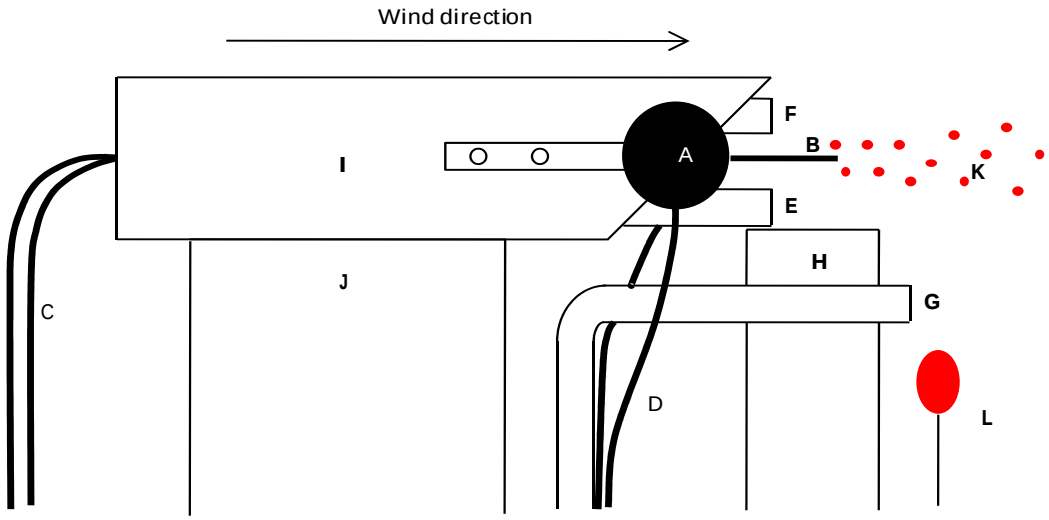

Figure 3.2: Magnified sideview of the odour release system. Two piezo nebulizer discs (**A**) were mounted on each side of the head (**I**) that held a glass capillary with a drawn-out tip (**B**), PTFE microtubes (**C**) and electric wire (**D**). Gases such as CO_2 or breath were released from tube **E** for experiment "Breath versus CO_2". In experiment "The pulse of breath", CO_2 was released from tube **E** and breath was released from tube G outside the aspiration flow (**H).** The aerosol (**K**) and CO_2 were sucked through tube **H** out of the wind tunnel when not required. The production of the aerosol was monitored with a web cam filming the diffraction of red light produced by a LED (**L**) in the aerosol (**K**). Another tube (**F**) was added in prevision for other experiments but was not used.

The pulse of breath

In this experiment, fed and starved flies were exposed to 3 odour treatments: a continuous flow of breath, a 2s pulse of breath immediately followed by a continuous

flow of carbon dioxide and a 2s pulse of breath. The pulse of breath and the continuous flow of breath were released from an additional tube (4,25 mm i.d., 6 mm o.d.) situated outside the aspiration flow and connected to a three-way solenoïd valve (Z723 A, Sirai, Steinhausen, Zug, Switzerland) which allowed the injection of breath at defined intervals into the air stream (Figure 3.1 & Figure 3.2). The aspirator and the two valves were connected to a timer programmed as in Figure 3.3. The amount of CO_2 released was similar to that delivered in breath (Table 3.1 & Figure 3.4).

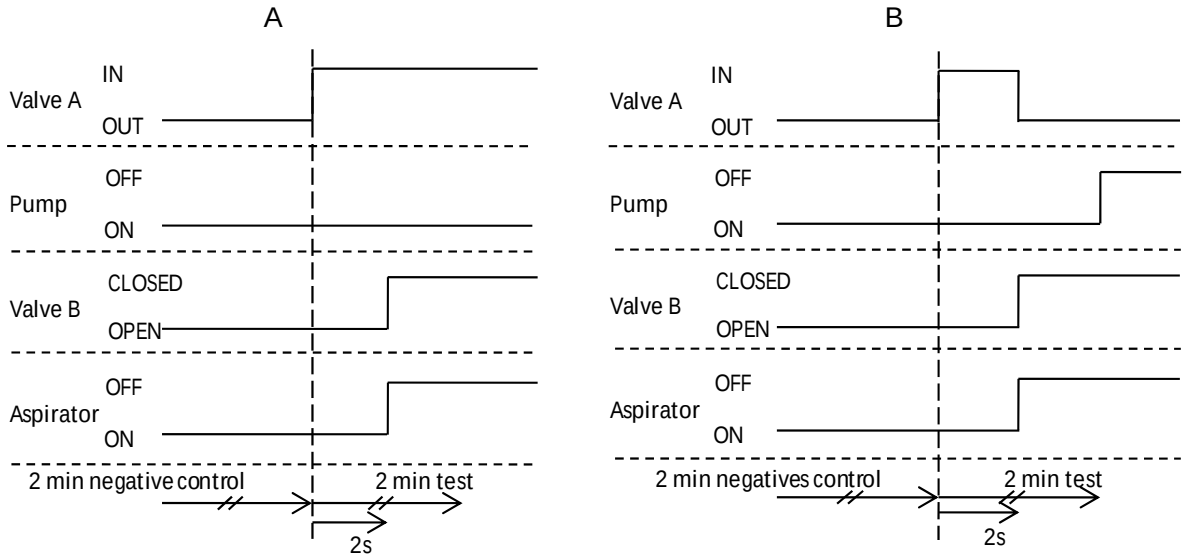

Figure 3.3: Outline of the 2 timer programs producing the 3 different stimulation regimes. **A**: program for continuous breath delivery. The pump is switched on 5s before stimulation. **B**: Program for 2s pulse of breath followed by CO_2 and for the 2s pulse of breath followed by pure air. The difference between these 2 treatments is in the fact that first CO_2 is injected into the wind tunnel and in the second case no gas is injected. Valve A corresponds to the three-way solenoïde valve controlling entrance of breath into the wind-tunnel. Pump corresponds to the apparatus to inject breath into the wind tunnel. Valve B is the solenoïde valve that closes the aspirator canal.

Table 3.1: Mean and standard deviation of CO_2 measurements. Measurements were repeated 3 times for each stimulation types and were done in the opened release cage in experimental condition (with the sphere).

Treatment	Mean of CO_2 jump [ppm]	Standard deviation
Breath in continuous	73.06	3.18
Pulse of breath followed by carbon dioxide	76.15	8.10
Pulse of breath followed by pure air	70.97	0.93

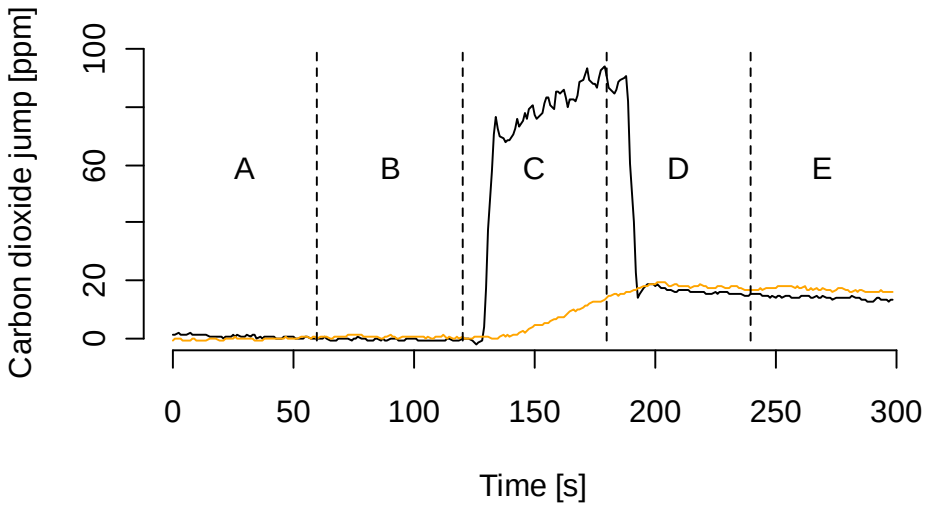

Figure 3.4: CO_2 measurements in the release cage (black line) and in the center of an insect cage on the floor of the wind tunnel (orange line). CO_2 was released at 220 ml/min. Dashed lines mark events such as: **A** aspirator was switched on; **B** CO_2 valve was opened; **C** aspiration flow was closed and aspirator was switched off; **D** aspirator was switched on; **E** CO_2 valve was closed. No jump in CO_2 was observable at the cage on the floor. However, CO_2 levels of the wind tunnel increased linearly as the wind tunnel is a closed circuit and charcoal filters do not remove CO_2.

Responses to test products

Flies were exposed to a mixture of CO_2 supplemented by synthetic compounds found in human breath and listed in Table 3.2 & Table 3.3. They were released by the piezo nebulizer described in Chapter 2. CO_2 was released as in experiment “Breath versus CO_2”.

Table 3.2: List of synthetics tested in the wind tunnel.

Products	Suppliers	Purity [%]
Geranylacetone	Flucka	98
Acetone	Acros	99.8
Heptane	Flucka	99.5
Isoprene	Flucka	99.5
Hexane	Merck	97
Ether tert-butyl-methyl	Sigma-Aldrich	99.8
Ethanol	Honeywell	Abs.

Table 3.3: Release rates of synthetics released by piezo nebulizer and accompanied by CO_2 at the same dose of that released in experiment "Breath versus CO_2". Concentrations were calculated as if the plume was homogenous.

Products	Release rate [µl/min], [pl/min]*	Concentration [ppm], [ppt]*	Solvent	Release rate of solvent [µl/min]
Geranylacetone	0.01* 0.1* 1* 10* 100* 1000*	0.00061* 0.0061* 0.061* 0.61* 6.06* 60.55*	Ethanol	10
Isoprene	0.2	0.027	Acetone	2
Acetone	2 10 100	0.36 1.81 18.10	None	
Heptane	1 10 100	0.091 0.91 9.15		
Hexane	10	1.03		
Ether tert-butyl-methyl	10	1.05		
Ethanol	10	2.28		

Test Procedure

Six flies (a batch) were transferred into 6-7 plastic release cages (transparent PVC cylinders 15 cm × 10 cm). Flies with wing damage were discarded. The cages were placed on the floor at the downwind end of the wind tunnel. After 15 min for fly acclimatization to the wind-tunnel conditions the first cage was placed horizontally on a stand at 50 cm from the floor and at 5 cm from the downwind end of the wind-tunnel.

The tracking system and a stopwatch were launched (time 0) and the door of the cage was lifted within 15s. The fly was successively exposed for an equal period of time to odour-free air (negative control) and then to the test odour. Stimulation began at time 75s and lasted 60s for experiments “Breath versus CO_2” and “Responses to synthetics”. In experiment “The pulse of breath”, stimulation began at time 135s and lasted 120s. Each fly was removed from the wind tunnel at the end of the experiment and the empty cage was placed on the floor of the wind tunnel. The same procedure was followed for the following five flies. Flies that sat in cages on the floor of the wind tunnel were not exposed to odours delivered from the plume generator (Figure 3.4). The potential effect of the progressive increase in CO_2 level inside the wind tunnel (Figure 3.4) or the potential effect of different acclimatization periods between flies in a batch was tested for my data and no behavioural difference was found between the first and the last fly in a batch. For experiments “Breath versus CO_2” and “Responses to synthetics”, flies in a batch received the same treatment, i.e. CO_2, breath or test compounds mixed with CO_2. For experiment “The pulse of breath”, each fly of 1 batch was tested randomly with 1 of the 3 treatments. As the downwind end of the wind tunnel was not visible to the cameras of the tracking system the time to activation of a fly was measured by observing flies with a web cam (SPCI 1300NC, Philipps Electronics, Amsterdam, Nordholland, Netherland). A fly activated during the negative control was not exposed to a test stimulus but had the possibility to fly for 1 min or 2 min in experiments “Breath versus CO_2”, “Responses to synthetics” and “The pulse of breath” to permit quantification of behavioral responses of flies not stimulated by odours (spontaneously activated flies).

3.2.3.Behavioural responses analysis

Behavioural analysis

Data analysis were made with R (R Development Cor Team, 2012). The level of significance was set at 0.05. The behavioral criteria used were: “activation”, the fly moved in the cage; “activation time”, time between the onset of stimulation and the first movement of the fly; “exit”, the fly exited the cage; “flight up to target”, the fly flew upwind for 1m at an altitude lower than 0.7m. The proportion of flies activated, that

exited the cage and flew up to the target was calculated as the proportion of responding flies of the total flies tested. Proportions for each behavioral criterion were analyzed separately using the Tuckey post hoc multicomparison of treatments following a generalized linear model (GLM) with a logit link function. Each step of reducing a GLM was controlled by a deviance analysis following a X^2 distribution. A non responding teneral female for the breath treatment was added to make the analysis possible. Flies activated during the negative control were not taken in account. Attention was paid to the "flight to target" criterion to determine if this variable depended on the odour treatment. In this case the proportion was calculated as the number of flies that reached the target of the number of flies that exited the cage (flies that flew out of the cage during the negative control were taken into account).

Flight track analysis

A first account of flights was made by quantifying the distance covered, time spent flying around the target, velocity. Data acquired with the tracking system were first filtered as follows. All points with a precision of 6 mm or more were discarded to avoid taking into account tracking system errors. Then only points that were inside a defined volume around the sphere were included. The cubic volume was limited as follows: X_{upper} = 2.2 m, X_{lower} = 0.3 m, Y_{upper} = 0.45m, Y_{lower} = -0.45 m, Z_{lower} = 0.05 m, Z_{upper} = 0.7 m. Parts of tracks where flies hit the ceiling or the walls of the wind tunnel were discarded with this filtering method. Only consecutive points with a maximal difference of 0.1 s (corresponding of 1 point skipped) and resulting in a minimal velocity of 0.1 m/s (removing walking behavior on the floor of the wind tunnel) were used to calculate distance covered, time spent flying around the target, velocity. Distance was calculated as follows:

$$D = \sum ||Pn + 1 - Pn||$$

where Pn is a point with coordinates (x,y,z) and Pn+1 is the next point of the track. Time was calculated as follow:

$$T = \sum (\mathrm{Tn} + 1 - \mathrm{Tn})$$

where Tn is the time recorded for point Pn. A first approximation of the Velocity (Va) was calculated according to Crenshow (2000):

$$\mathrm{Va} = \frac{||\mathrm{Pn} + 1 - \mathrm{Pn}||}{\mathrm{Tn} + 1 - \mathrm{Tn}}$$

These 3 parameters were analysed by ANOVA when the variance was homogeneous (tested with Bartlett's test) and with GLM with a reciprocal link function (Gamma family) when variance was inhomogeneous. Each step of reducing a GLM was controlled by a deviance analysis following a X^2 distribution. When necessary, post-hoc tests were made with Tukey's Honest Significant Difference test (Tukey HSD) following ANOVA or with a Tukey matrix of contrast in the GLHT function for GLM.

An attempt to detail flight trajectories was also made for experiment "Breath versus CO_2" and "The pulse of breath". Flights were cut into two parts: first approach of the target and local search. The first approach was defined as the first points in the track to the nearest point from the sphere which preceded at least 3 points that took the fly away from the sphere. The rest of the flight within the volume mentioned above was defined as the local search. To compare the effect of treatments on either first approach and local search, the distribution of points was estimated for each treatment in 3 dimensions with a kernel smoothing method (function kde, *ks* package). Velocity, curvature and torsion of the 4-dimensional trajectories were estimated following the finite helix fit technique described by (Crenshaw et al., 2000), from the discret sampling (24 Hz) of the tracking system. The tracking system did not follow the fly through the entire volume of the wind tunnel as the fly was lost when it passed under the sphere for example. This resulted in records of incomplete flight trajectories, rendering impossible the estimation of velocity, curvature and torsion with a greater window than a 1-point window (Crenshaw et al., 2000).

To compare the effect of treatments on the first approach of the target, flights were retained if they fulfilled the following criteria: began at least 0.7m from the center of the

sphere and arrived within at least 0.35m from the center of the sphere, still contained 90% percent of the points after filtering them by a maximum time between consecutive points of 0.1s and a maximum window size of 0.5 to avoid alising effect (Crenshaw et al., 2000). Velocity, curvature and torsion were analysed as a function of the distance to the center of the sphere by generalysed additive model (GAM, *mgcv* package). As efforts to remove pseudoreplications with generalized additive mixed models (GAMM) failed, two 0.2m bins were created with the centers at 0.3 and 0.6m from the sphere. The mean of each parameter was calculated for each individual of each treatment. Factorial ANOVA was used to compare the effect of treatments and to verify the dependency of parameters with the distance to the target.

For local search in experiment “The pulse of breath”, points were filtered by the volume mentioned above. The time between 2 consecutive points had to be less than 0.1s and the maximal window size was 0.5. Parameters were averaged for each individual in each treatment in cubic bins of 0.1m*0.1m*0.1m. Factorial ANOVA was used to compare the effect of treatments and to verify the dependency of parameters with the 3D space. As the local area search for flies stimulated with the pulse + CO_2 or the pulse was smaller than that of flies stimulated with breath, only bins that contained points of at least 3 individuals and that were shared by the 3 treatments, were considered. This led to a reduction of the volume analysed. Only the altitude from 0.2m to 0.5, the distance from 1.1 to 1.6m along the x-axis and the distance from -0.2 to 0.3 along the y-axis could be examined. The interactions between the 3 dimensions were not tested as different numbers of bins were different according to the altitude or along the x-axis.

3.2.4. Tsetse landing behaviour

During the experiments described here, landings on the blue sphere were observed. The setup used here was not designed to quantify landings with precision as these landings occurred most of the time on the lower part of the sphere, a zone hidden from the cameras. Despite this, landings could be roughly quantified by considering as a landing each time the fly stayed more than 2s in the zone beneath the sphere. In this manner, 75% of tsetse tested in experiment “The pulse of breath” was estimated to have landed at least once on the sphere or on the column supporting it. This represents

an opportunity to study factors that induce alighting behaviour in *G. pallidipes*, taking into account such factors as the size and the shape of the sphere.

Wind tunnel experiments were made where 2 day-old teneral *G. pallidipes* were presented with different objects accompanied by human breath to attract them. Using the experimental set-up described in experiment “The pulse of breath”, teneral *G. pallidipes* were exposed to both olfactory and visual stimuli. The olfactory stimulus was the 1min breath treatment described in experiment “The pulse of breath”. Tsetse were visually stimulated with 7 different objects used one at the time and placed in the wind tunnel as in experiment “The pulse of breath”. The objects were made of 2 separate parts: a black column holding a blue object standing on a 5mm thick Plexiglas® plate 300mm*300mm in dimensions. Two supporting columns 370mm in length were used: one 30mm in diameter was made of Plexiglas® painted black (Dupli-color, Migro, Marin, Neuchâtel, Switzerland), the second 105mm in diameter made of cardboard painted in black as mentioned above. Objects painted in gentiane blue (Dupli-color, Migro, Marin, Neuchâtel, Switzerland) placed at the top of the columns were 120mm and 250mm diameter polystyrene spheres, a complex object composed of 14 polystyrene spheres 60mm in diameter linked to central sphere of 60mm diameter by 16mm diameter columns 35mm in length, horizontal and vertical oriented 250mm diameter discs made of 4mm thick cardboard. The landing behavior was observed with a camera (432*320 pixels at 240 frames/s, Casio Exilim EX-ZR100, Shibuya, Tokyo, Japan) placed on the floor of the wind tunnel 60cm downwind from the object and tilted to hold the object in the center of the field of view. Only flies that entered the camera’s field of view during the 1min test were taken into account. Proportions of flies that landed at least once on the different objects were analyzed with a generalized linear model (GLM) with a logit link function. Sex was added to the model as an explanatory variable and each step of reducing the GLM was controlled by a deviance analysis following a X^2 distribution. The level of significance was set at 0.05. One female and male was added to fly numbers landing on the column tested alone to make the analysis possible.

3.2.5.Analysis of the breath content

Extraction of VOCs present in human breath were achieved by passing 120L of breath contained in Tedlar® bags at 2L per min through two glass cartridges (76.2 mm long, 6.35 mm o.d. borosilicate volatile collection trap, Analytical Research System, Gainesville, Florida, USA) carrying 25mg Porapak Super Q (Altech Assoc., Deerfield, Illinois, USA) 80-100 mesh size. The Porapak phase was conditioned in N_2 at 200°C for 90min before extractions. VOCs were desorbed in approximately 20µl of dichloromethane (DCM, 99.8% purity, Merck Serono, Geneva, Switzerland) and the extracts were maintained at -20°C in closed vials. The breath of two individual was extracted, one male and one female of about 30 years old. To ensure the endogenus production of identified compouds, extractions of the experimental room air and of the Tedlar bags filled with charcoal-purified air were made according to the protocol described above. VOCs present in human breath were analysed using electroantennogram (EAG) responses of tsetse antenna in tandem with gas chromatography (GC-EAG).

The electroantennogram introduced by (Schneider, 1957) allows the measurement of the electrophysiological response of insect antennal receptor cells to stimulation with an odour. Electroantennograms from *Glossina* spp. antennae were recorded as described in (Guerin and Visser, 1980) and in (Syed and Guerin, 2004). The tsetse fly head was placed between the tips of two drawn out glass capillary electrodes (2mm o.d.) filled with a 0.1M KCl solution, with the tips of the electrodes placed at the base and the tip of the antenna with micro manipulators. The antenna was held in a humidified charcoal-filtered air stream (90-100% RH, 23±2°C) delivered at $1ms^{-1}$ in a glass water-jacketed tube (7mm i.d.) whose outlet was about 1cm from the preparation (Steullet and Guerin, 1994). The antennal preparation was connected to a computer via a high impedance preamplifier (X10, Syntech, NL), a DC amplifier (UN-03, Syntech) and to an analog-digital converter (USB-IDAC box, Syntech). The antenna so mounted can be used as a biological detector (Arn *et al.*, 1975). Electroantennographic responses were recorded in parallel with the flame ionisation detector of the GC (5300, Carlo Erba Instruments, Milan, Italy). Extracts or solutions of standards were injected directly onto an apolar

capillary column (ZB 5, L 30 m, ID 0.32, df 0.25; BGB Analytik, Böckten, Switzerland) using on-column injection to separate VOCs present in breath extracts. Carrier gas was H_2 and set at 50 cm sec-1.The identification of volatiles was achieved by three processes: gas chromatography linked mass spectrometry (GC-MS) in the Analytical Service of the University of Neuchâtel, retention time comparisons of active peaks with standards and EAG activity comparisons.

3.3. Results

3.3.1. Proportion of flies responding and flight duration

3.3.1.1. Breath versus CO_2

In this experiment fed and starved and teneral flies were exposed to human breath and to a concentration of CO_2 similar to the breath treatment. Human breath decreased significantly by 6.8 times the time to activation compared to CO_2 (Cox regression model, $p<0.001$, Figure 3.5). This difference was independent of the feeding status or the sex of the flies tested. However fed and starved flies activated 1.3 times faster than teneral flies (Cox regression model, $p=0.049$) and females activated 1.3 times faster than males (Cox regression model, $p=0.037$). The proportion of flies that activated during the negative control was 14% and was not correlated with the time to activation as Figure 3.6 shows a quasi straight line. In other words, the probability of a fly being activated was equal between the moment of the opening of the cage and the onset of stimulation. This means that there was no bias during this control. In term of proportions, significantly more flies were activated, exited the cage and arrived at the target when exposed to breath as compared to CO_2 alone (Figure 3.7A). No significant difference was found between fed and starved and teneral flies or between males and females for these 3 parameters, except that the proportion of females exposed to CO_2 that exited the cage was significantly higher than the proportion of males (36% and 24% respectively, GLM, $p=0.03$). The percentage of flies that exited the cage and flew up to the target was significantly higher for the breath treatment than for CO_2 (Figure 3.7B). This proportion was strongly reduced to 15% when flies were activated spontaneously during the negative control. Breath was also a better treatment than CO_2 in eliciting flight in fed and starved tsetse as those exposed to breath flew a distance 2.6 time greater (GLM, $p<0.001$) and 2.2 time longer in time (GLM, $p<0.001$) than flies exposed to CO_2 alone (Figure 3.8). The distance and time parameters for teneral flies exposed to breath are not significantly different from those of fed and starved flies (GLM, $p=0.16$ for the distance covered and $p=0.49$ for the time spent flying). However, teneral flies stimulated with CO_2 flew a distance 1.6 time greater and 1.5 time longer in time than fed

and starved flies (GLM, $p<0.01$, Figure 3.8). The difference between the breath and the CO_2 treatments was thus smaller for teneral flies than for fed and starved flies but was still significant (GLM, $p=0.021$ for the distance covered and 0.017 for the time spent flying). No significant difference between males and females was found. The treatments had also significant effects on the average velocity as flies exposed to breath flew 0.14m/s faster than flies exposed to CO_2 (TukeyHSD multicomparison test following ANOVA, $p<0.01$). The factor sex was not significant in fed and starved flies (difference of 0.0018m/s) but was significant in teneral flies where females flew 0.24m/s faster than males (Figure 3.9).

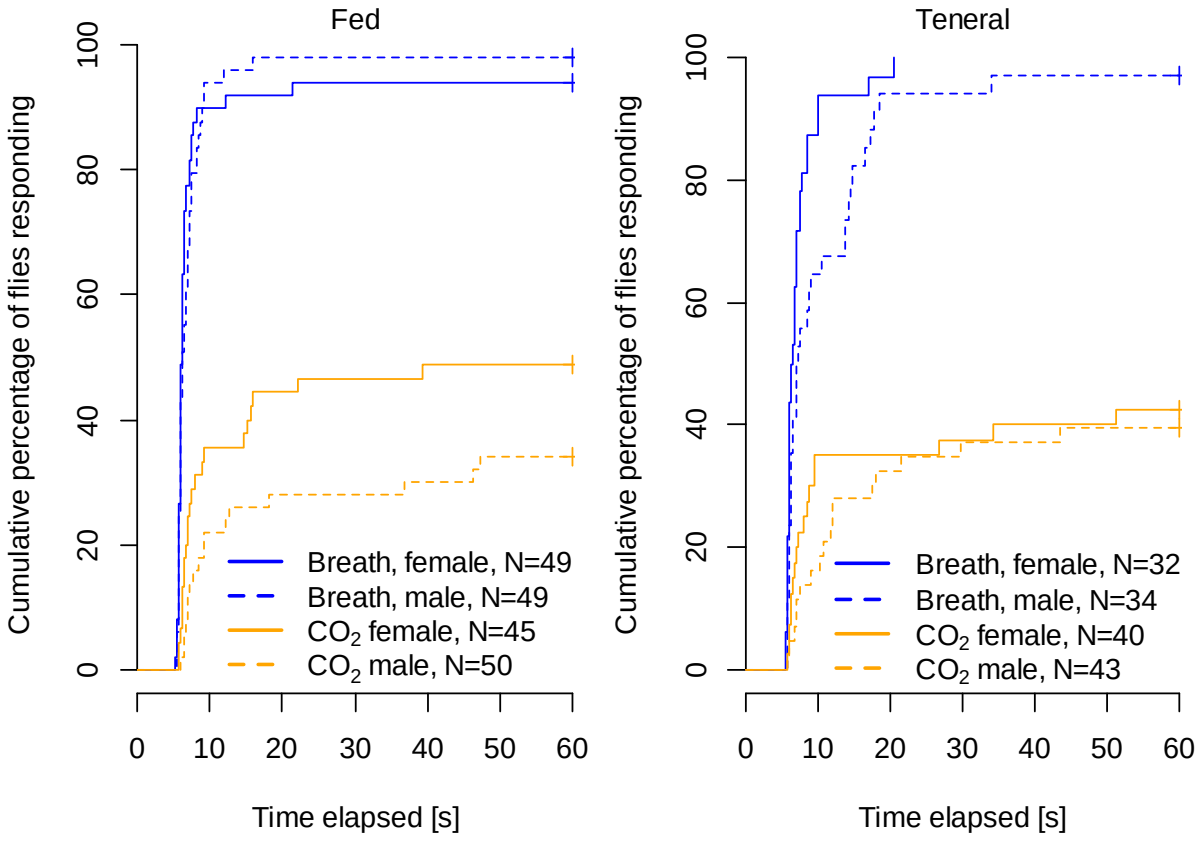

Figure 3.5: Cumulative percentage of fed and starved (indicated as Fed) and teneral (indicated as Teneral) *G. pallidipes* responding to CO_2 and to breath as a function of time elapsed until activation. Zero on the abscissa is the beginning of treatment release into the wind tunnel. The treatment carried by the air stream needs about 5s to reach the insect at the downwind end of the wind tunnel. N is the number of flies tested.

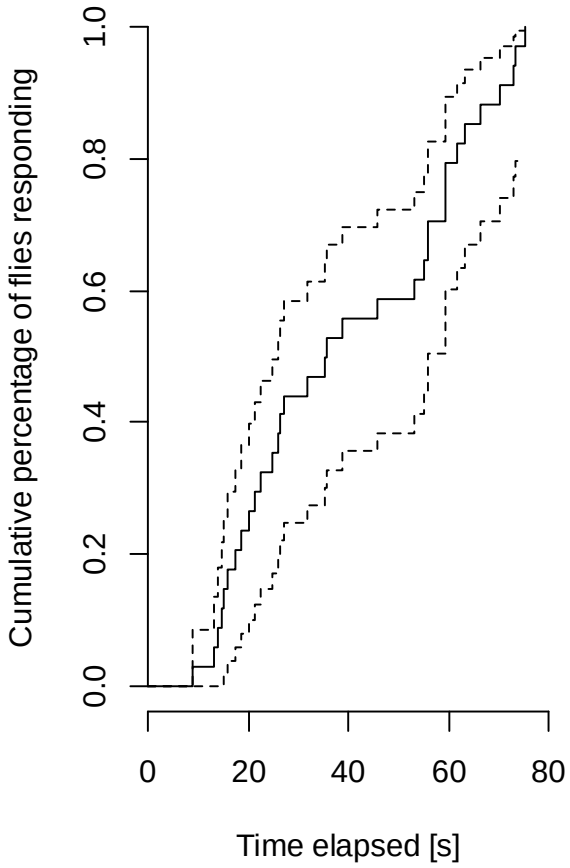

Figure 3.6: Cumulative percentage of fed and starved and teneral *G. pallidipes* activated during the 1 minute pure air control as a function of time elapsed until activation, with 95% confidence intervals (dashed lines). These responses show a certain degree of linearity indicating that flies have the same chance to be activated at any time during the negative control.

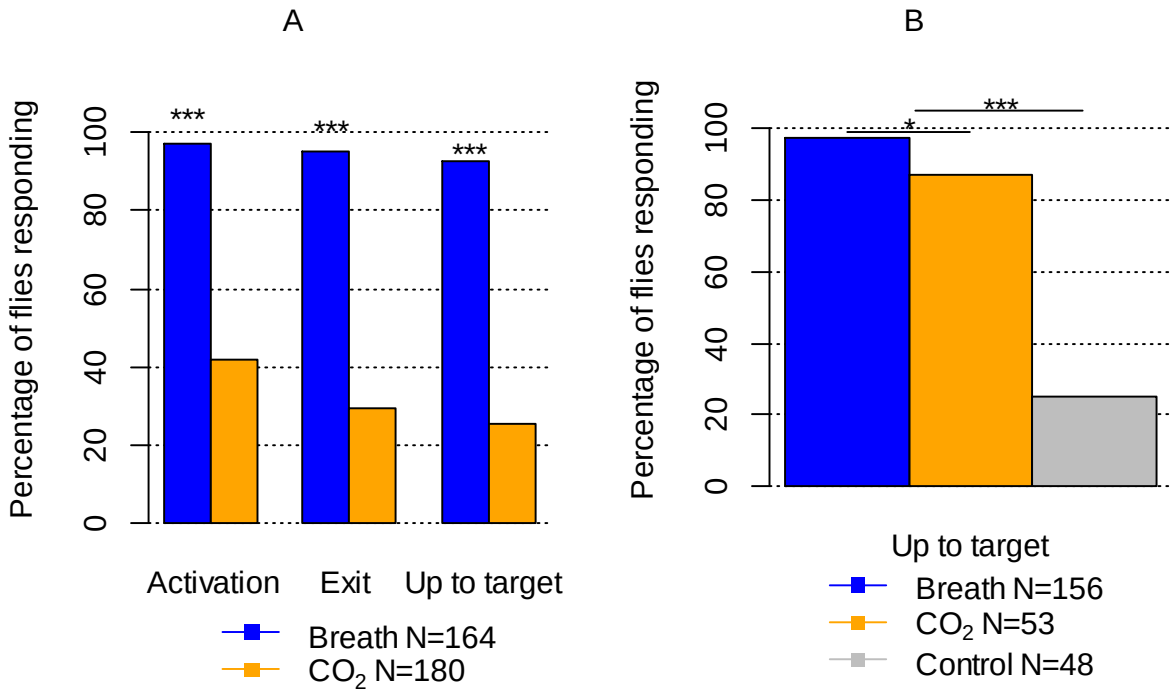

Figure 3.7: Proportion of flies that were activated (Activation), exited the cage (Exit) and flew up to the target (Up to target) in presence of CO_2 and breath. **A** The percentage response for the 3 criteria as calculated from the number of flies tested (N). Asterisks indicate that the percentage response for a behavioural criterion is significantly different from the CO_2 control at $p<0.05$(*) and $p<0.001$ (***) using GLM with a logit link function. **B** The percentage response as calculated from the number of flies that exited the cage (N). Asterisks indicate that the percentage response for a behavioural criterion is significantly different among treatments at $P<0.05$(*) and $P<0.001$ (***) using the Tuckey post hoc multicomparison of treatments following GLM with a logit link function.

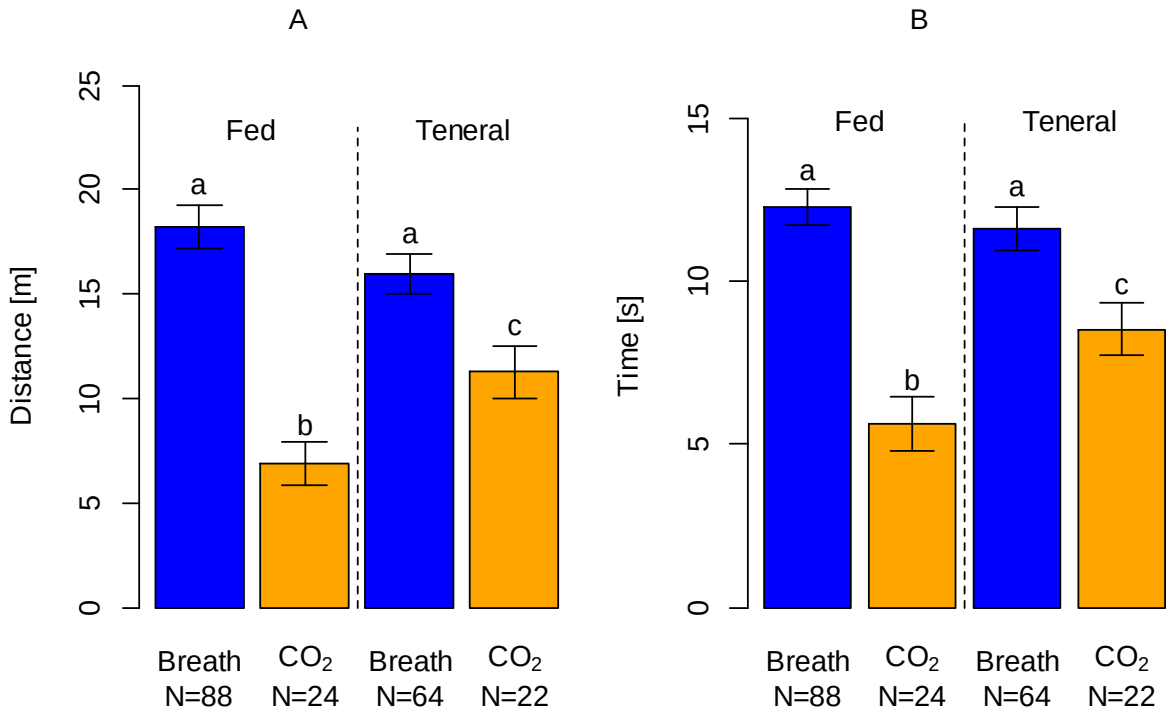

Figure 3.8: Mean ± SEM of distance covered (**A**) and time spent flying (**B**) of fed and starved (indicated as Fed) and teneral (indicated as Teneral) *G. pallidipes* exposed to CO_2 and Breath. Statistical analysis was performed using GLM with a reciprocal link function (Gamma distribution). Bars with different letters are significantly different ($p<0.05$). N is the number of flies that flew to the target.

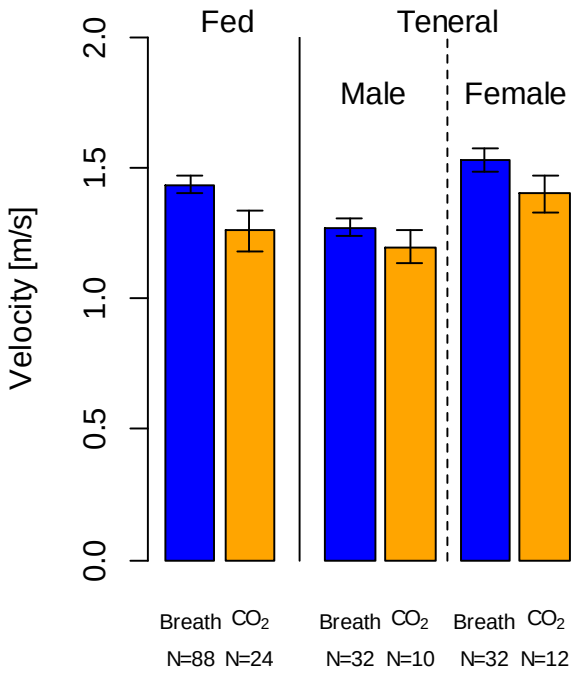

Figure 3.9: Mean ± SEM of the average velocity of fed and starved (indicated as Fed) and teneral (indicated as Teneral) *G. pallidipes*. N is the number of flies that flew to the target.

To ensure that responses of teneral flies to breath compared to CO_2 was not due to possible contaminants from the Tedlar bag itself into the breath sample, the breath treatment was compared in a further experiment to a mix of CO_2 and samples from Tedlar bags filled with charcoaled purified air (indicated as CO_2 + air). This dataset was compared with the previous dataset where the response of teneral flies to breath and to CO_2 was compared. For all parameters no significant difference was found between breath from the new dataset (indicated as *) and the previous experiment nor between CO_2 and CO_2 + air. Breath was always significantly better in activating flies (Figure 3.10), to induce exit or up to target flights (Figure 3.10B) and to increase the distance covered and time spent flying (Figure 3.11). No change in velocity was observed compared to the previous experiment with breath and CO_2, *i.e.* females flew faster than males (TukeyHSD multicomparison test, $p<0.001$, Figure 3.12).

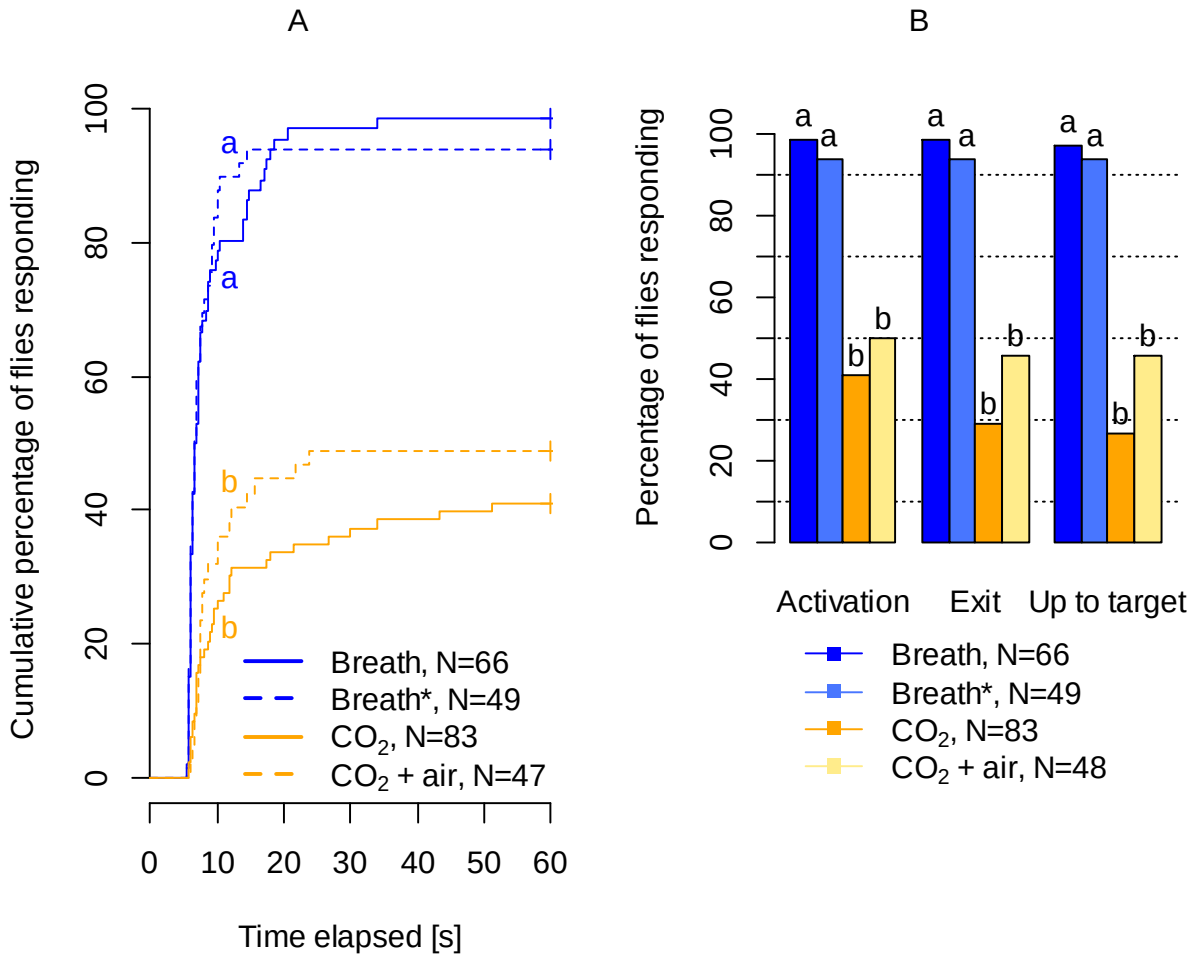

Figure 3.10: **A** Cumulative percentage of teneral *G. pallidipes* responding to breath, CO_2 and CO_2+air from a Tedlar bag, as a function of time elapsed until activation. Curves with different letters are significantly different using Tukey multicomparison test following Cox regression at $p<0.05$. **B** Proportion of flies that activated, exited the cage and flew up to target. Bars with different letters are significantly different using Tukey multicomparison test following GLM with logit function at $p<0.05$. A second breath control (*) was compared with data obtained previously. N is the number of flies tested.

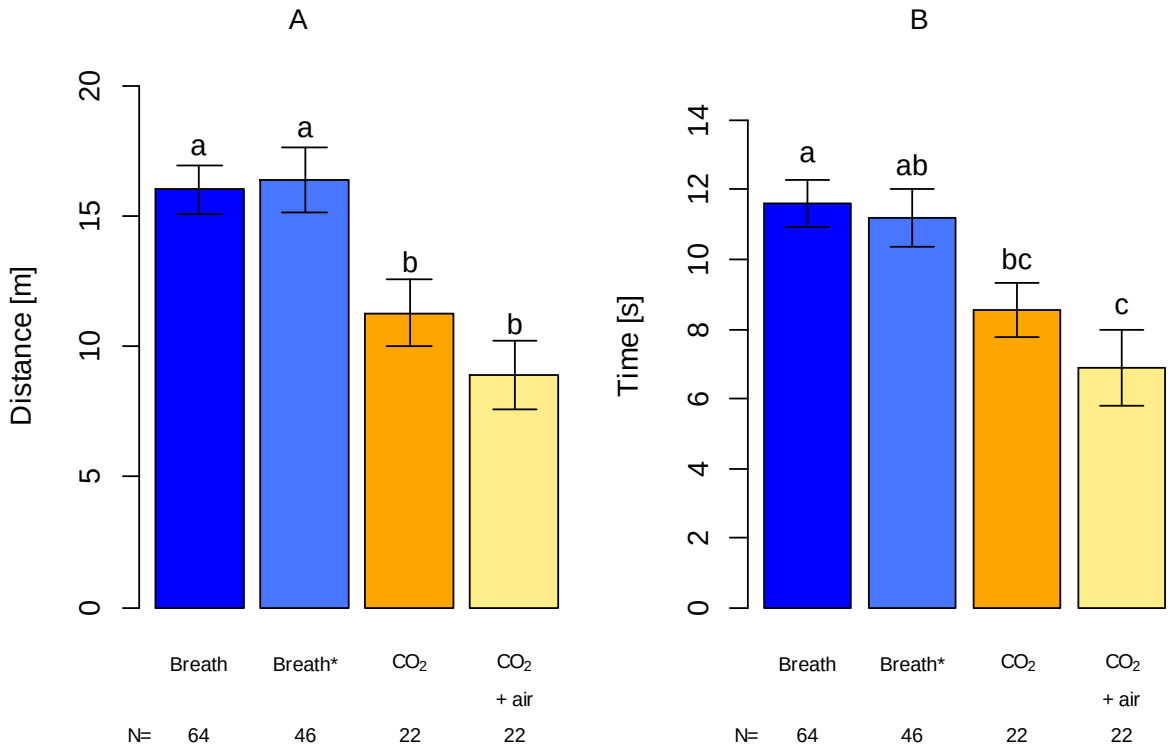

Figure 3.11: Mean ± SEM of the distance covered (**A**) and the time spent flying (**B**) by teneral G. pallidipes responding to breath, CO_2 and CO_2+air from a Tedlar bag. Statistical analysis was performed using GLM with a reciprocal link function (Gamma distribution). Bars with different letters are significantly different at $p<0.05$. A second breath control (*) was compared with data obtained previously. N is the number of flies that flew to the target.

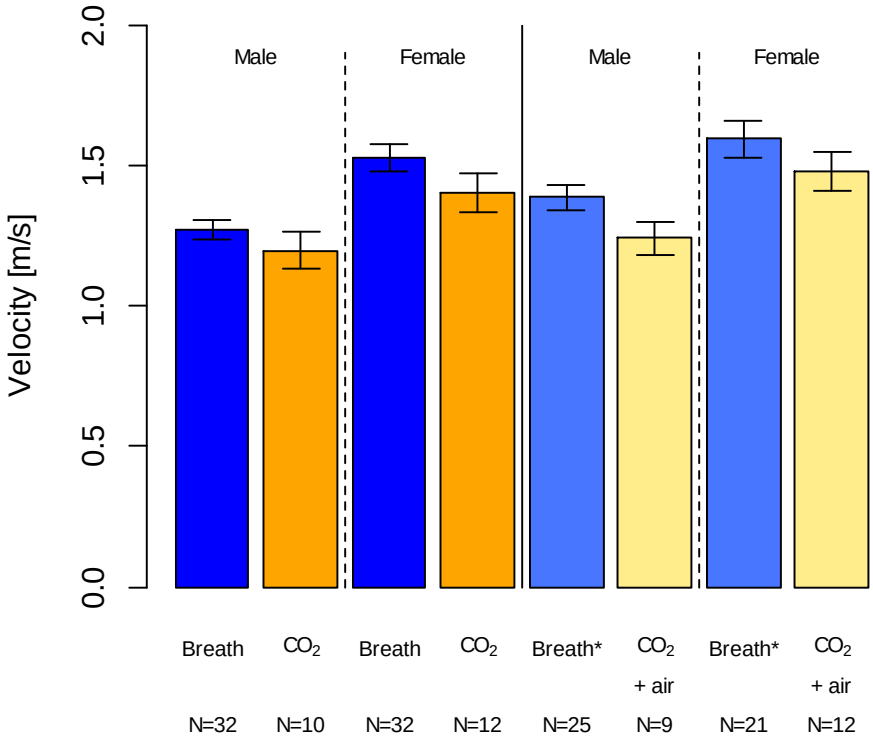

Figure 3.12: Mean ± SEM of the average velocity of teneral G. pallidipes responding to breath, CO_2 and CO_2+air from Tedlar bags. Data at left of the solid vertical bar are from a first experiment and at right from a subsequent experiment. Second breath control (*) is compared with data obtained previously. N is the number of flies that flew to the target.

3.3.1.2. The pulse of breath

In experiment "Breath versus CO_2", CO_2 did not activate half of the flies tested. This resulted in a strongly unbalanced dataset and added a qualification to the interpretation that breath increased distance covered and time spent flying by *G. pallidipes* compared to CO_2. CO_2 for example might activate only flies that had the lowest energy reserves, resulting in shorter flights. The length of flights would thus be in this experiment dependant on the physiological status of flies instead of the odour treatment. I endeavoured to overcome this problem by making a new set of experiments where fed and starved flies were stimulated by a continuous flow of breath (indicated as breath) or by a 2s pulse of breath followed immediately by CO_2 (indicated as pulse + CO_2) or by pure air (indicated as pulse). Moreover, this experiment provided important information on how tsetse flies manage the loss of an odour plume as they approach a target. The percentage of flies activated, exited the cage and flew up to the target was near 100% for the breath treatment and lower for the pulse treatments but no significant difference

was found with the Tukey multicomparison test following GLM with logit link function (Figure 3.13A). The 2s pulse of breath was sufficient to activate 85% of the flies tested and to allow 81% of flies to fly up to the target. Adding CO_2 immediately after delivering the pulse of breath did not change these proportions (Figure 3.13A). The 3 treatments had no significant effect on the proportion of flies that exited the cage and flew up to the target (Figure 3.13B). Flies that were activated during the negative control (indicated as control) represented 22% of the total, comparable to the 14% recorded in experiment "Breath versus CO_2" taking into account that the length of experiment "The pulse of breath" was twice the length of experiment "Breath versus CO_2". Moreover, as in experiment "Breath versus CO_2", only 23% of flies that were activated and exited the cage during the negative control flew up to the target (Figure 3.13B). This proportion is significantly different from those obtained with breath, the pulse or the pulse + CO_2 treatments (Figure 3.13B).

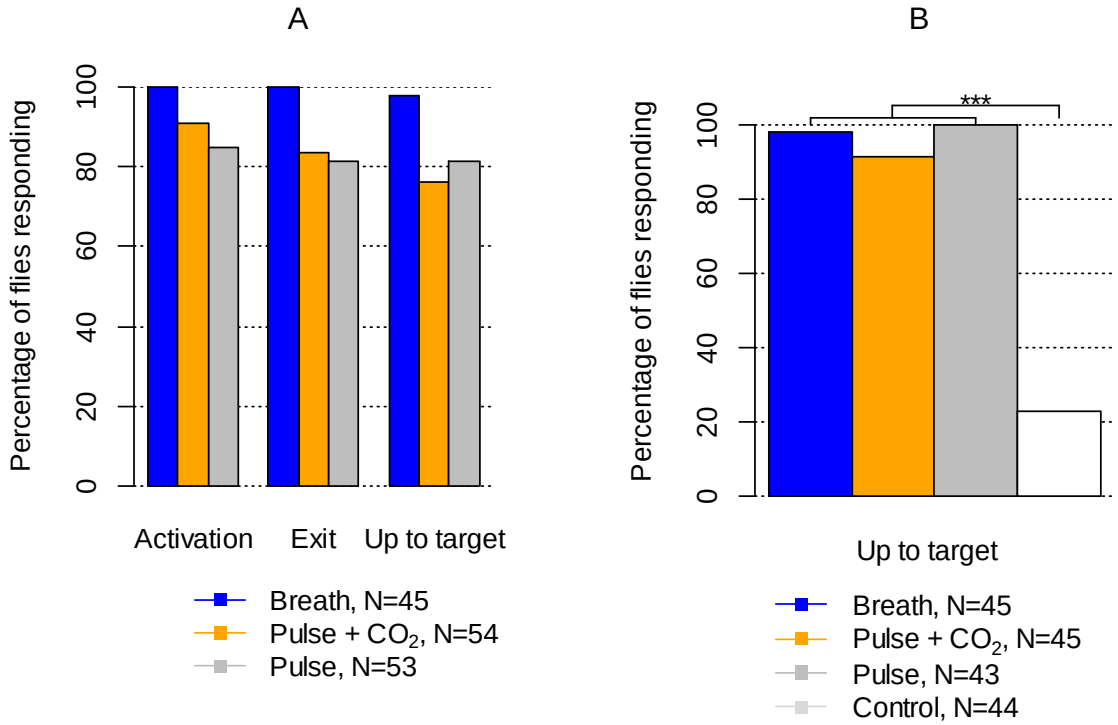

Figure 3.13: Proportion of flies that activated (Activation), exited the cage (Exit) and flew up to target (Up to target) in presence of a continuous stimulation with breath (breath), a 2s pulse of breath (pulse) and a 2s pulse of breath immediately followed by CO_2 (pulse+CO_2). **A** The percentage response for the 3 criteria as calculated from the number of flies tested (N). **B** The percentage response as calculated from the number of flies that exited the cage (N). Asterisks indicate that the percentage response for a behavioural criterion was significantly different among treatments at $p<0.001$ (***) using the Tuckey multicomparison test following on GLM with a logit link function.

Figure 3.14 summarises the distance flown and the time that flies spent flying when exposed to the 3 treatments. Flies exposed to breath flew 2.7 times further ($p<0.001$) and 2.3 times longer in time ($p<0.001$) than flies exposed to pulse + CO_2 (Examples of flight tracks are shown in Appendix B.2). The immediate addition of CO_2 following the pulse of breath increased the flight distance by an insignificant factor of 1.3 ($p=0.16$), and the time spent flying by flies exposed to the pulse by a significant factor of 1.4 ($p=0.037$). Flies stimulated with the pulse flew 1.7 times further and 1.8 times longer in time than flies that flew during the negative control. These differences were not significant, but only 9 flights were obtained during the negative control, not adequate for a valid analysis. The discrepancy in distance and time for the pulse+CO_2 treatment is explained by the fact that the average velocity for the pulse+CO_2 treatment was lower than that for pulse treatment (Figure 3.15). Although differences in average velocities between treatments were not significant (ANOVA, $F_{3,133}$, $p=0.14$), flies stimulated with the pulse+CO_2 flew at a lower speed than flies exposed to breath, as in experiment "Breath versus CO_2".

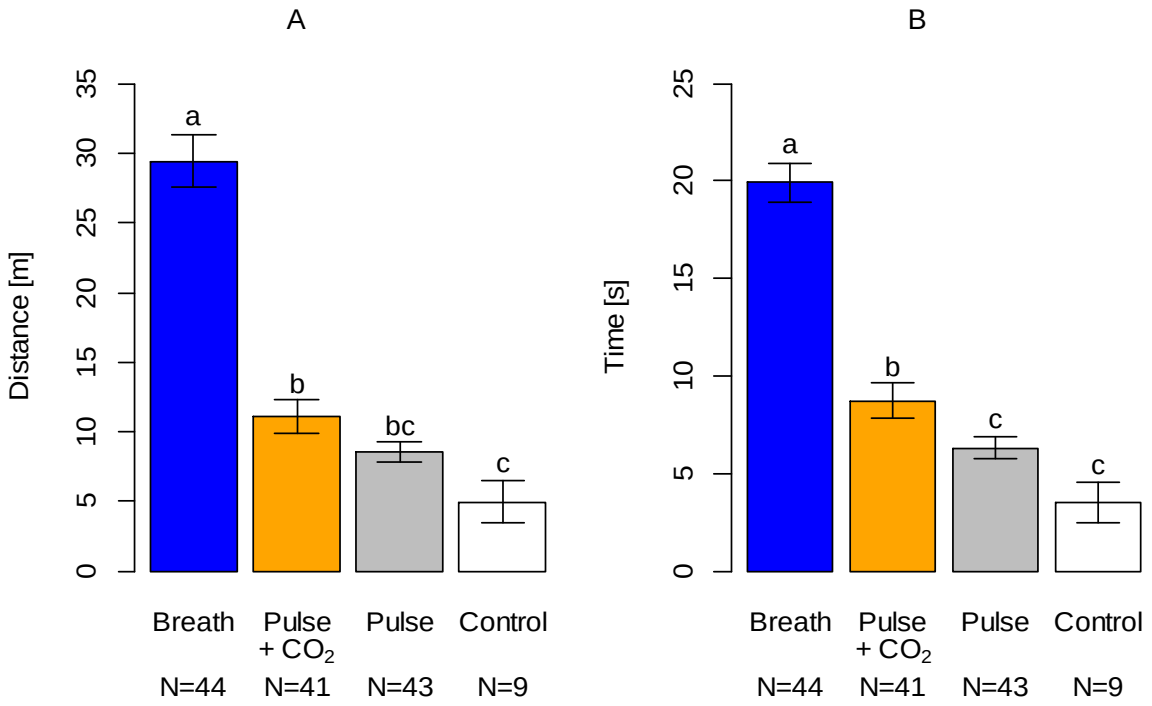

Figure 3.14: Mean ± SEM of the distance covered (**A**) and the time spent flying (**B**) by fed& starved *G. pallidipes* responding to of a continuous stimulation of breath (breath), a 2s pulse of breath (pulse) and a 2s pulse of breath immediately followed by CO_2 delivery (pulse+CO_2). Statistical analysis was performed using the Tukey multicomparison test following GLM with a reciprocal link function (Gamma distribution). Bars with different letters are significantly different at $p<0.05$. N is the number of flies that flew to the target.

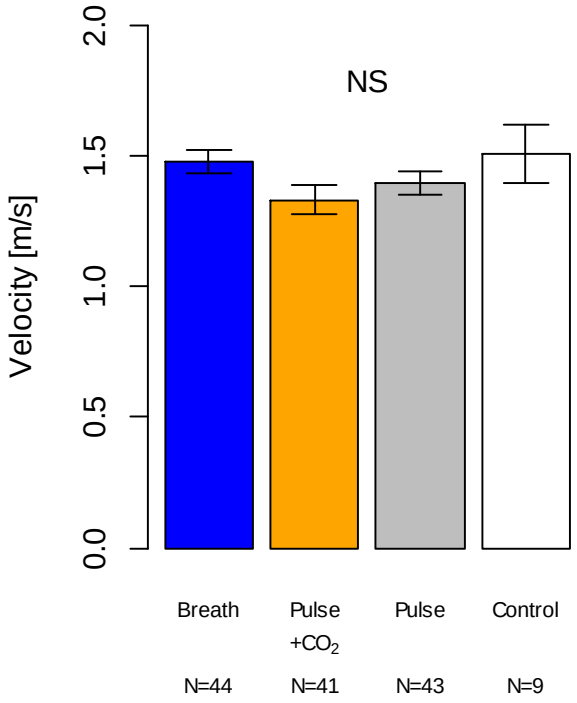

Figure 3.15: Mean ± SEM of the average velocity of fed *G. pallidipes*. Breath is a continuous stimulation of breath, pulse is a 2s pulse of breath and pulse + CO_2 is a 2s pulse of breath immediately followed by CO_2 delivery. N is the number of flies that flew to the target.

3.3.1.3. Responses to test products

Acetone heptane and isoprene

As demonstrated in experiment "Breath versus CO_2" and confirmed in experiment "The pulse of breath", the stronger behavioural response to human breath indicated that it contained compounds acting on *G.pallidipes*. Acetone, heptane and a mixture of isoprene in acetone were added to CO_2 to test the hypothesis that the strong response of fed and starved *G.pallidipes* to human breath could be explained partially or entirely by the addition of these 3 compounds to CO_2. For this new controls with CO_2 alone were made. To check if compounds tested remained in circulation in the wind tunnel, females were exposed to CO_2 alone at different times during the experimental period. The first batch of controls with CO_2 (indicated as Be) was made at the beginning of the experimental periods, so no compounds were released into the wind tunnel for a minimum of 3 hours before the start of the control. A second control with CO_2 (indicated as Af) was made in the middle or at the end of the experimental period, such that 15 to

30min separated the beginning of the control to the last release of compounds. As can be seen in Figure 3.16 & Figure 3.17, females of the “Af” batches responded significantly more to CO_2 than those of “Be” batches. The control with CO_2 alone for males was done only over 2 days where only CO_2 was tested. During the first part of experiments an exceptional response was observed for males (indicated as Su), so contamination was suspected. The odor release system and the statif for the target was cleaned with ethanol and new control experiment were made (indicated as No). Males of the “Su” batch responded significantly more to CO_2 than those of the “No” batch (Figure 3.16 & Figure 3.17).

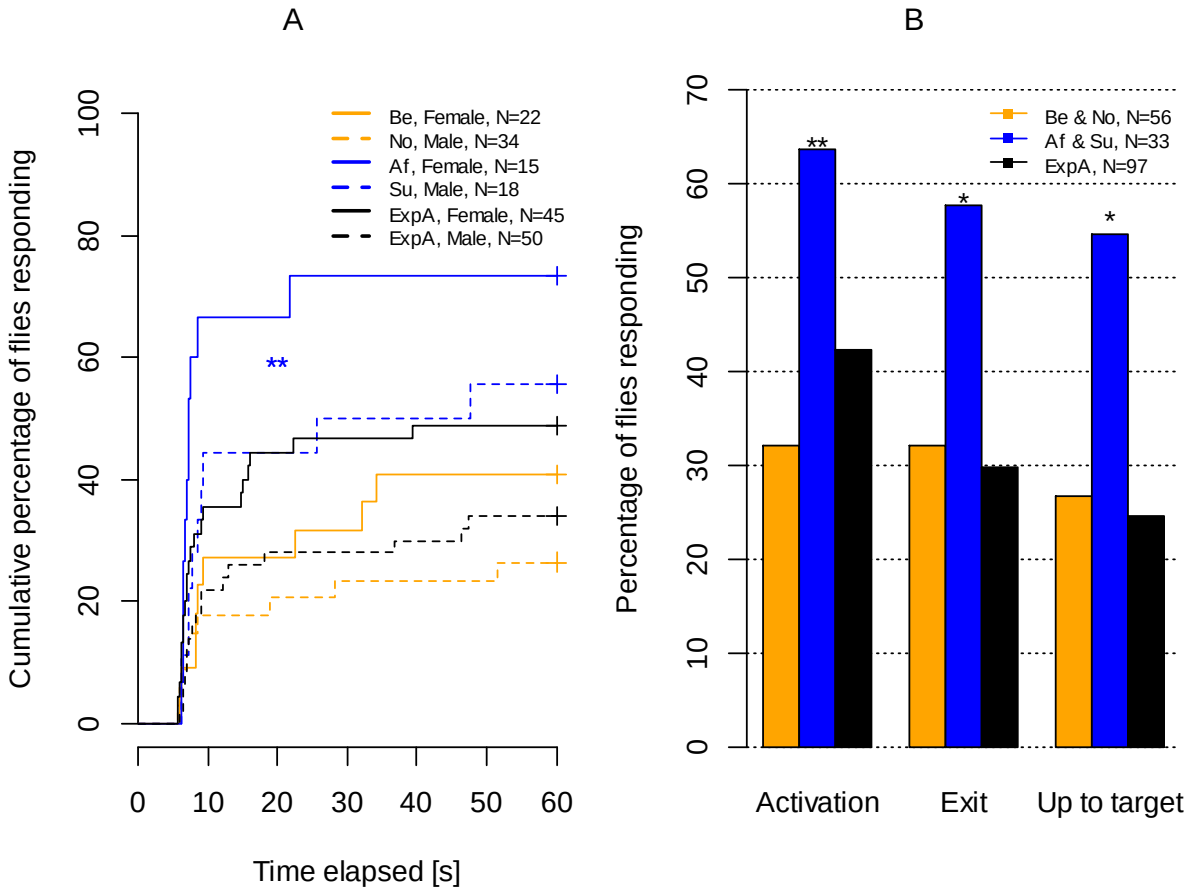

Figure 3.16: **A** Cumulative percentage of fed *G. pallidipes* responding to CO_2 as a function of time elapsed until activation. Curves with astericks are significantly different from the Be and No batches following Cox regression at $p<0.01$. **B** Proportion of flies that activated, exited the cage and flew up to target. Bars with astericks are significantly different from Be and No batches at $p<0.05$(*) and $p<0.01$(**) following GLM with logit function. N is the number of flies tested. Be, females stimulated with CO_2 at the beginning of the experimental periods. Af females stimulated with CO_2 in the middle or at the end of experimental periods. Su and No correspond to males stimulated with CO_2 before and after cleaning the target and the release system.

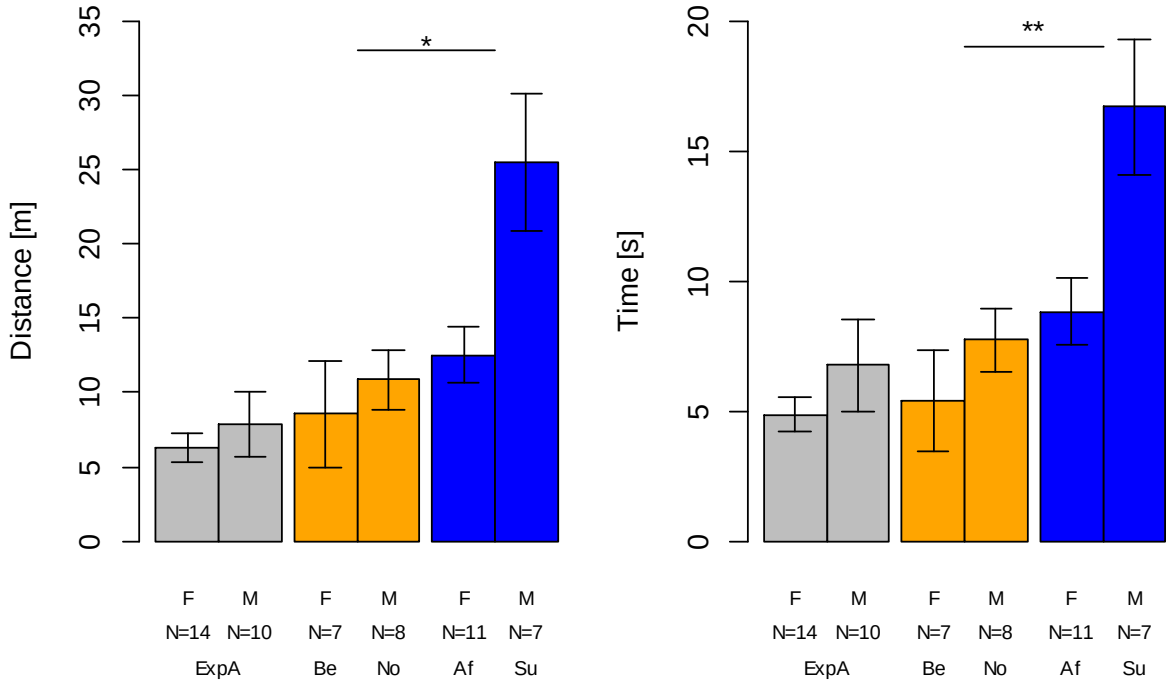

Figure 3.17: Mean ± SEM of the distance covered and the time spent flying of fed and starved *G. pallidipes* responding to CO_2. Statistical analysis was performed using GLM with a reciprocal link function (Gamma distribution). Bars with asterisks are significantly different from the Be and No batches at $p<0.05$(*) and $p<0.01$(**). N is the number of flies that flew to the target. M = males and F = females. Be females stimulated with CO_2 at the beginning of the experimental periods. Af females stimulated with CO_2 in the middle or at the end of experimental periods. Su and No correspond to males stimulated with CO_2 before and after cleaning the target and the release system.

No significant difference was found between responses to CO_2 obtained in experiments "Breah vs. CO_2" (noted as "ExpA"), "Be" and "No". As compounds released 15-30min before a "negative" control still had effects on females and contamination occurred for males, data of "Af" and "Su" experiments were removed and data from "Be" and "No" experiments were pooled with data of experiment "Breath versus CO_2" to obtain a true estimate of parameters for CO_2. Pooling these data did not significantly change the deviance.

When acetone or heptane was added to CO_2, time to activation was inversely proportional to the dose added (Figure 3.18). Acetone decreased significantly time to activation of CO_2 by factors 1.9 and 2, respectively, at doses of 10 and 100µl/min. A 2µl/min release rate of acetone had no significant effect on time to activation. Heptane significantly decreased time to activation of CO_2 by factors of 2, 3.5 and 5.3,

respectively, at doses of 1, 10 and 100 µl/min (Figure 3.18). The proportion of flies activated, that exited the cage and flew up to the target was significantly increased by the addition of any dose of acetone or heptane to CO_2, except for 1µl/min heptane (Figure 3.19). Proportions for acetone did not show a dose-response pattern whereas they did so for heptane. No significant difference was found between males and females.

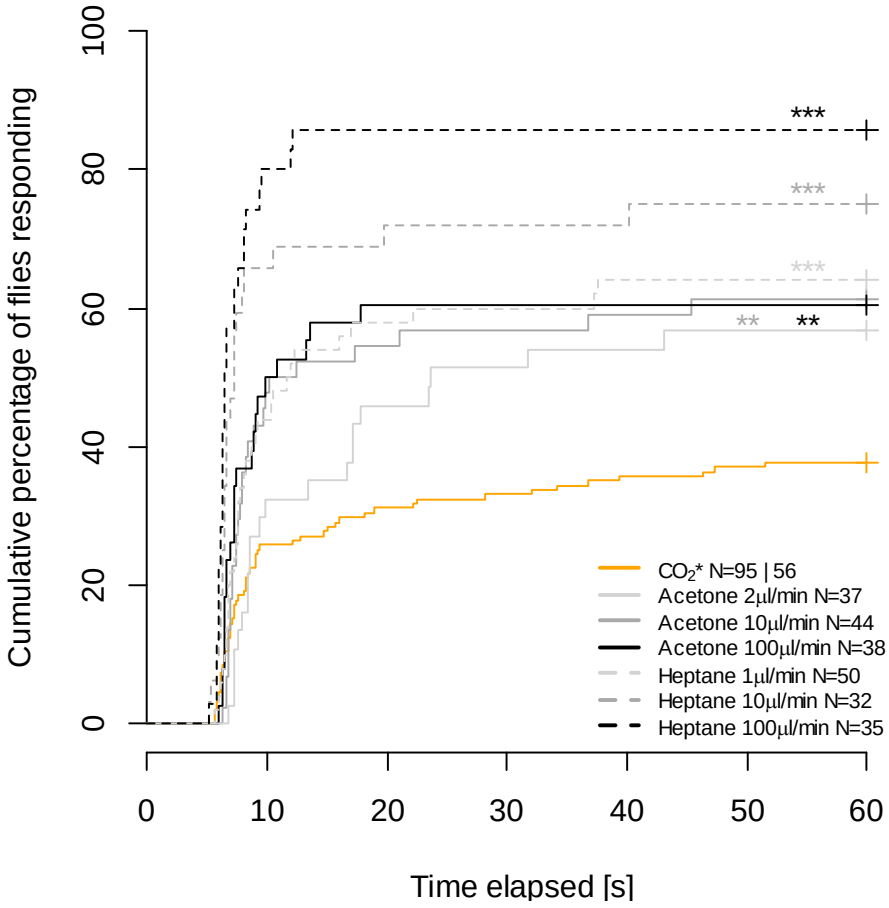

Figure 3.18: Cumulative percentage of fed and starved *G. pallidipes* responding to CO_2, acetone and heptane, as a function of time elapsed until activation. Curves with asterisks are significantly different to the CO_2 control at $p<0.05$ (*), $p<0.01$(**) and $p<0.001$(**) according to Cox regression. CO_2 from experiment "Breath versus CO_2" and the control for this experiment were pooled and noted as CO_2* (Figure 3.5 & Figure 3.16, and see text). N is the number of flies tested.

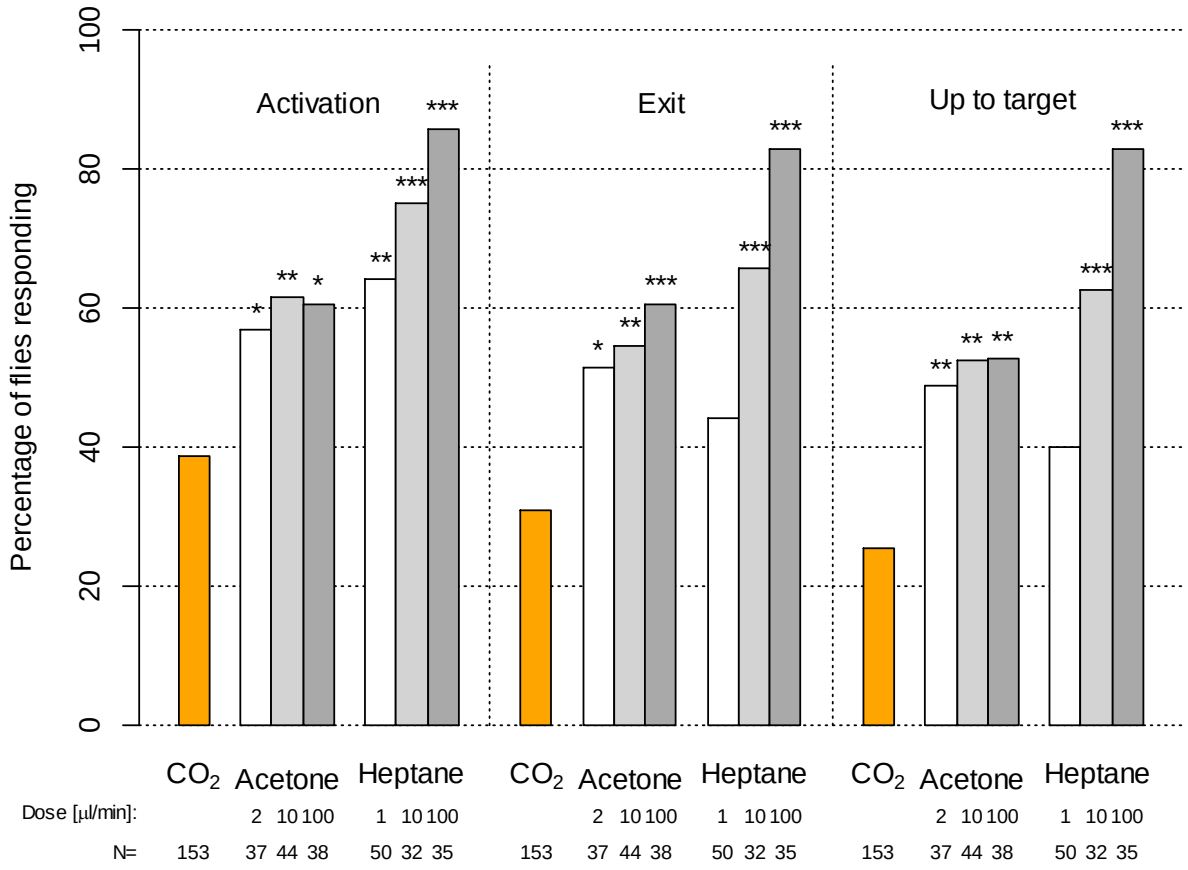

Figure 3.19: Proportion of flies that activated (Activation), exited the cage (Exit) and flew up to target (Up to target) in response to CO_2, acetone and heptane. Asterisks indicate that the percentage response for a behavioural criterion was significantly different to the CO_2 control at $p<0.05$(*), $p<0.01$(**) and $p<0.001$ (***) using GLM with a logit link function. N is the number of flies tested.

In terms of distance covered and time spent flying, males and females did not respond in the same manner (Figure 3.20). Only the small dose of acetone and heptane significantly increased the flight distance, whereas females significantly increased their flight distance when stimulated with the highest dose of acetone and by the medium and the highest dose of heptane (Figure 3.20). The same pattern was observed for the time spent flying, except that no significant effect was found for males (Figure 3.20). The highest velocity was recorded with the smallest dose of acetone (Figure 3.21). As in experiment "Breath versus CO_2", no difference in velocity was found between fed and starved males and fed and starved females.

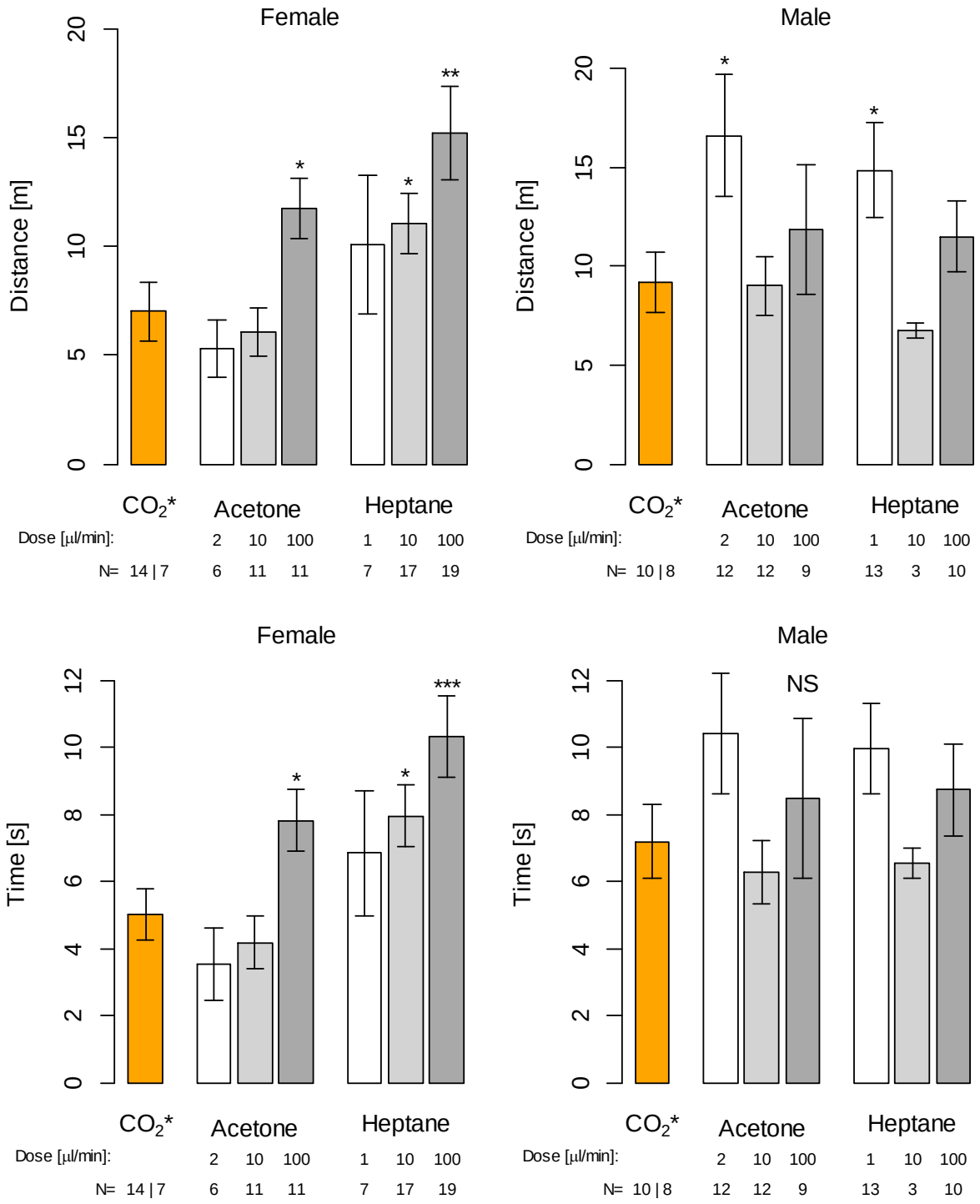

Figure 3.20: Mean ± SEM of the distance covered and the time spent flying by fed and starved *G. pallidipes* in presence of CO_2, acetone and heptane. Bars with asterisks differ significantly from the CO_2 control at $p<0.05$(*), $p<0.01$(**) and $P<0.001$ (***) using GLM with a reciprocal link function. N is the number of flies that flew to the target.

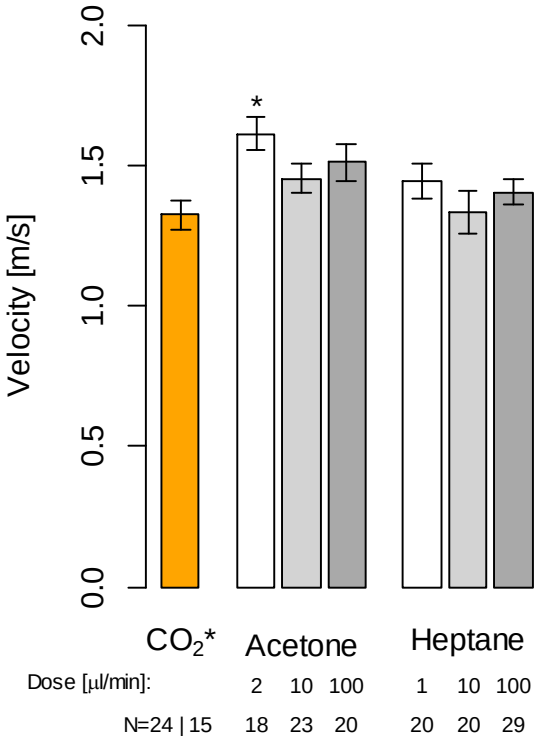

Figure 3.21: Mean ± SEM of the average velocity of fed and starved *G. pallidipes* in presence of CO_2, acetone and heptane. Bars with asterisks differ significantly from the CO_2 control at $p<0.05$ following ANOVA. N is the number of flies that flew to the target.

For both sexes, isoprene diluted in acetone significantly decreased the time to activation by a factor of 2.6 compared to acetone (Figure 3.22A). Isoprene in acetone also significantly increased proportion of flies activated, that exited the cage and flew up to target compared to acetone (Figure 3.22B). However no significant effect was found in the distance and time parameters of flights according to GLM with reciprocal function (Figure 3.23). Flies exposed to the mixture of isoprene and acetone flew 0.17m/s slower than flies exposed to acetone (ANOVA, $F_{1,55}$, $p=0.039$).

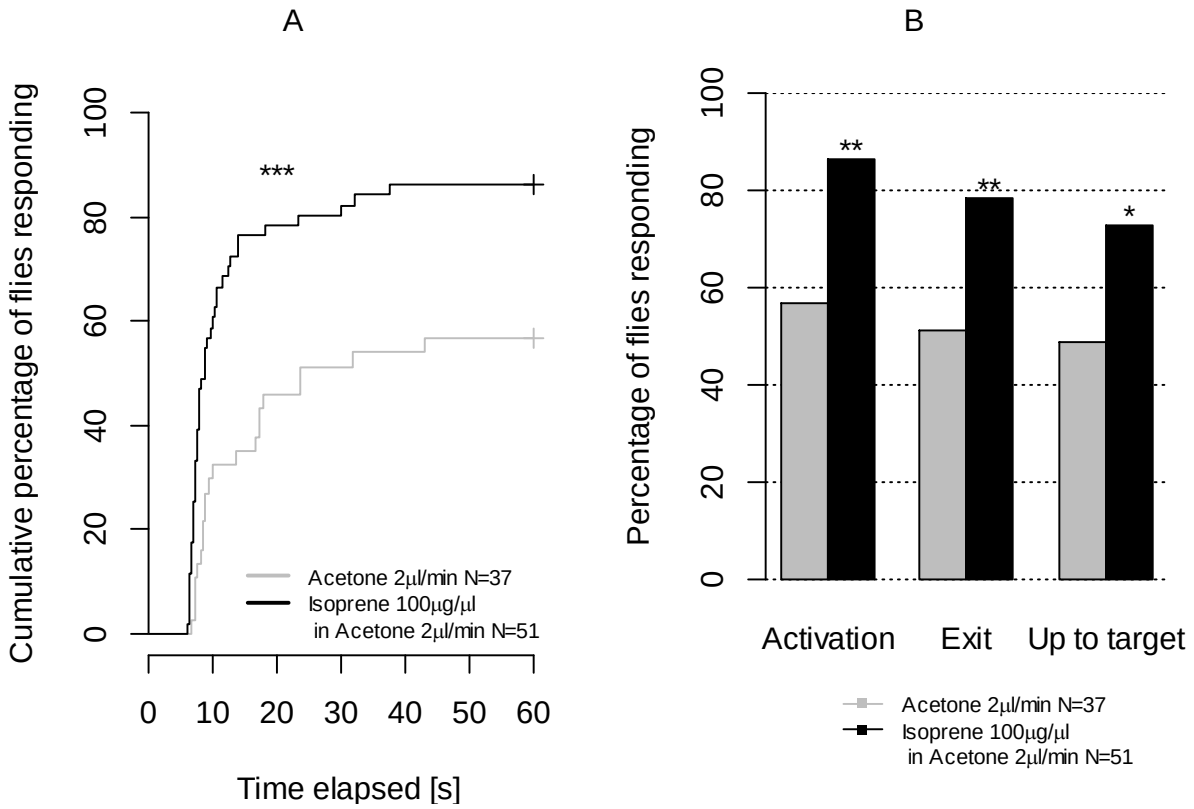

Figure 3.22: **A** Cumulative percentage of fed and starved *G. pallidipes* responding to a mixture of acetone plus CO_2 (indicated as Acetone) and to a mixture of isoprene diluted in acetone plus CO_2 (indicated as Isoprene in Acetone) as a function of time elapsed until activation. Curves with astericks are significantly different from the control following Cox regression at $p<0.001$. **B** Proportion of flies that activated, exited the cage and flew up to the target. Bars with astericks are significantly different from the control at $p<0.05$(*) and $p<0.01$(**) following GLM with logit function. N is the number of flies tested.

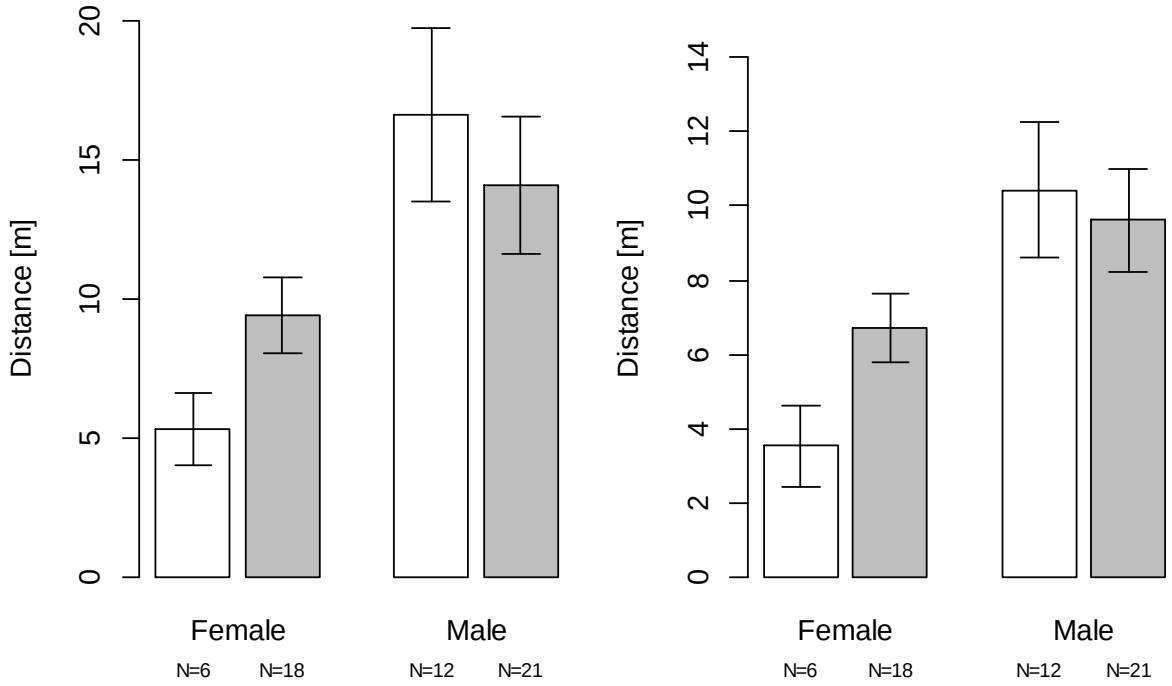

Figure 3.23: Mean ± SEM of the distance covered and the time spent flying by fed and starved *G. pallidipes* responding to a mixture of acetone plus CO_2 and to a mixture of isoprene diluted in acetone plus CO_2. Open bars and grey bars represent the 2µl/min of acetone plus CO_2 treatment, and the addition of isoprene to acetone plus CO_2 treatment, respectively. N is the number of flies that flew to the target.

Geranylacetone

For teneral flies, only the release rate 0.1 ng/min geranylacetone significantly decreased the time to activation compared to ethanol (Figure 3.24). Flies exposed to the higher doses 10 and 100 ng/min had a tendency to respond faster (Figure 3.24). A similar tendency is visible in Figure 3.25 where proportions of flies activated, that exited the cage and flew up to the target also tended to be higher. Only the 0.1ng/min release rate significantly increased these proportions except for the exit parameter where geranylacetone had no significant effect.

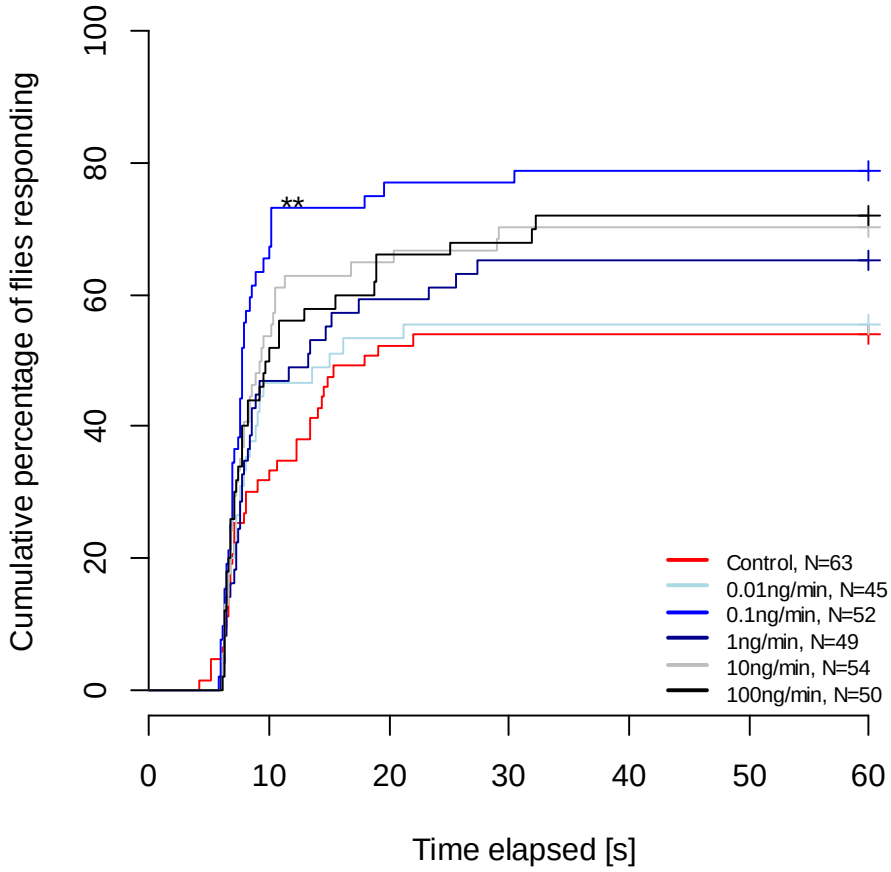

Figure 3.24: Cumulative percentage of teneral *G. pallidipes* responding to different release rate of geranylacetone as a function of time elapsed until activation. The curve with an asterisks is significantly different following Cox regression at $p<0.01$. N is the number of flies tested. Control was a mixture of CO_2 and ethanol.

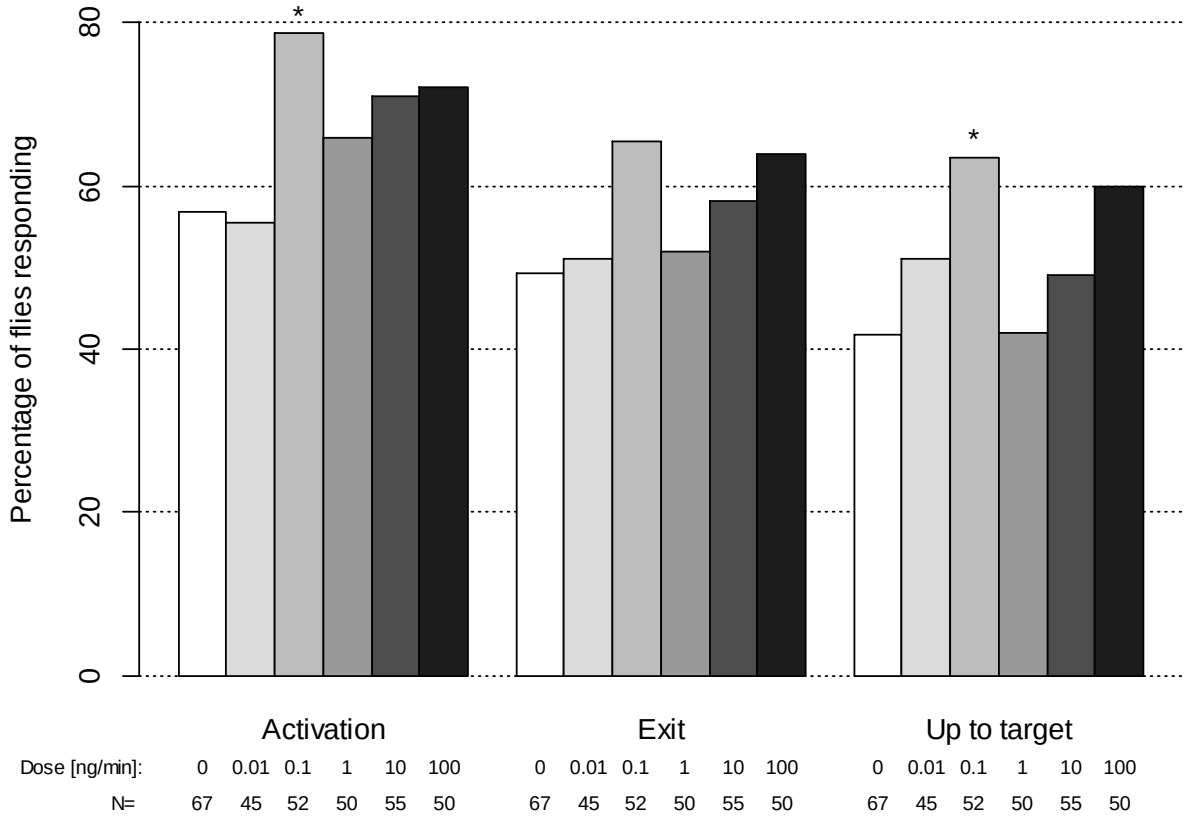

Figure 3.25: Proportion of teneral flies that activated (Activation), exited the cage (Exit) and flew up to target (Up to target) in response to different release rates of geranylacetone. Asterisks indicate that the percentage response for a behavioural criterion was significantly different to the control at $p<0.05$ using GLM with a logit link function. N is the number of flies tested.

Teneral females exposed to a release rate of 0.01 ng/min geranylacetone flew a distance significantly higher than those exposed to CO_2 and ethanol and the 0.1ng/min release rate was significant for males (Figure 3.26). The other doses did not significantly affect the flight distance. No significant difference was found in the time spent flying by females, whereas males exposed to the release rate of 0.1 ng/min geranylacetone flew significantly longer in time than those exposed to ethanol (Figure 3.26). Females flew 0.13m/s faster than males (ANOVA, $p<0.001$) and flies exposed to a release rate of 100ng/min geranylacetone flew slower than flies exposed to CO_2 and ethanol (Figure 3.27).

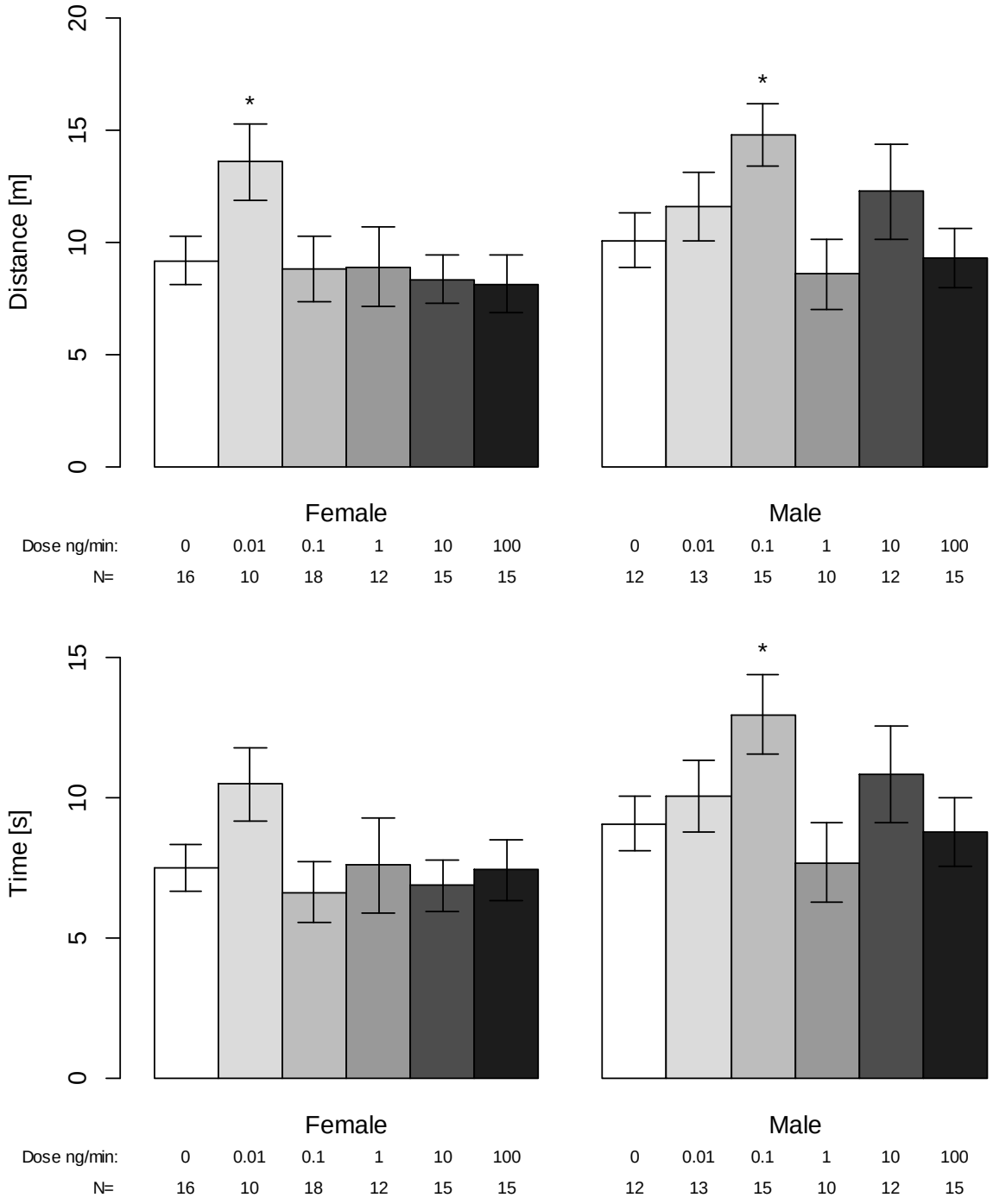

Figure 3.26: Mean ± SEM of the distance covered and the time spent flying by teneral *G. pallidipes* exposed to different doses of geranylacetone. Bars with asterisks differ significantly from the control at $p<0.05$(*) using ANOVA. N is the number of flies that flew to the target.

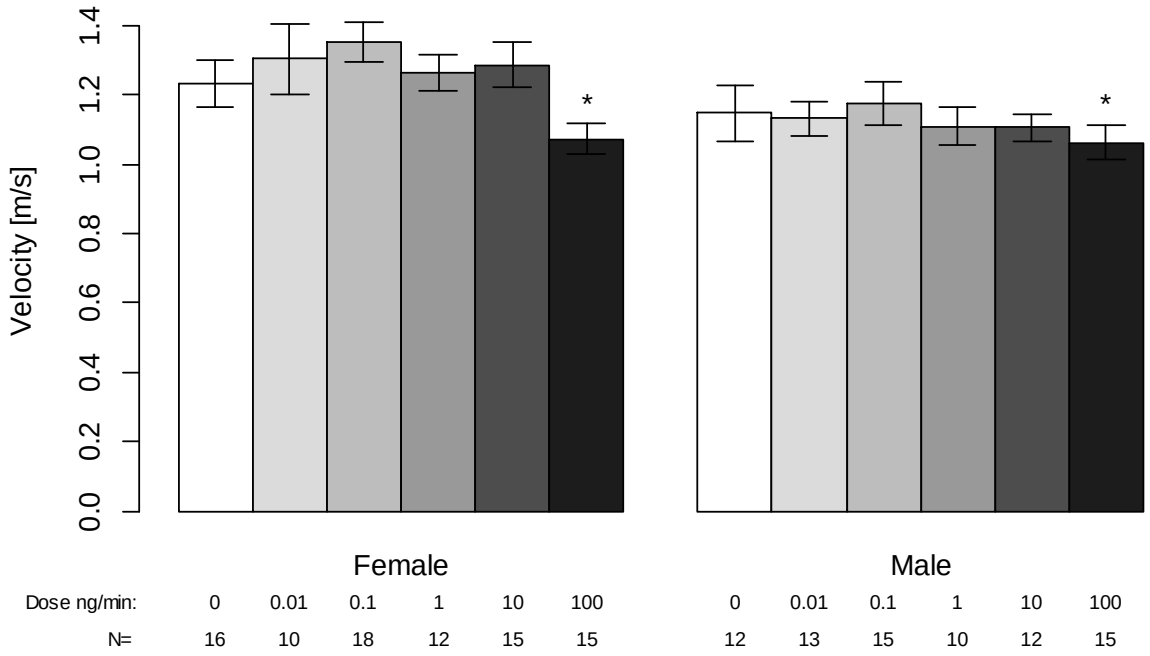

Figure 3.27: Mean ± SEM of average velocity of flies responding to different doses of geranylacetone. Bars with asterisks differ significantly from the control at $p<0.05$ following ANOVA. N is the number of flies that flew to the target.

Some tests were made with fed and starved flies to compare with teneral flies. No significant difference for all behavioural parameters was found with females exposed to 0.1, 100 and 1000ng/min of geranylacetone (Figure 3.28, Figure 3.29, Figure 3.30 & Figure 3.31). Numbers of males tested were not sufficient to make a statistical analysis. The response to ethanol of fed and starved flies was too strong to use it as solvent. An attempt to reduce the effect of the control was made by reducing the CO_2 amount at about 22ppm noted here as LD for low dose. Other solvents such as hexane and TBME were also tested at 10µl/min each. Compared to CO_2 alone, ethanol and TBME significantly decreased time to activation (Figure 3.32). Lowering the dose of CO_2 mixed with ethanol also significantly reduced the time to activation compared to a higher dose of CO_2 alone but not to the same extent as for the mix of ethanol and the high dose of CO_2 (Figure 3.32). Ethanol with the high or low doses of CO_2 significantly increased the proportion of flies activated and that exited the cage but not the proportion of flies that flew up to the target, compared to the high dose of CO_2 alone (Figure 3.33). TBME also

increased these proportions but not significantly. The addition of these solvents to CO_2 increased the number of flies that activated and exited the cage but it did not increase the number of flies that reached the target (Figure 3.33). Hexane produced similar results as for CO_2 alone. For the parameters such as distance covered, time in flight and velocity, no significant difference was found between treatments. However, ethanol with the high dose of CO_2 increased the time spent flying and the distance flown (Figure 3.34), thus decreasing the differences recorded between CO_2 alone and the best treatment, which was breath. Velocity was not significantly affected (Figure 3.35).

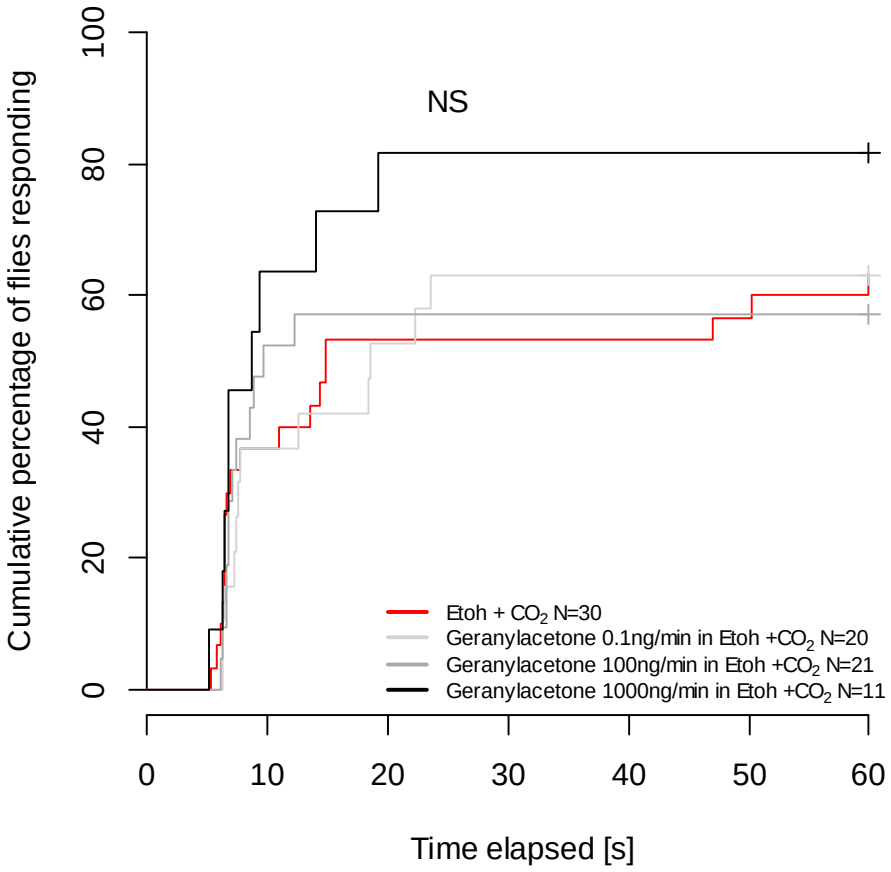

Figure 3.28: Cumulative percentage of fed and starved females responding to geranylacetone released at different doses as a function of time elapsed until activation. No significant difference was found following Cox regression at $p>0.05$ (NS). N is the number of flies tested.

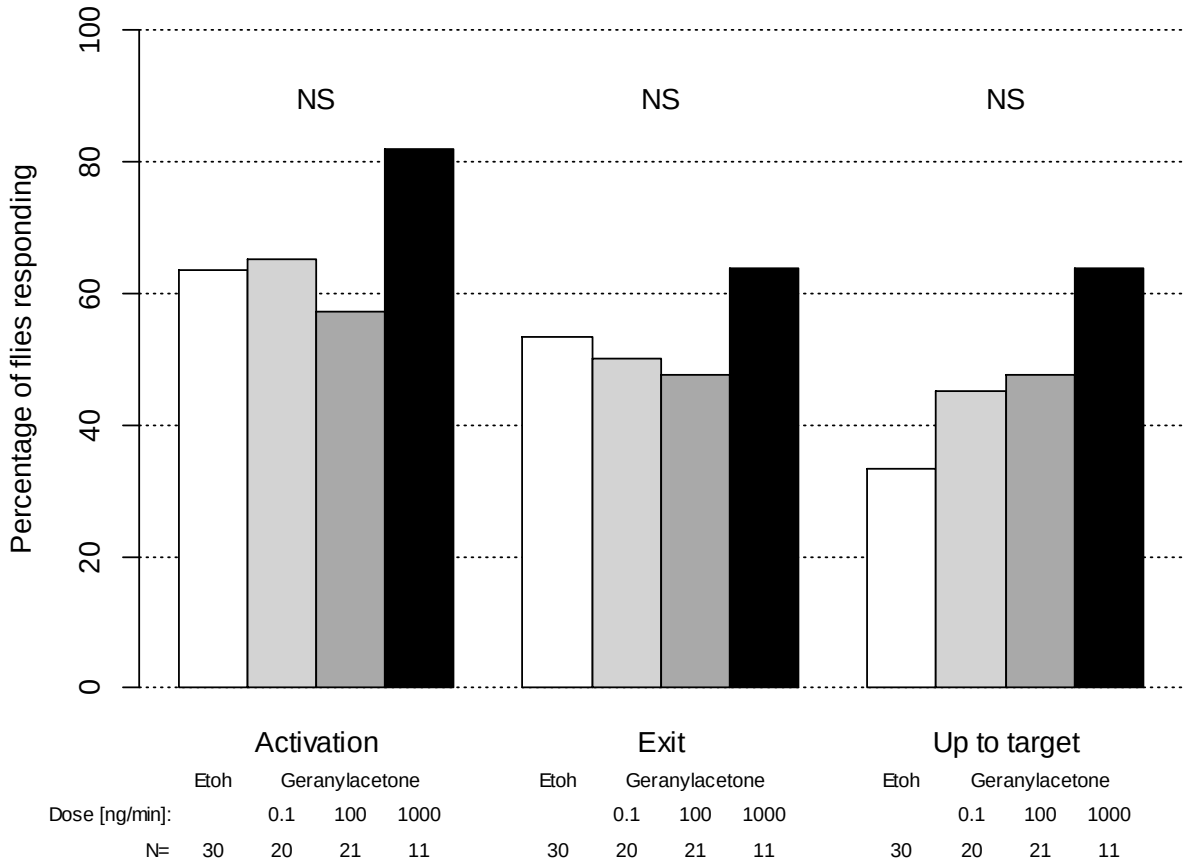

Figure 3.29: Proportion of fed and starved females that activated (Activation), exited the cage (Exit) and flew up to target (Up to target) in response to ethanol (control) and to geranylacetone at different release rates. No significant difference was found at $p<0.05$ using GLM with a logit link function. N is the number of flies tested.

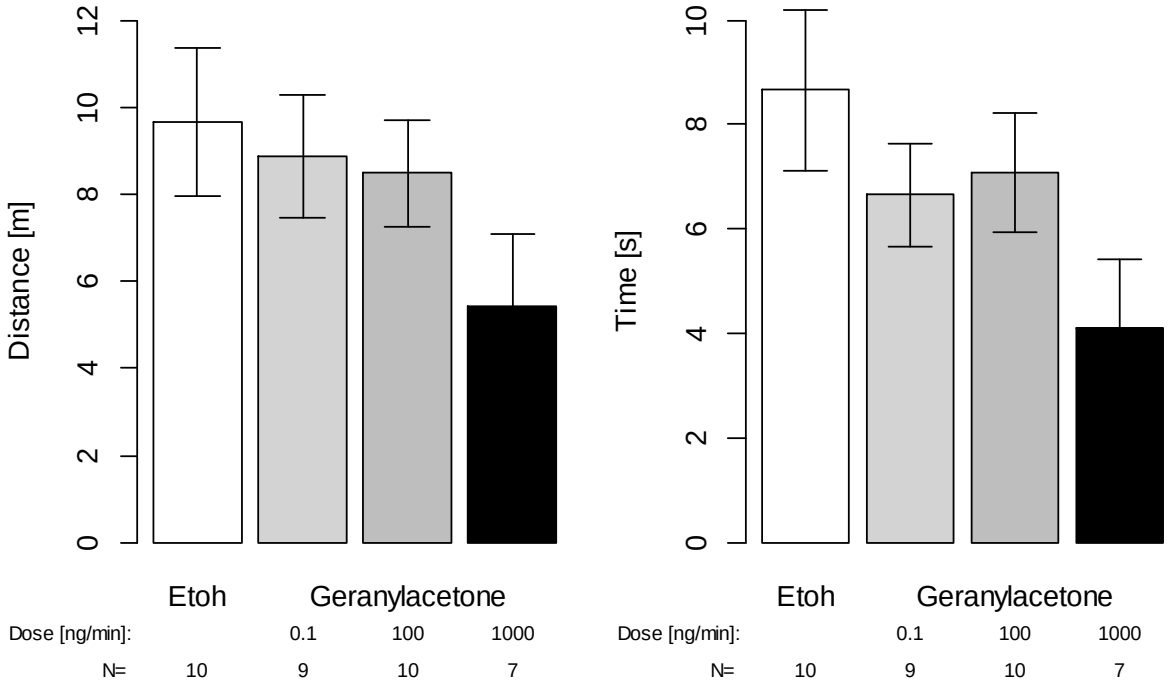

Figure 3.30: Mean ± SEM of the distance covered and the time spent flying by fed and starved females exposed to different doses of geranylacetone. No significant difference was found at $p<0.05$ following ANOVA. N is the number of flies that flew to the target.

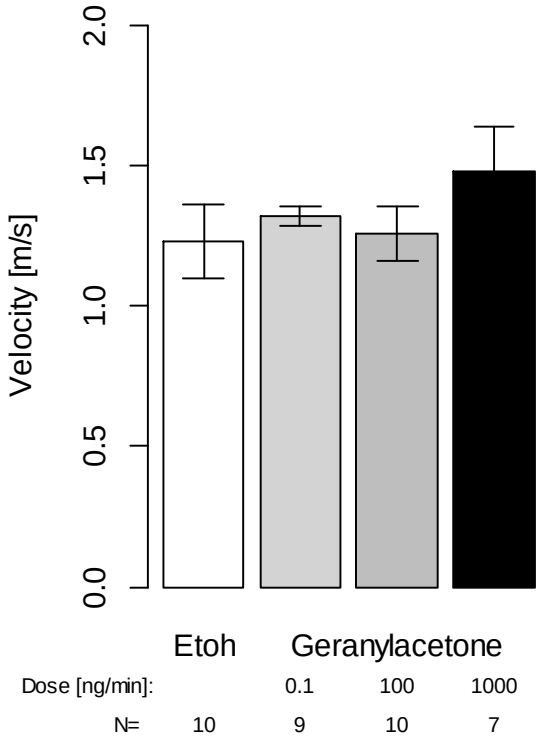

Figure 3.31: Mean ± SEM of average velocity of fed and starved females responding to different doses of geranylacetone. No significant difference was found at $p<0.05$ following ANOVA. N is the number of flies that flew to the target.

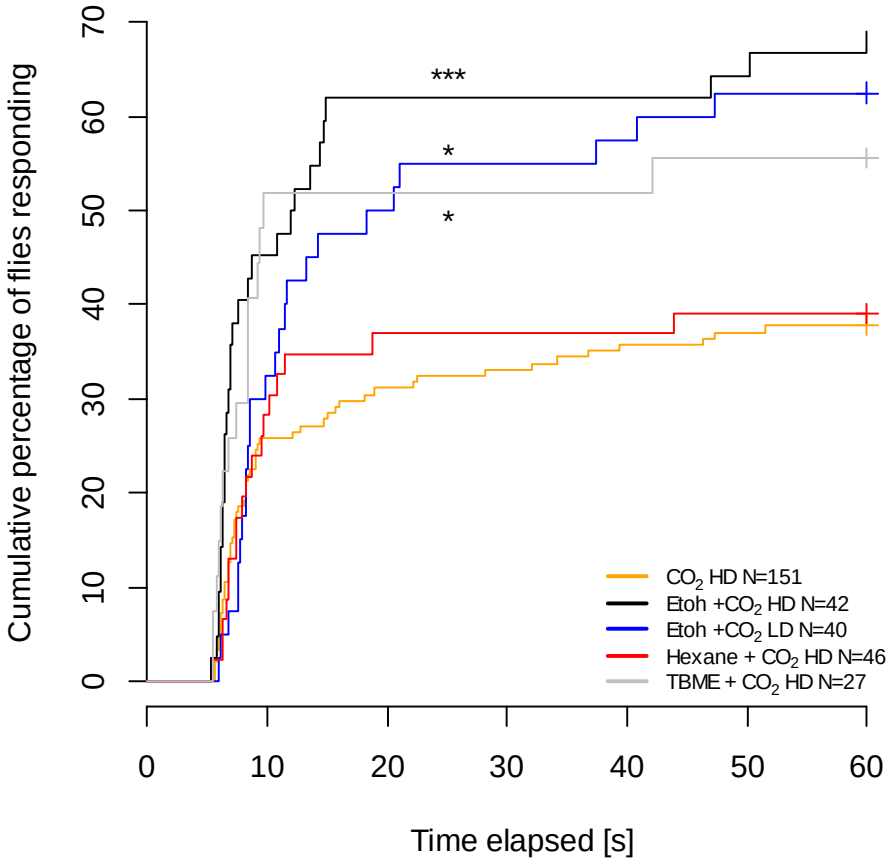

Figure 3.32: Cumulative percentage of fed and starved flies responding to different solvents released at 10µl/min, as a function of time elapsed until activation. Curves with asterisks are significantly different from the CO_2 control following Cox regression at $p<0.05$(*) and $p<0.001$(***). HD indicates a release rate of CO_2 at 220ml/min (70ppm) and LD a release rate of CO_2 at 60-80ml/min (22ppm). N is the number of flies tested.

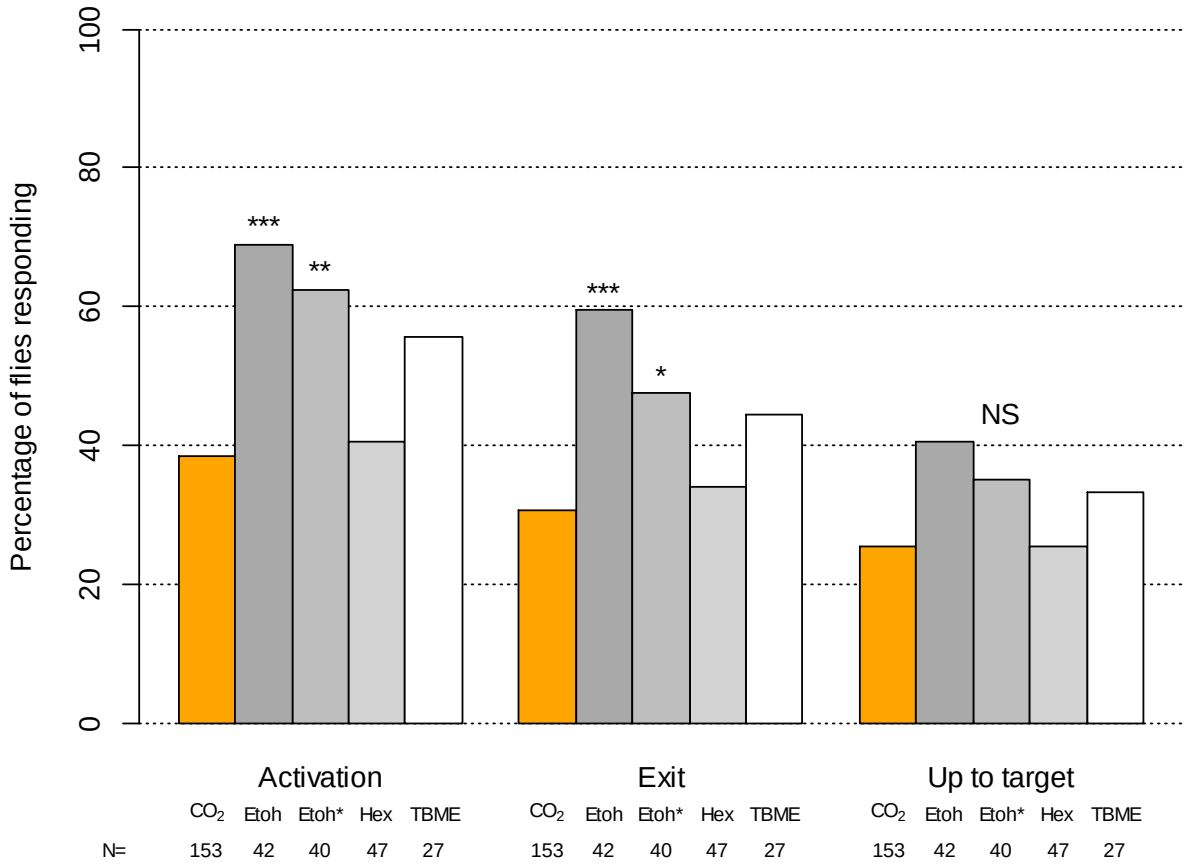

Figure 3.33: Proportion of flies that activated (Activation), exited the cage (Exit) and flew up to the target (Up to target) in response to CO_2, ethanol, hexane and methyl tert-butyl ether (TBME). Asterisks indicate that the percentage response for a behavioural criterion was significantly different to the CO_2 control at $p<0.05$(*), $p<0.01$(**) and $p<0.001$(***) using GLM with a logit link function. The release rate of CO_2 was 220ml/min (70ppm) for all treatments except for Etoh* where the CO_2 release rate was 60-80ml/min (22ppm). N is the number of flies tested.

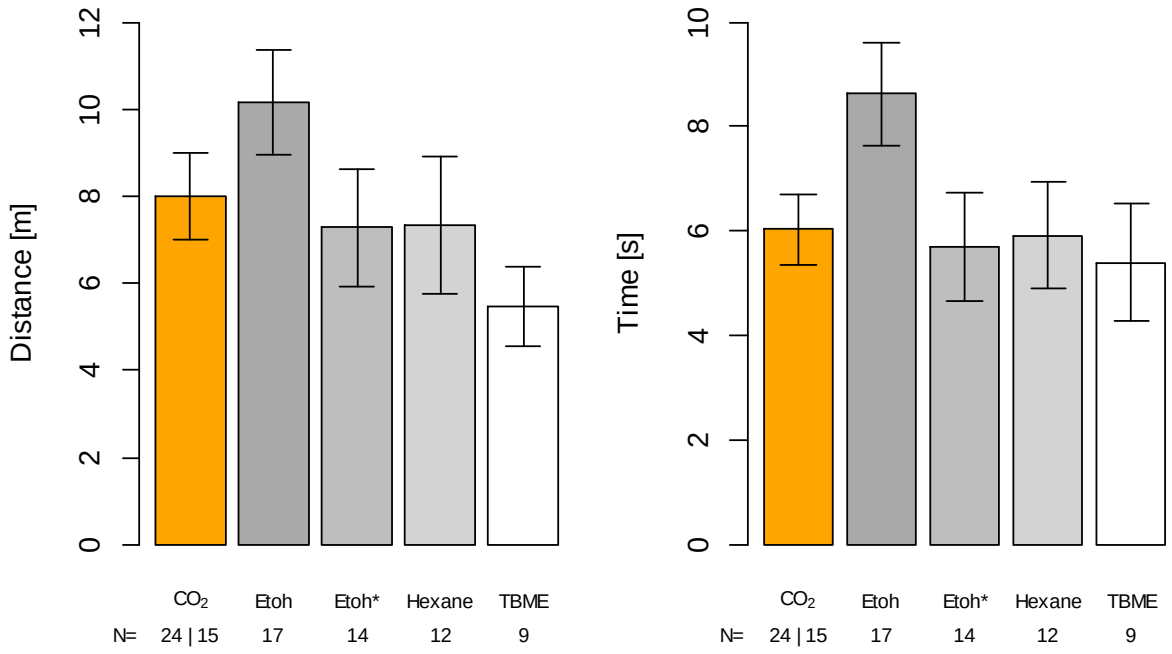

Figure 3.34: Mean ± SEM of the distance covered and the time spent flying by fed and starved flies exposed to different solvents released at 10µl/min with CO_2. Asterisks indicates significant difference from the control at $p<0.05$ following ANOVA. Etoh with the asterisk indicates that the release rate of CO_2 was 60-80ml/min (22ppm) in that treatment; it was 70ppm for all other treatments. N is the number of flies that flew up to the target.

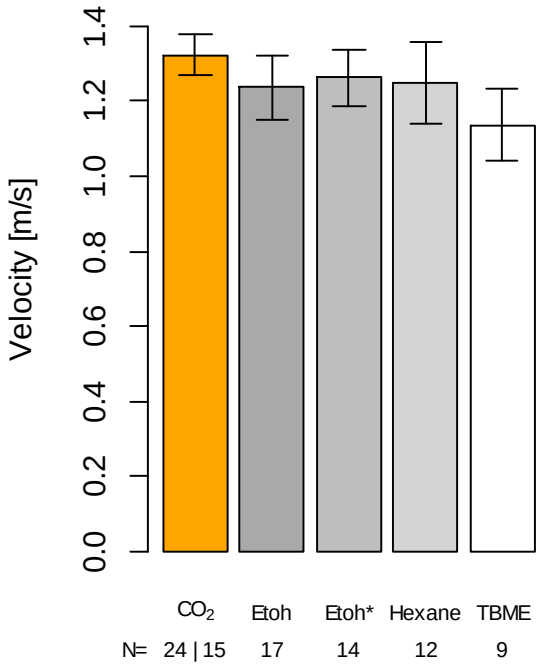

Figure 3.35: Mean ± SEM of average velocity of fed and starved flies responding to different solvents at a release rate of 10µl/min. No significant difference was found following ANOVA. Etoh with the asterisk indicates that the release rate of CO_2 was 60-80ml/min (22ppm); it was 80ppm for all other treatments in that treatment. N is the number of flies that flew up to the target.

3.3.2. Tracks analysis

3.3.2.1. Point distribution

The distance covered and the time spent flying parameters do not give any information about the spatial distributions of the fly positions. This lack can be filled by making, in 3-dimensions, kernel smoothed distributions of points. Then volumes that contain 95, 75, 50, 25 and 5% of the probability mass can be drawn in 3-dimensions. The probability mass corresponds to the probability for each point in the 3D-space and is used for discrete variables. It can be compared to the density in a continuous variable represented by the area under the curve of a distribution and can be interpreted as the probability to encounter a point of given spatial coordinates. So a volume, representing 5% of the probability mass, is the smallest region where the probability of finding a point is 5%. Taking into account the fact that points are recorded at regular time intervals (~40ms), the most dense regions can be interpreted as the spatial positions where flies

spent most of their time flying. Figure 3.36 shows similar points distribution of up to target flights for fed and starved and teneral flies exposed to breath or CO_2 (experiment "Breath versus CO_2"). Flies flew at an altitude of 0.35m and turned to the right or to the left of the target at about 1.2m along the x-axis. Until the turning point, the 95% and the 50% of the probability mass were within 0.4 and 0.2-diam. Cylinders, respectively. Moreover, layers are very close to each other, indicating a high degree of straightness and a poor variation between individuals. Distributions for teneral flies exposed to CO_2 looked different to those for breath. The distribution was biased on one side of the target. This could be explained by the fact that few data are available for CO_2 as it is a poor activator. In the local search behaviour, the flight was concentrated at 0.28m altitude in a croissant shaped volume around the sphere (Figure 3.37). Treatments did not drastically change the shape of these distributions. However, the distribution for fed and starved flies exposed to breath was spread wider than those exposed to CO_2, especially for the two layers containing 95 to 75% and 75 to 50% of the probability mass (Figure 3.37). These two layers were not smooth at both their downwind and upwind ends. Structures were deeper for the breath treatments than for the CO_2 treatment meaning that the number of downwind, up to target and upwind flights were greater in breath treatment than in CO_2 treatment (Figure 3.37). Similar effects of these two treatments was also found in teneral flies but the difference was narrower as teneral flies responded more to CO_2 than fed and starved flies (see above, Figure 3.37).

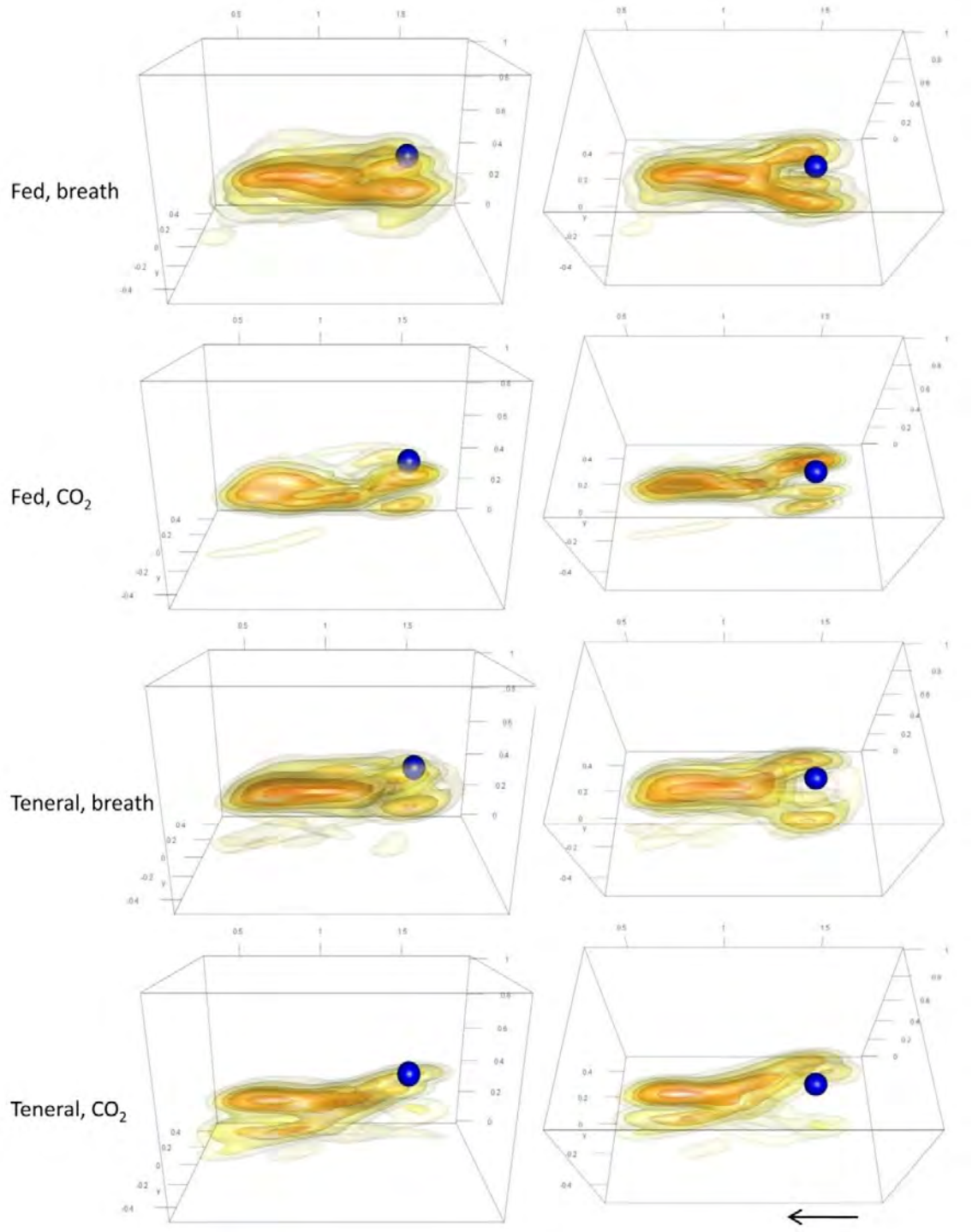

Figure 3.36 Kernel-smoothed distributions in 3D of up to target flight positions recorded with fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath and CO_2. To visualize the entire distribution, two viewing angles are represented in two columns, one at 70° (left) and the other at 30° (right). The blue sphere is the target placed at X=1.5m, Y=0m, Z=0.5m. The box delimits the border of the wind tunnel (to scale). The black arrow indicates wind direction. The colors correspond to regions with the respective probability masses: red=5%, red+orange=25%, red+orange+dark yellow=50%, red+orange+dark-yellow+light-yellow=75% and red+orange+dark-yellow+light-yellow+clear-yellow=95%.

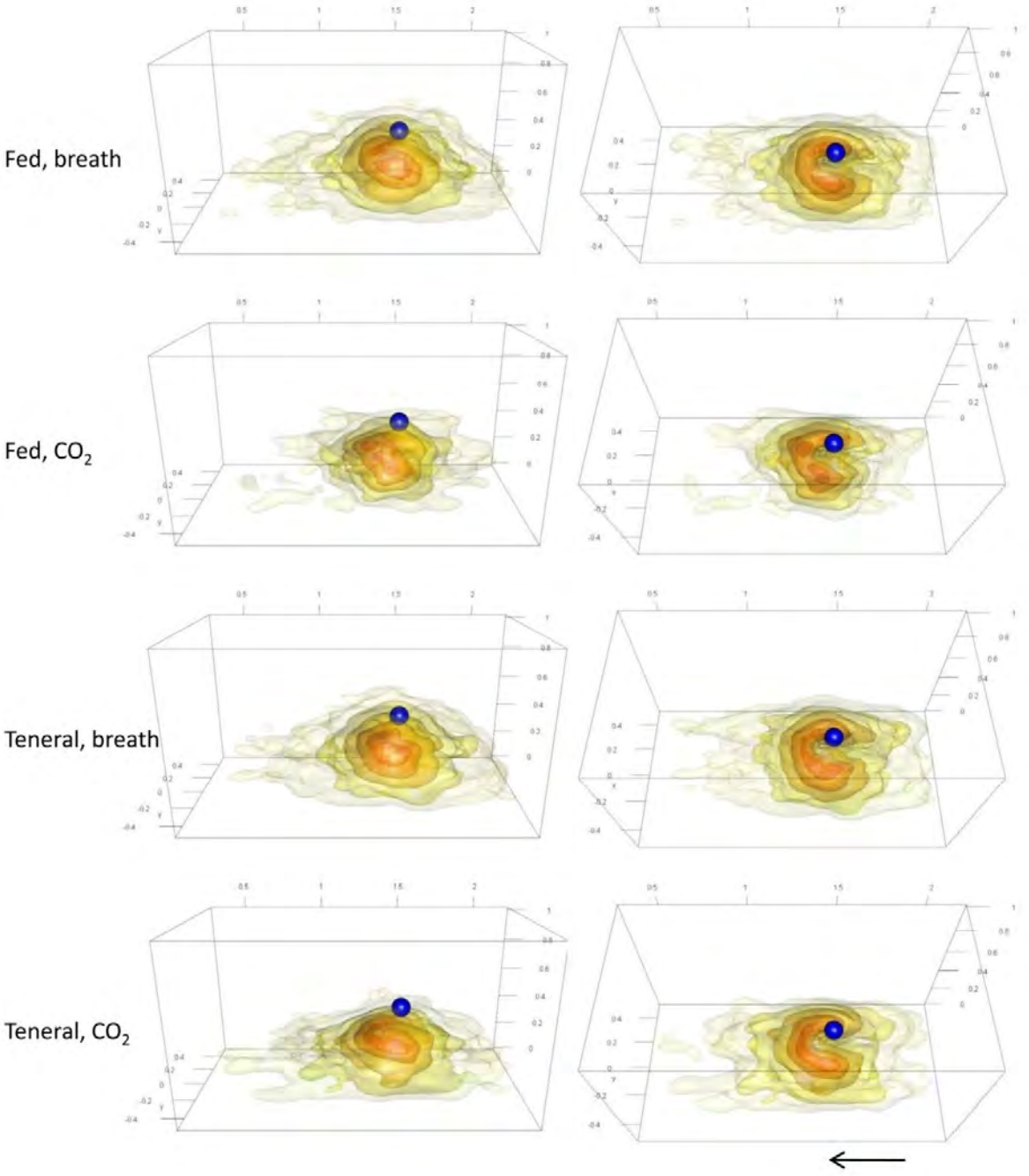

Figure 3.37: Kernel-smoothed distribution in 3D of local search positions recorded with fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath and CO_2. To visualize the entire distribution, two viewing angles are represented in two columns, one at 70° (left) and the other at 30° (right). The blue sphere is the target placed at X=1.5m, Y=0m, Z=0.5m. The box delimits the border of the wind tunnel (at scale). The black arrow indicates wind direction. The colors correspond to regions with the respective probability masses: red=5%, red+orange=25%, red+orange+dark yellow=50%, red+orange+dark-yellow+light-yellow=75% and red+orange+dark-yellow+light-yellow+clear-yellow=95%.

The up to target flights obtained in experiment “The pulse of breath” were similar to those obtained in experiment “Breath versus CO_2” (Figure 3.38). Flies flew at an altitude of 0.35m and turned to the right or to the left of the target at about 1.2m along the x-axis. Until the turning point, 95% and 50% of the probability masses were within 0.4 and 0.2-diam. Cylinders, respectively. The treatments, breath in a continuous flow, pulse + CO_2 and pulse, did not affect drastically the points distributions except for the pulse treatment. Indeed the 3 layers containing 95 to 75%, 75 to 50% and 50 to 25% of the probability masses came very close to the target (Figure 3.38; examples of flight tracks are shown in Appendix B.2). This means that flies exposed to the 2s pulse of breath approached closer to the sphere before turning than flies exposed to the other treatments. Treatments also affected the points distribution during the local search (average altitude: 0.30m). Distribution for the breath treatment was wider than that for the pulse + CO_2 treatment and had the same structures in external layers found in the breath treatment in experiment “Breath versus CO_2” (Figure 3.39). This indicated that the breath treatment elicited more flights along the x-axis than CO_2. Moreover, flies stimulated with just a 2s pulse of breath, meaning that no or hardly any odour was present around the sphere during the local search, flew higher in altitude and less in the upwind part of the wind tunnel (Figure 3.39).

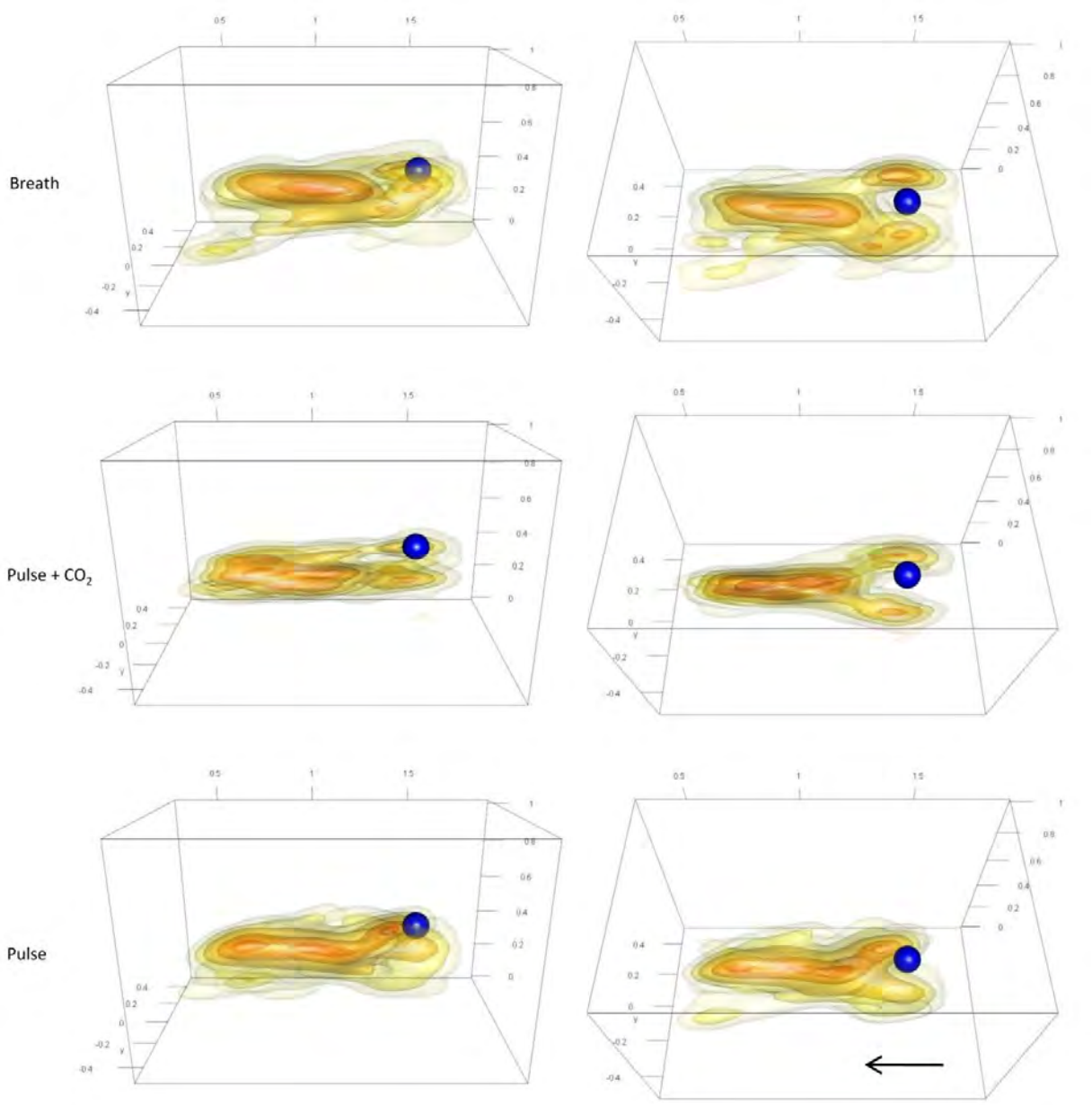

Figure 3.38: Kernel-smoothed distribution in 3-D of the up to target flights positions recorded with fed and starved flies exposed to a continuous flow of breath (breath), a 2s pulse of breath followed by CO_2 (pulse + CO_2) and a 2s pulse of breath (pulse). To visualize the entire distribution two viewing angles are represented in two columns, one at 70° (left) and the other at 30° (right). The blue sphere is the target placed at X=1.5m, Y=0m, Z=0.5m. The box delimits the border of the wind tunnel (to scale). The black arrow indicates wind direction. The colors correspond to regions with the respective probability masses: red=5%, red+orange=25%, red+orange+dark yellow=50%, red+orange+dark-yellow+light-yellow=75% and red+orange+dark-yellow+light-yellow+clear-yellow=95%.

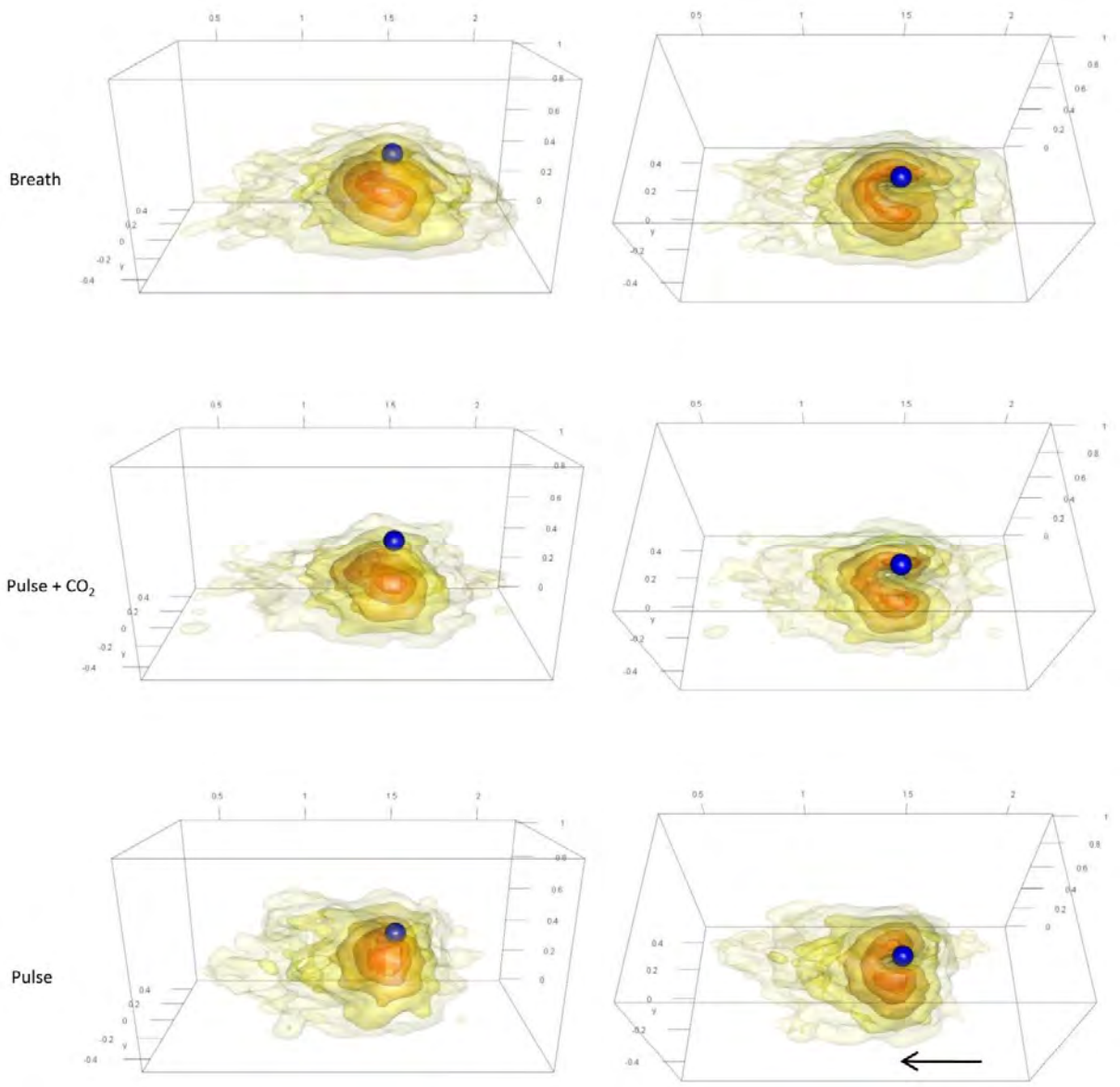

Figure 3.39: Kernel-smoothed distributions in 3D of local search flight positions recorded with fed and starved flies exposed to a continuous flow of breath (breath), a 2s pulse of breath followed by CO_2 (pulse + CO_2) and a 2s pulse of breath (pulse). To visualize the entire distribution, two viewing angles are represented in two columns, one at 70° (left) and the other at 30° (right). The blue sphere is the target placed at X=1.5m, Y=0m, Z=0.5m. The box delimits the border of the wind tunnel (to scale). The black arrow indicates wind direction. The colors correspond to regions with the respective probability masses: red=5%, red+orange=25%, red+orange+dark yellow=50%, red+orange+dark-yellow+light-yellow=75% and red+orange+dark-yellow+light-yellow+clear-yellow=95%.

3.3.2.2. Velocity, curvature and torsion

Breath versus CO_2

The velocity was correlated with the distance to the sphere for teneral or fed and starved flies exposed to CO_2 or breath (Figure 3.40). Flies accelerated to reach the maximal velocity at about 0.6m from the sphere and decelerated gradually approaching the sphere. ANOVA on two 0.2m portions of the flight at 0.2-0.4m and 0.5-0.7m from the sphere (see Figure 3.41) revealed that flies exposed to breath flew on average 0.25m/s faster than flies exposed to CO_2 ($p<0.001$) and confirmed that flies decelerated approaching the sphere as the average velocity in the portion 0.2-0.4m from the sphere was 0.41m/s lower than the portion 0.5-0.7m from the sphere ($p<0.001$, Figure 3.41). The velocity reduction while approaching the sphere was equal among treatments, sexes or feeding status (Figure 3.40 & Figure 3.41). No difference between both sexes or between fed and starved and teneral flies was found except that fed and starved males flew 0.21m/s faster than teneral males ($p=0.029$, Figure 3.41).

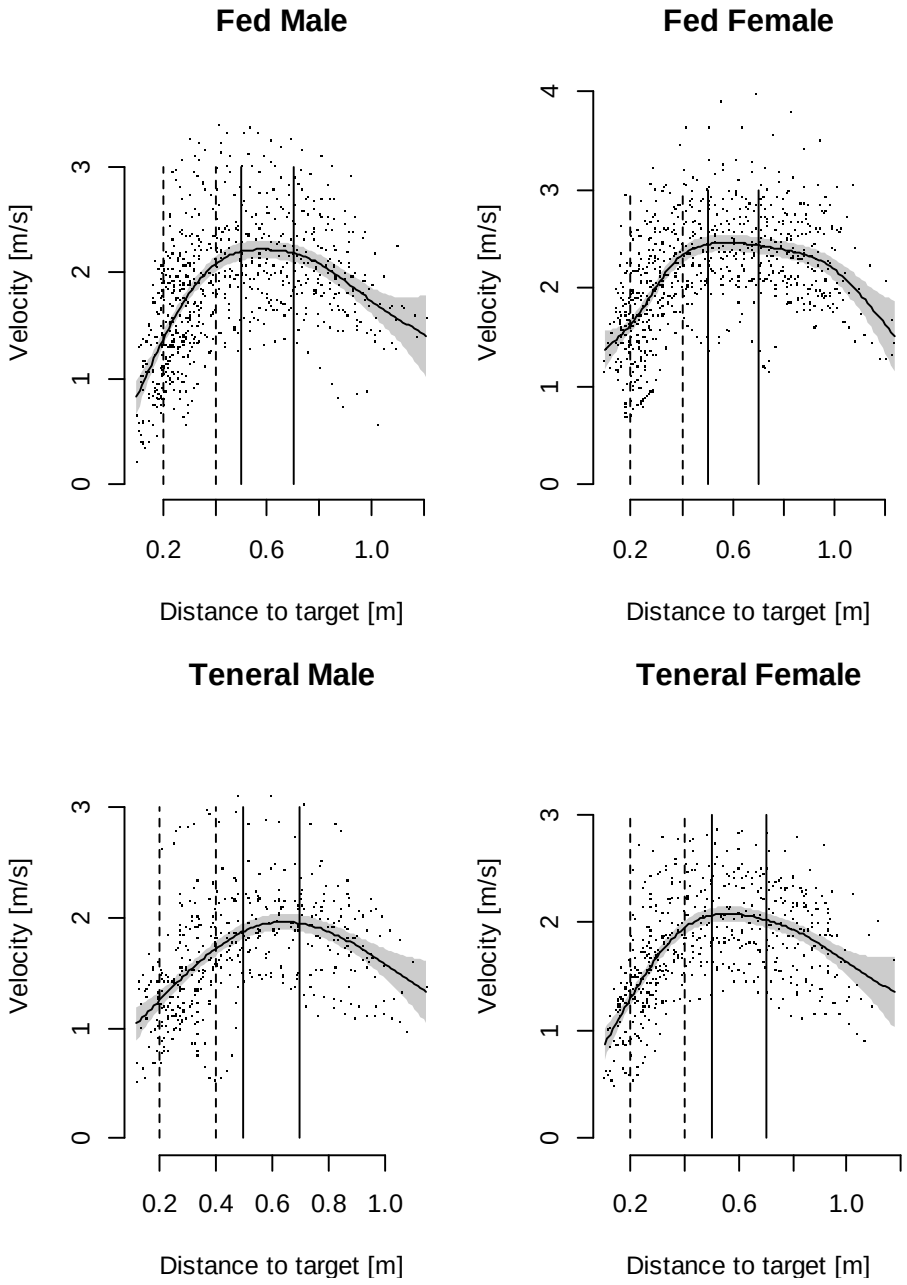

Figure 3.40: Velocity for fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath and CO_2, as a function of the distance to the center of the target. The curves represent the fitted model, GAM whose linear smoothing estimates were significant ($p<0.001$) for each graph presented here. The gray area shows the SEM. Vertical and dashed solid lines delimit the bins within which means were estimated (0.2-0.4m and 0.5-0.7m).

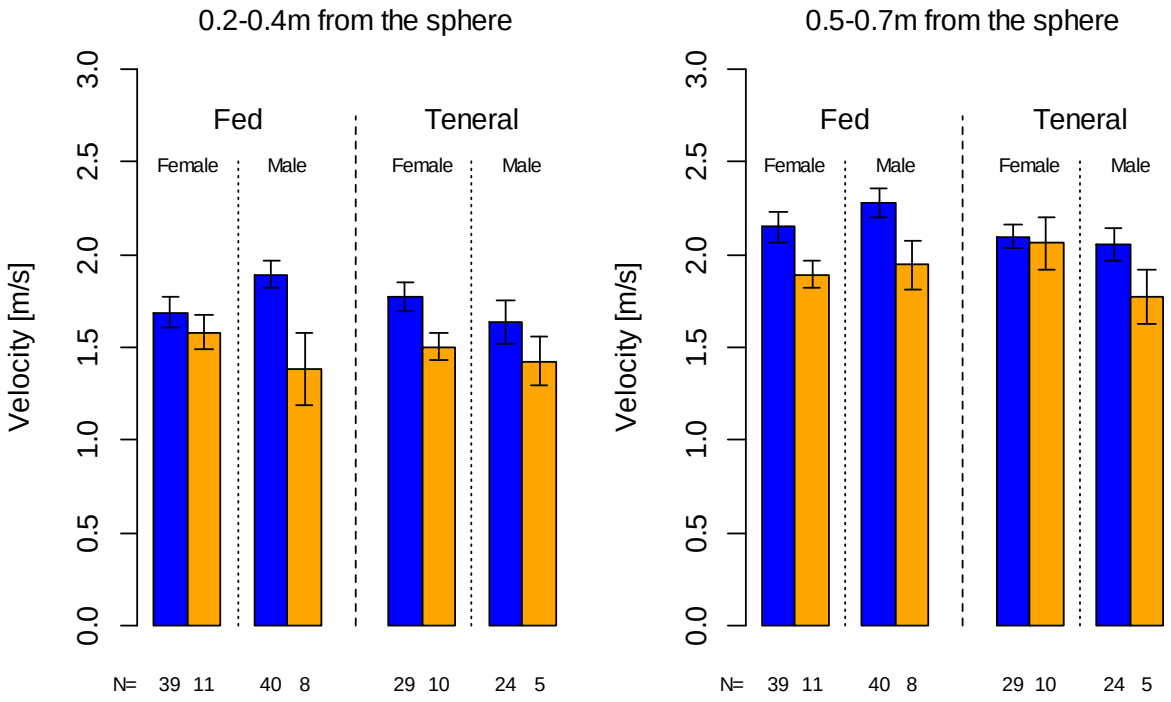

Figure 3.41: Mean velocity ± SEM for fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath (blue) and CO_2 (orange) as a function of the 2 bins (shown in Figure 3.40). N is the number of flies that flew up to target after filtering (see Materials and Methods).

Curvature was also correlated with the distance to the sphere but at the contrary of velocity, the curvature increased approaching the sphere (Figure 3.42). ANOVA on the same two portions described above confirmed that the average curvature in the portion 0.2-0.4m from the sphere was 1.56rad/m higher (detransformed mean ,$p<0.001$) than in the portion 0.5-0.7m (3.22rad/m, detransformed mean, Figure 3.43). Neither the treatment or the sexes or the feeding status had a significant influence on the curvature. No conclusion could be draw for the torsion as it was very noisy and oscillated near zero (Figure 3.44). This noise came from a window size that was too small for smoothing the torsion (see Crenshaw et al. (2000)). No analysis was performed on the mean velocity, curvature and torsion of local search because not enough flies responded to CO_2 resulting in strongly unbalanced data.

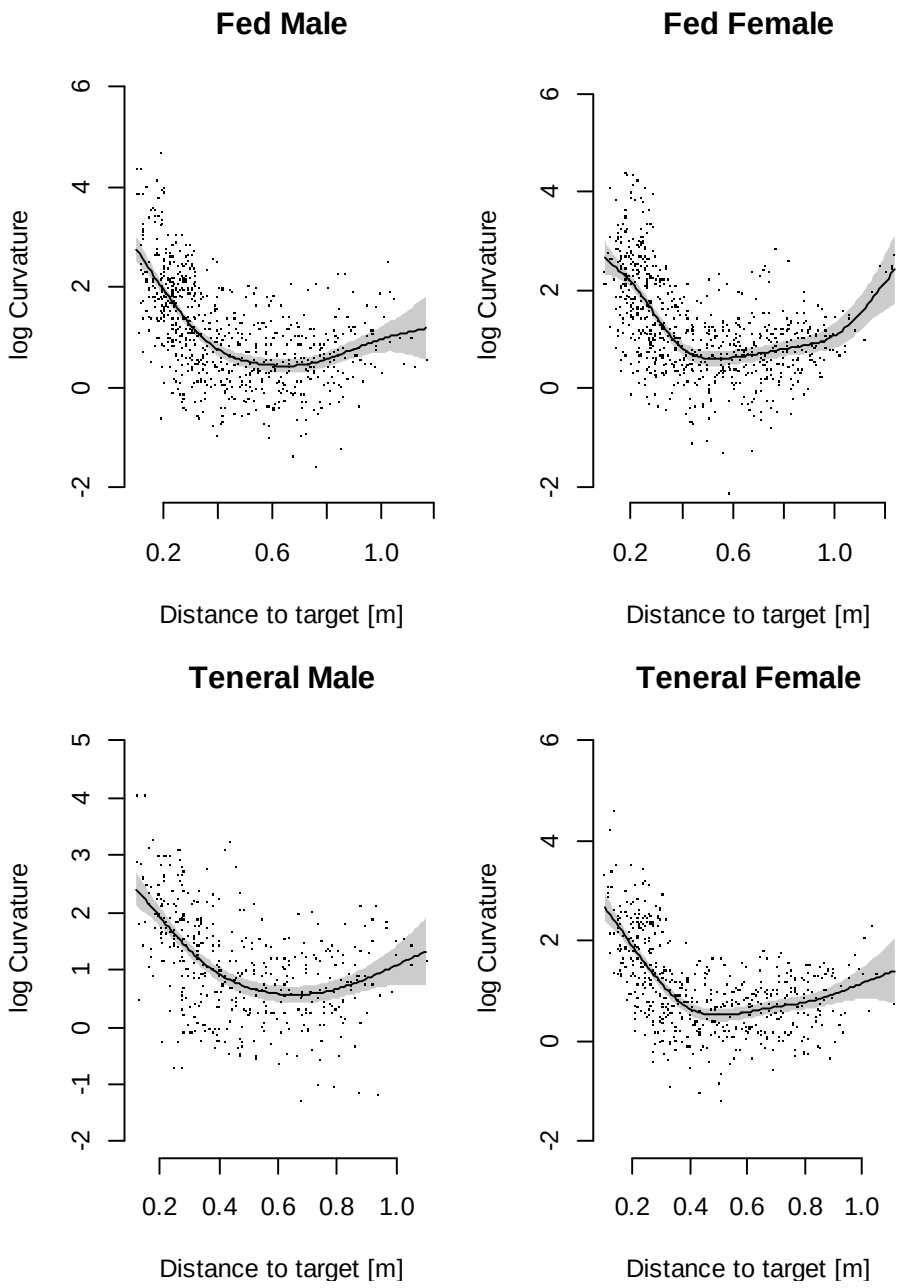

Figure 3.42: Log transformed curvature parameters for fed and starved (indicated as Fed) and teneral (indicated as Teneral) exposed to breath and CO_2, as a function of the distance to the center of the target. The curves represent the fitted model, GAM whose linear smoothing estimates were significant ($p<0.001$) for each graph presented here. The gray area shows the SEM.

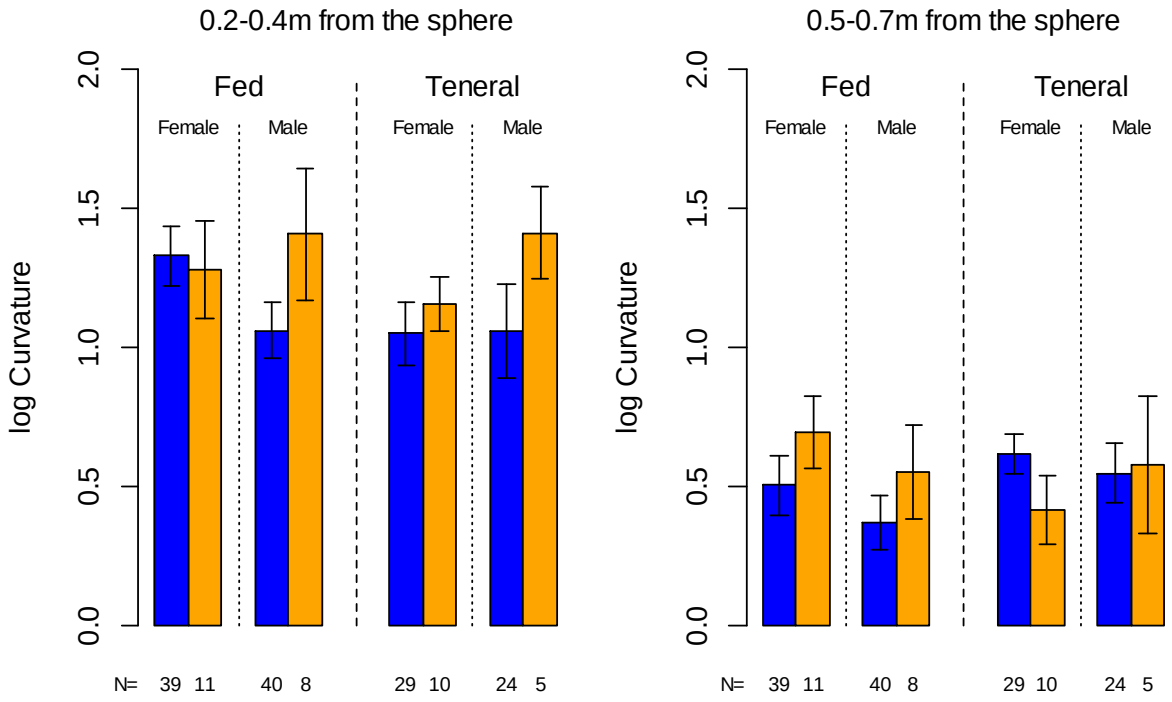

Figure 3.43: Mean of the log transformed flight curvature ± SEM for fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath (blue) and CO_2 (orange), as a function of the 2 sampling bins (shown in Figure 3.40). N is the number of flies that flew up to target, after filtering (see Materials and Methods).

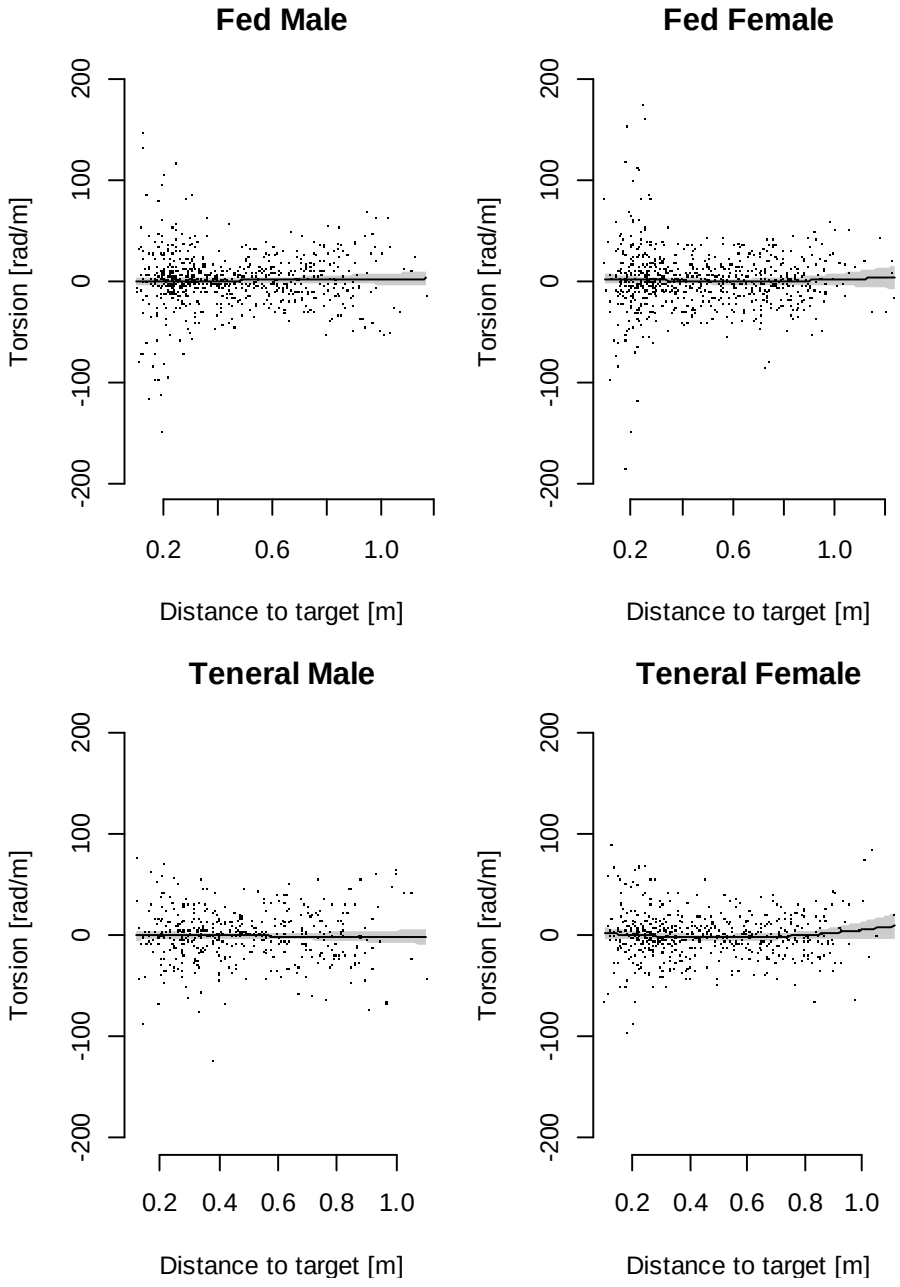

Figure 3.44: Flight torsion in fed and starved (indicated as Fed) and teneral (indicated as Teneral) flies exposed to breath and CO_2, as a function of the distance to the center of the target. The curves represent the fitted model, GAM whose linear smoothing estimates were not significant for each graph presented here. The gray area shows the SEM.

The pulse of breath

The velocity and the curvature were correlated with the distance to the target in the same manner as in experiment “The pulse of breath” (Figure 3.45 & Figure 3.46). The nearer the fly approached the target the more velocity decreased and the more curvature increased. ANOVA on two 0.2m portions of the flight at 0.2-0.4m and 0.5-0.7m (see Figure 3.47 & Figure 3.48) confirmed that velocity was reduced and curvature was increased as the average velocity and the average curvature in the portion 0.2-0.4m from the sphere was 0.32m/s lower and 0.53rad/m higher (detransformed mean) than in the portion 0.5-0.7m from the sphere (for both parameters: $p<0.001$, Figure 3.47 & Figure 3.48). Males flew 0.17m/s faster than females ($p<0.01$). Although no significant difference was found between the sexes in experiment “Breath versus CO_2”, fed and starved males flew 0.12 m/s faster than females in experiment “The pulse of breath”. No significant difference in curvature was found between in sexes.

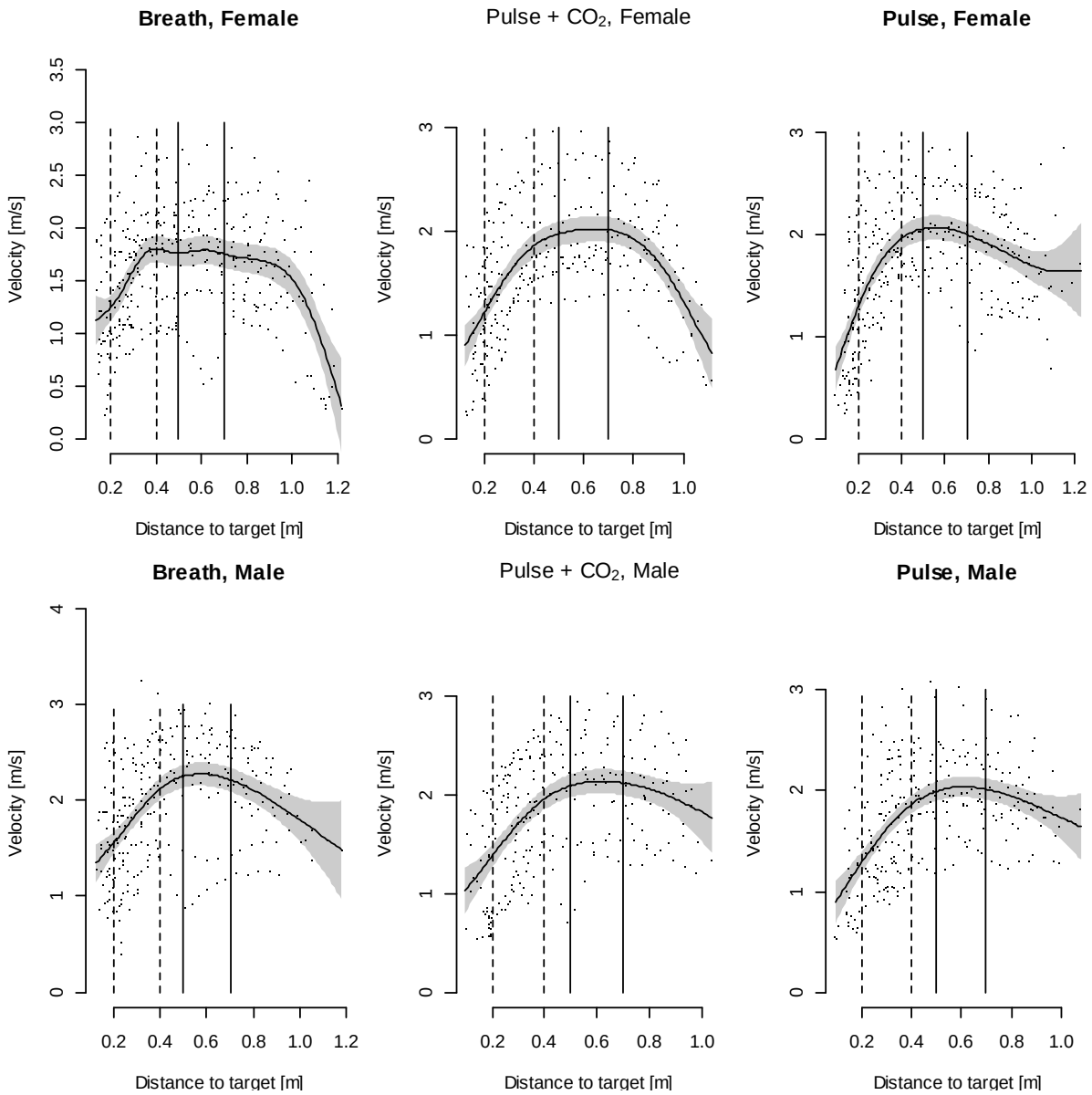

Figure 3.45: Velocity for fed and starved flies exposed to breath, pulse + CO_2 and pulse treatments, as a function of the distance to the center of the target. The curves represent the fitted model, GAM whose linear smoothing estimates were significant ($p<0.001$) for each graph presented here. The gray area shows the SEM. Vertical and dashed solid lines delimit the bins to estimate means at 0.2-0.4m and 0.5-0.7m from the sphere.

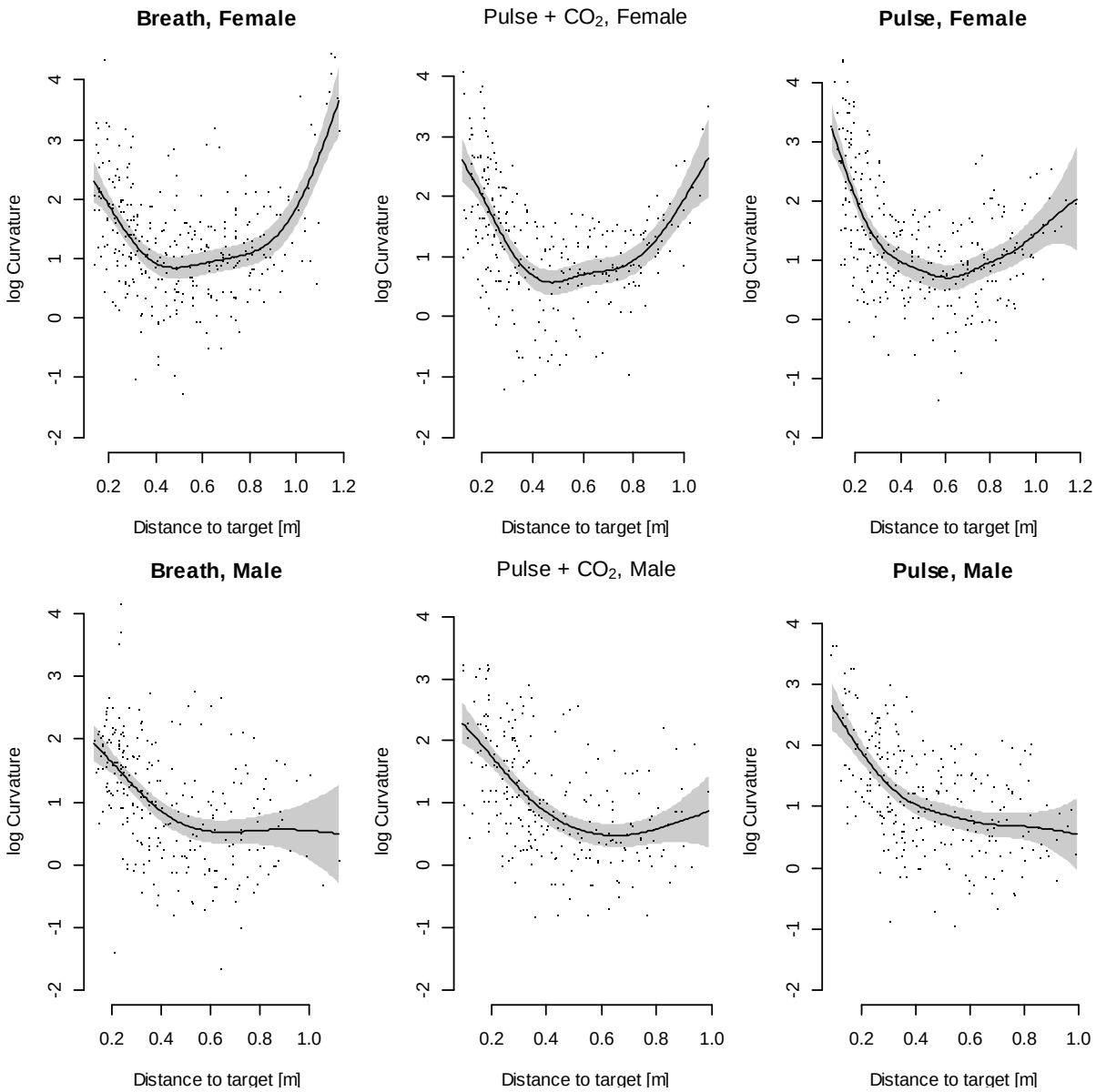

Figure 3.46: Log transformed flight curvature for fed and starved flies exposed to breath, pulse + CO_2 and pulse treatments, as a function of the distance to the center of the target. The curves represent the fitted model, GAM whose linear smoothing estimates were significant ($p<0.001$) for each graph presented here.

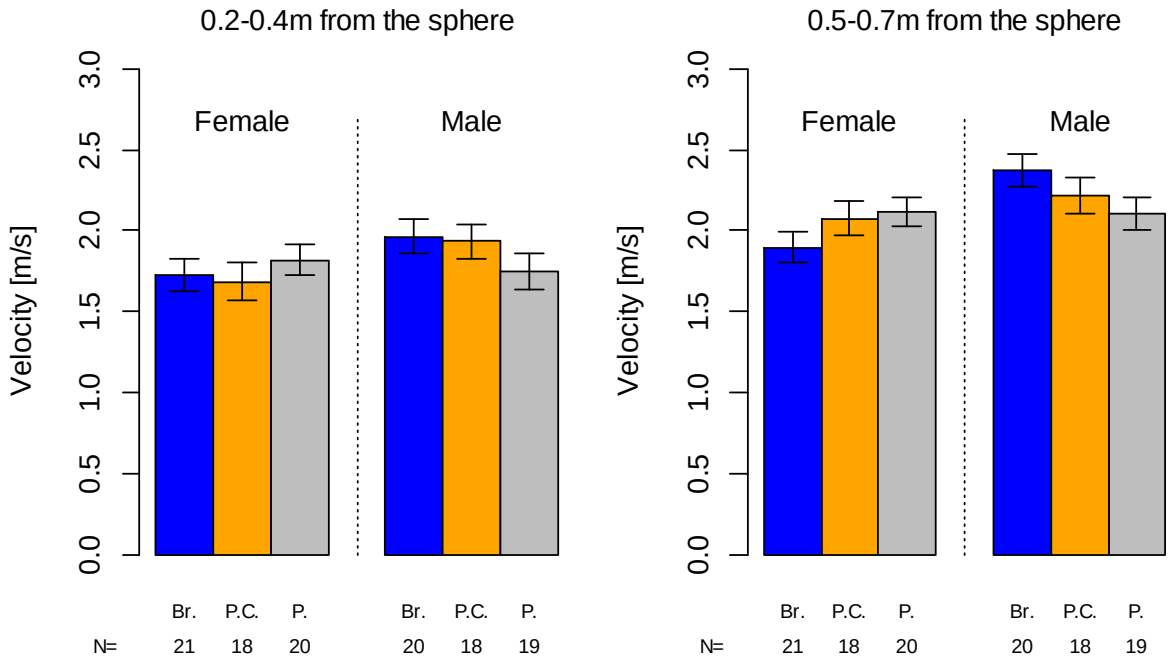

Figure 3.47: Mean velocity ± SEM for fed and starved flies exposed to breath (Br.), pulse + CO_2 (P.C.) and pulse (P.) treatments, as a function of 2 sampling bins (shown in Figure 3.45). N is the number of flies that flew up to target after filtering the data (see Materials and Methods).

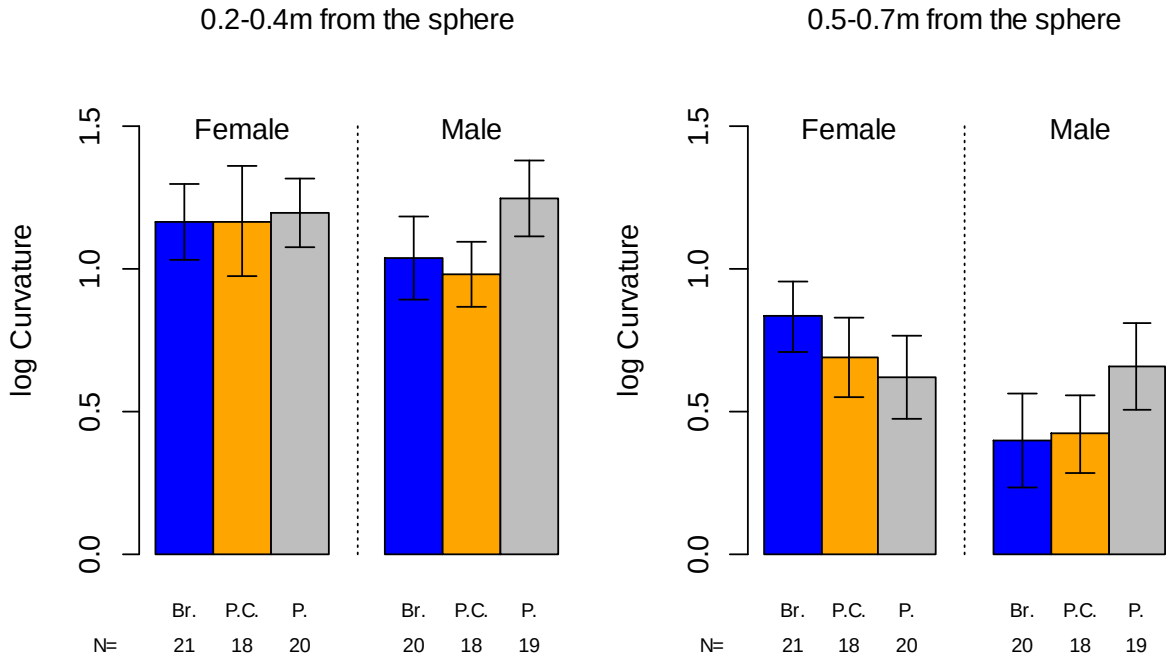

Figure 3.48: Mean of the log transformed flight curvature ± SEM for fed and starved flies exposed to breath (Br.), pulse + CO_2 (P.C.) and pulse (P.) treatments, as a function of 2 sampling bins (shown in Figure 3.45). N is the number of flies that flew up to target after filtering the data (see Materials and Methods).

The odour treatment had no significant effect on the velocity or the curvature during the up to target flight but had a significant effect during the local search. Indeed flies exposed to the continuous breath treatment flew on average 0.15m/s ($p<0.001$), 0.22m/s ($p<0.001$) faster and 0.60rad/m ($p<0.001$), 0.93rad/m ($p<0.001$) less in curvature than flies exposed to the pulse and the pulse + CO_2 treatment, respectively (Figure 3.49 & Figure 3.50). The velocity was correlated with the x-axis: at 1.1m the average velocity was 1.51m/s and was significantly reduced to 1.05m/s at 1.6m ($p<0.001$). The curvature did the opposite: at 1.1m the average curvature was 4.8rad/m and was significantly increased to 10.08rad/m at 1.6m (Figure 3.49 & Figure 3.50). On the y-axis, the velocity did not significantly change on average between -0.1and 0.1m but it was 0.31m/s ($p<0.001$) and 0.17m/s ($p<0.001$) greater at a distance greater than 0.2m or less than -0.1m, respectively. As for the x-axis, the curvature did the opposite to the velocity: on the y-axis, the curvature did not significantly change on average between -0.1m and 0.1m but was 1.42rad/m ($p<0.001$) and 0.98rad/m ($p<0.001$) greater from a distance higher than 0.2m or less than -0.1m, respectively (Figure 3.49 & Figure 3.50). The velocity and the curvature did not change significantly on average along the z-axis despite the fact that the area of the local search increased with decreasing altitude. Differences between the sexes were considered negligible as they were less than 0.1m/s and 0.1rad/m. As mentioned above, the interactions between axes could not be integrated in the ANOVA. However, according to Figure 3.49 & Figure 3.50 the velocity and the curvature were correlated with the distance to the sphere. The closer a fly was to the sphere the more it reduced its velocity to make sharper turns.

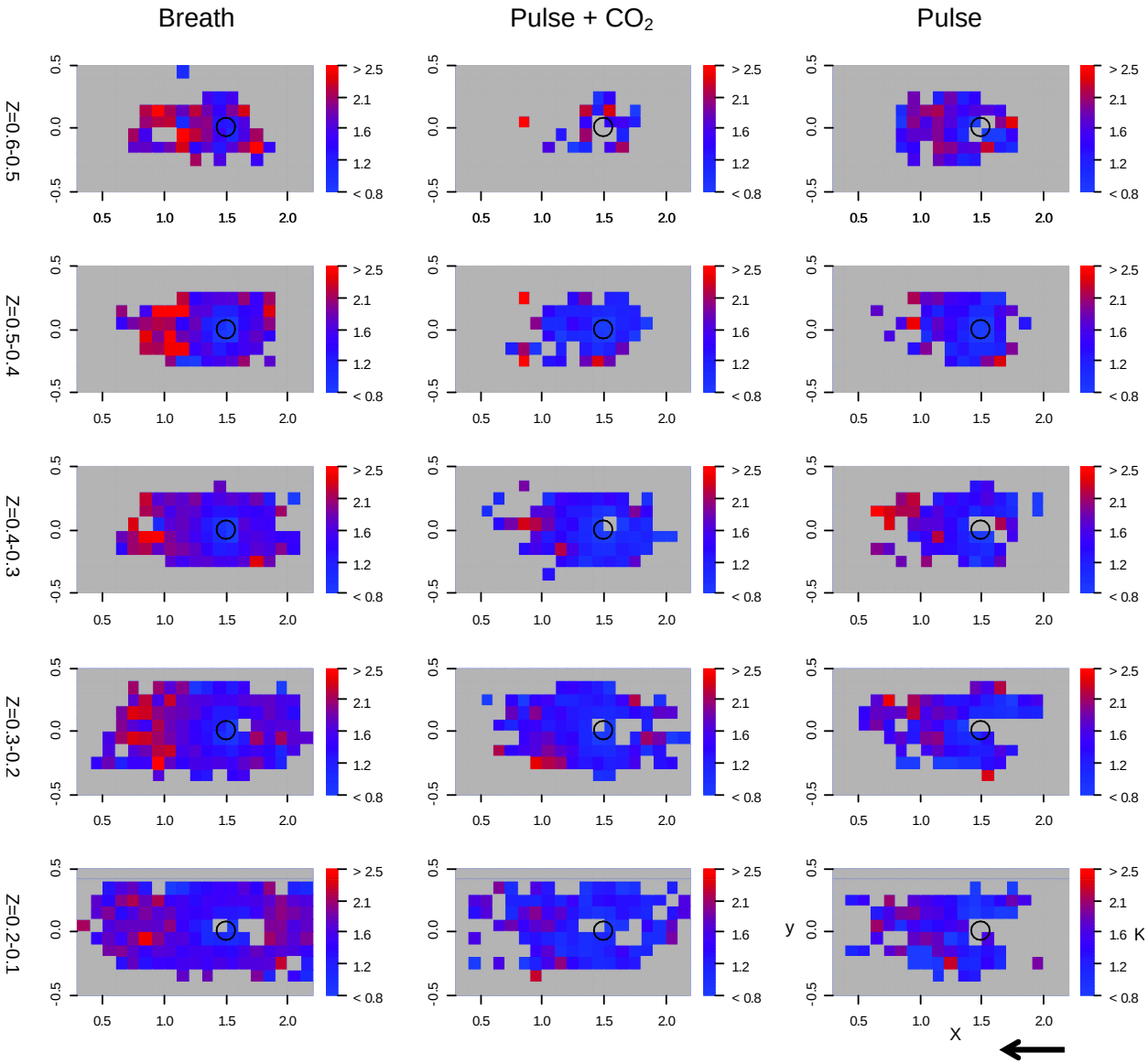

Figure 3.49: Mean velocity of fed and starved flies exposed to breath, pulse + CO_2 and pulse treatments as a function of spatial coordinates X,Y,Z. Color scale represents the mean velocity in bins of 0.1m*0.1m*0.1m in size (see Materials and Methods). The black circles represent the sphere. The black arrow indicates the wind direction.

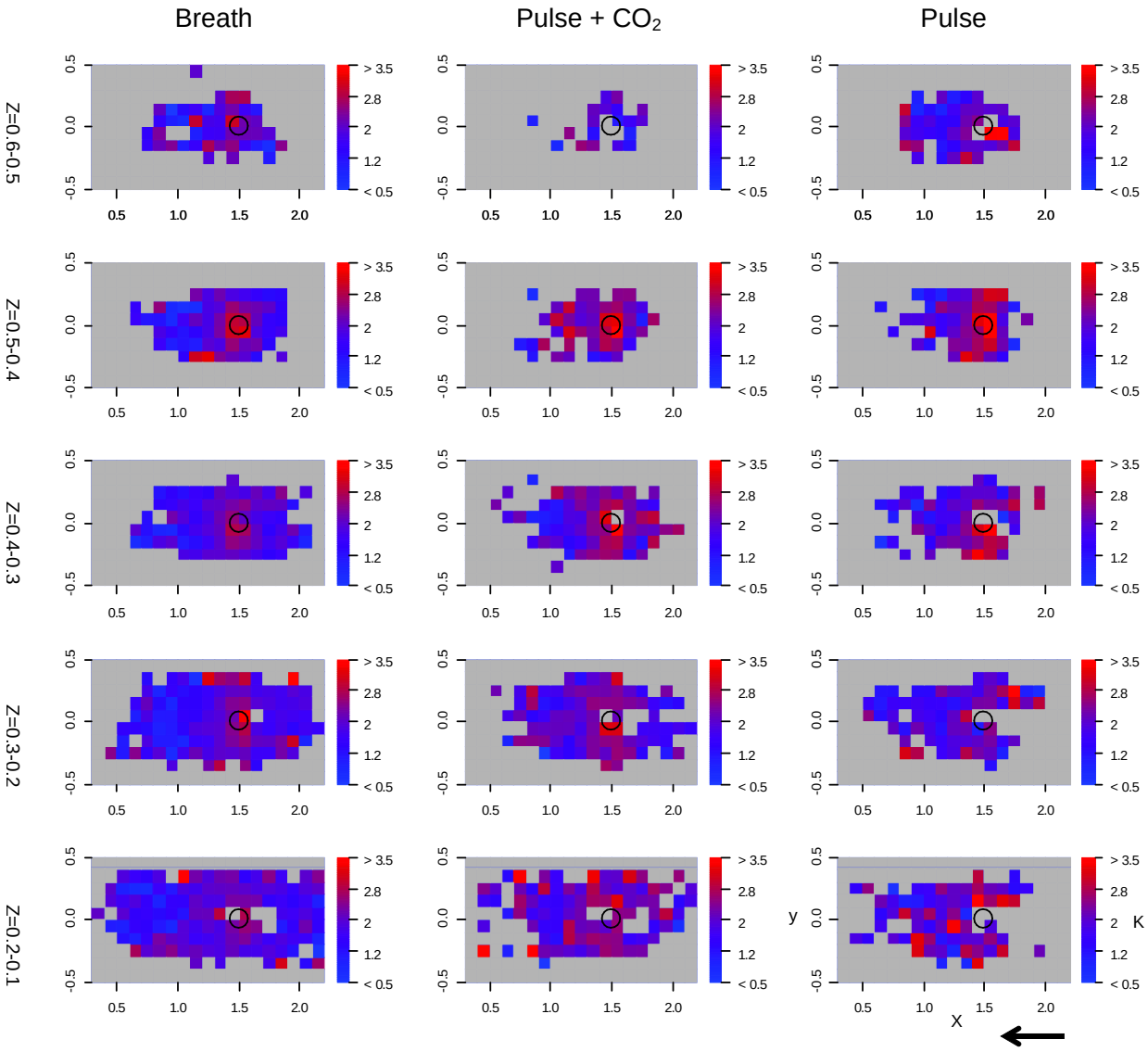

Figure 3.50: Mean curvature (log transformed) of fed and starved flies exposed to breath, pulse + CO_2 and pulse treatments as a function of spatial coordinates X,Y,Z. Color scale represents the mean curvature (log transformed) in bins of 0.1m*0.1m*0.1m in size (see Materials and Methods). The black circles represent the sphere. The black arrow indicates the wind direction.

3.3.3. Tsetse landing behaviour

Although the behavioural assays in the wind tunnel presented in experiment “The pulse of breath” were designed primarily to study the tsetse flight, I saw that the blue sphere provided a landing stimulus. Flies landing on the equator and on the upper part of the sphere were visible to the cameras allowing landings to be counted. I also observed that flies tend to land on the lower part of the sphere or on the stative but these landings could not be recorded as the cameras filmed the scene from above. In experiment “The

pulse of breath", the number of landings were approximated by counting the number of visible landings plus the number of times that flies remained at least 2s under the visual target. Overall, 75% of tsetse tested landed at least once on the sphere. The three treatments, continuous breath, 2s pulse of breath followed by CO_2 and the 2s pulse of breath, did not affect the proportion of landings by females, which was 82%, 80% and 90%, respectively. However, these treatments did have an effect on the landing behavior of males as 95%, 85% and 55% of males stimulated with continuous breath, the 2s pulse of breath followed by CO_2 and the 2s pulse of breath, respectively, landed on the target.

Seeing this, I tested different objects as landing stimulus. Figure 3.51 summarizes the scores for the objects tested as landing stimuli. The highest proportion of flies that landed at least once was obtained with the 250mm diameter sphere mounted on either the 30mm and 105mm diameter columns. Reducing the size of the sphere to a diameter of 120mm or reducing its 3D component to 2D (disc), reduced significantly by about half the proportion of flies that landed. No flies landed on the 30mm diameter column presented alone and adding just any object to the column did not increase the proportion of flies landing. Indeed, the complex object with one central and surrounding spheres induced few landing whereas adding the 120mm diameter sphere to the column significantly increased the proportion of flies that landed compared to the black bar alone (Figure 3.51). No interaction was found between the objects and sex variables, but significantly more males landed on the objects than females independently of the objects being tested: 57% of males landed whereas this proportion was reduced to 46% for females (GLM, p=0.043).

I was also interested to determine if tsetse preferred to alight on a particular part of an object. Results presented in the lower of (Figure 3.51) show that *G. pallidipes* alighted quasi-systematically on the lower parts of spheres and the horizontal disc. Furthermore, decreasing the surface ratio between the sphere and the column either by decreasing the size of the sphere or by increasing the width of the column, decreased the preference to land on the sphere and increased the preference for the bar (Figure 3.51). When the disc was placed vertically on the bar, tsetse did not show a clear preference

for a place to land. However, tsetse clearly preferred to land on the lower part of the horizontal disc, corresponding to results obtained with the sphere. On the horizontal disc, flies tended to land in the outer ring (Figure 3.51).

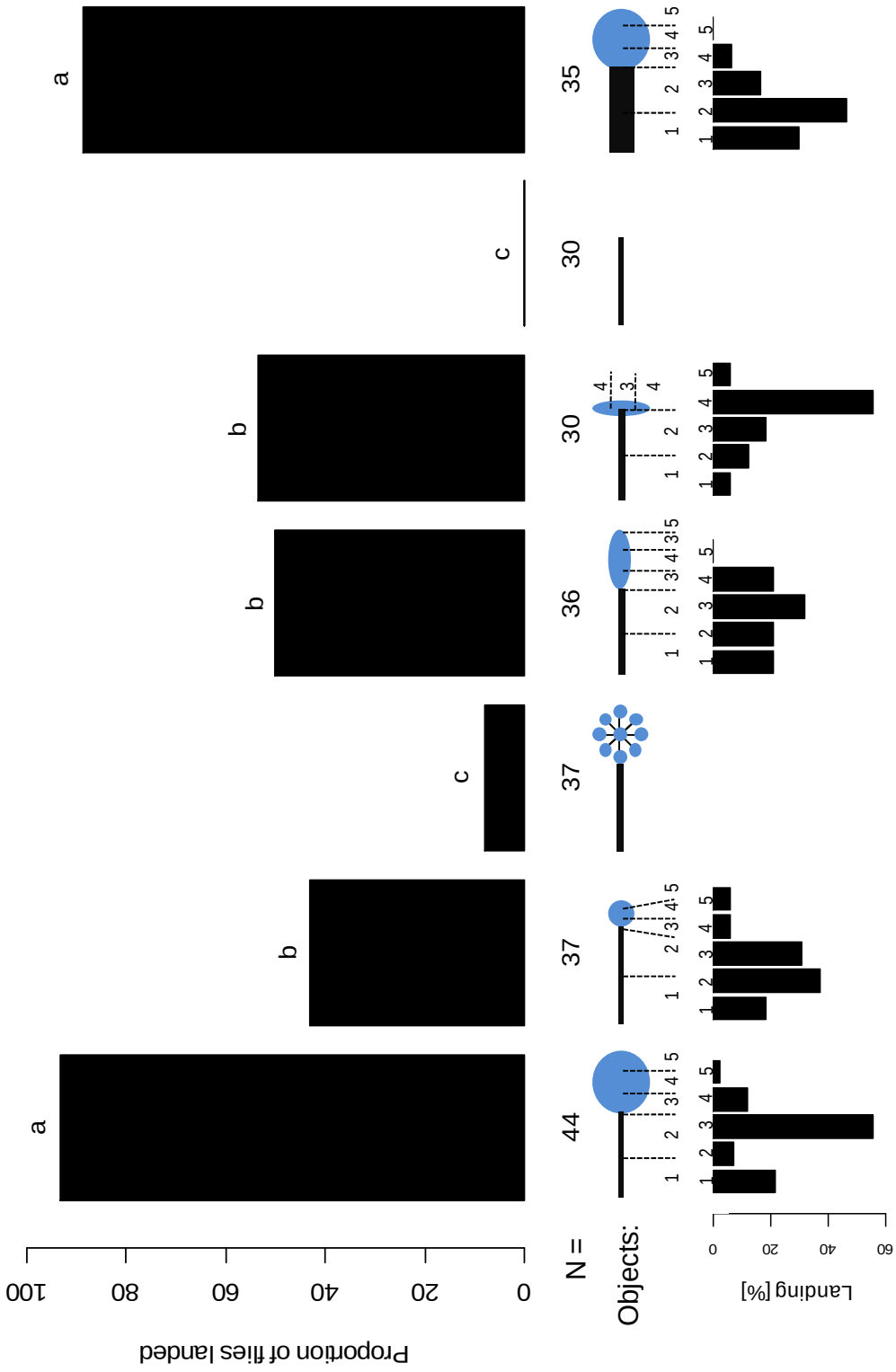

Figure 3.51: Landing response of *G .pallidipes* to objects differing in size and shape. Barplots at the top are the proportion of tsetse that landed at least once on the object. Different letters indicate that proportions are significantly different at P<0.05 according to a GLM with a logit link function. Barplots at the bottom show the preferred site for the first landing, designated by numbers on the object. For the vertical disc, the number 4 indicates the center, the number 3 the ring around the center and the number 5 the border of the disc. For the horizontal disc, the number 3 indicates the center of the lower side, the number 4 the ring around the center of the lower side, the number 5 (not indicated on the drawing) the upper face of the disc.

3.3.4.Analysis of the breath content

GC-EAG analysis revealed that *β*-caryophyllene and geranylacetone were present in both breath samples (Figure 3.52). I did not detect any flame ionization detector (FID) peaks corresponding to geranylacetone and β-caryophyllene neither in the extraction of air from the experimental room nor in purified air samples from Tedlar® bags. However, in the extract of air from Tedlar® bags a very weak EAG response at the retention time of geranylacetone but with no FID peak was detected (Figure 3.53). *β*-caryophyllene was already described as a tsetse attractant from rumen bolus (Harraca et al., 2009a) and found in extracts of *Lantana camara*, an invasive plant used by tsetse as a refuge (Syed and Guerin, 2004). To my knowledge, geranylacetone has never been described from human breath but it has been found in rumen bolus (Jeanbourquin and Guerin, 2007). Geranylacetone and *β*-caryophyllene both elicited EAG responses at low doses, comparable to those of 1-octen-3-ol, p-cresol, 3*n*-propyl-phenol (Figure 3.54). The latter three compounds elicit EAG responses (Bursell et al., 1988; Hall et al., 1984) and attract *G. pallidipes* in the field when combined with acetone (Torr et al., 2011). Geranylacetone has the trans-form of the double bond on the fifth carbon of 6,10-dimethyl-5,9-undecadien-2-one. I also tested the cis-isomer, nerylacetone. The retention time of nerylacetone was sufficiently distant from that of geranylacetone not to consider it as the compound in breath. Nerylacetone also elicited an EAG response but to a lower extent than geranylacetone (Figure 3.55). As shown by the GC trace in Figure 3.52, migration of compounds occurred from the Tedlar® bags into the purified air samples. These bags released several compounds, one of which is phenol that has been described in buffalo urine (Bursell et al., 1988; Vale et al., 1988). Phenol alone elicits EAG responses (Den Otter, 1991) but has no effect on the flight activity of *G. pallidipes* in the laboratory (Bursell et al., 1988) or on catches in the field when used to bait traps (Vale et al., 1988).

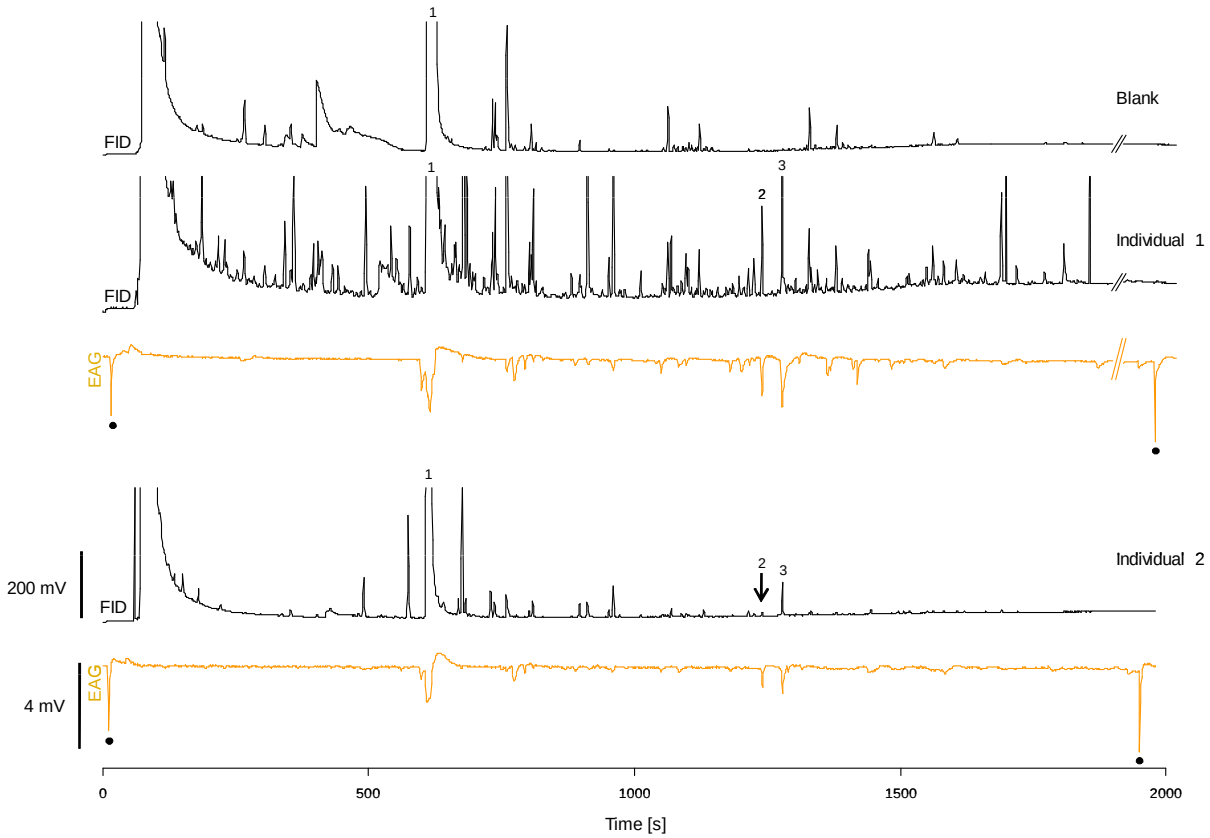

Figure 3.52: GC coupled EAG responses of *G. pallidipes* to breath extracts of a human male (individual 1) and female (individual 2) and a GC trace of an extract of air from Tedlar® bags (Blank). The GC traces are the upper ones (in black) and the lower ones are EAG responses (in orange). At the beginning and end of GC-EAG recordings, reference stimulations (marked by black dotes) were made with puffs of air from a filter paper strip with 1µg 1-octen-3-ol in a stimulus syringe. Identified peaks: 1 phenol, 2 β-caryophyllene, 3 geranylacetone.

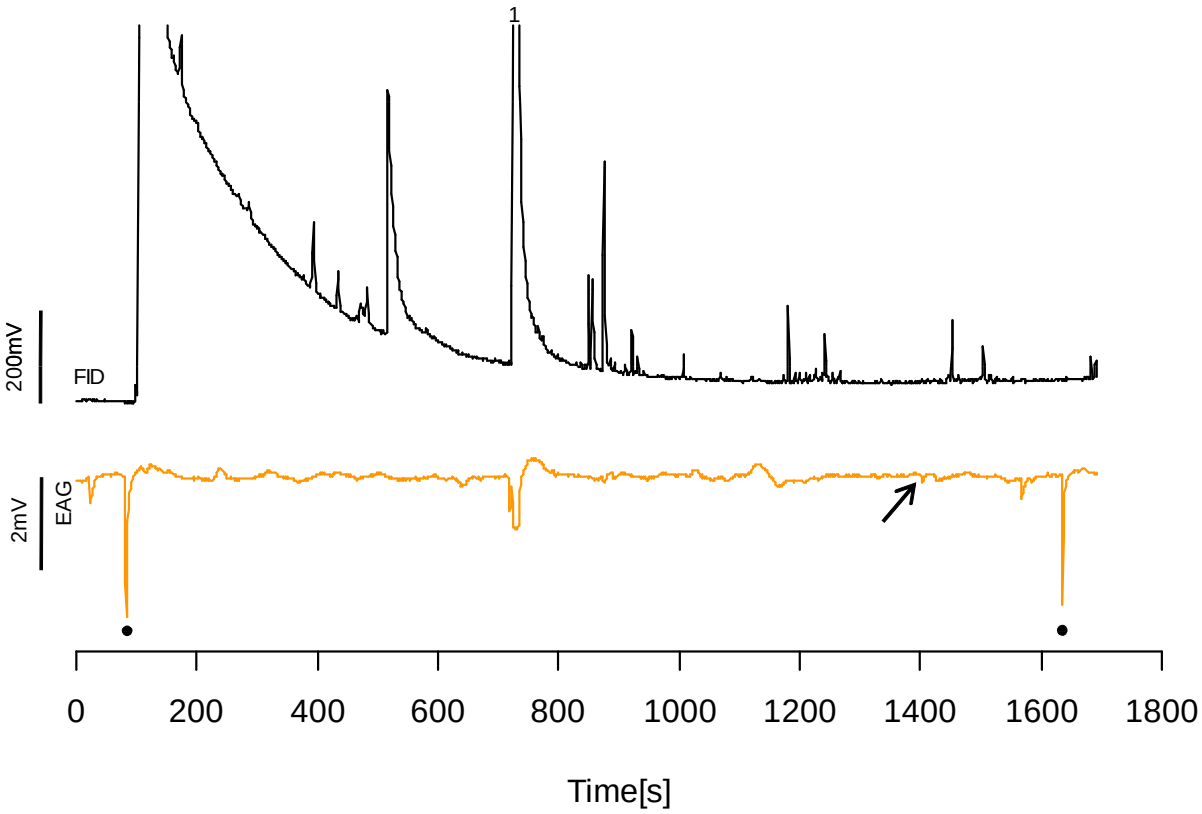

Figure 3.53: GC coupled EAG responses of *G. pallidipes* to an extract of air from a Tedlar® bags. The GC trace is the upper one (in black), the lower one is the EAG response (in orange). At the beginning and at the end of GC-EAG recordings, reference stimulations (marked by black dotes) were made with puffs of air from a filter paper strip with 1µg 1-octen-3-ol in a stimulus syringe. Identified peak: 1phenol. The arrow shows an EAG response to a chemostimulant which the retention time corresponding to that of geranylacetone but no FID peak was visible.

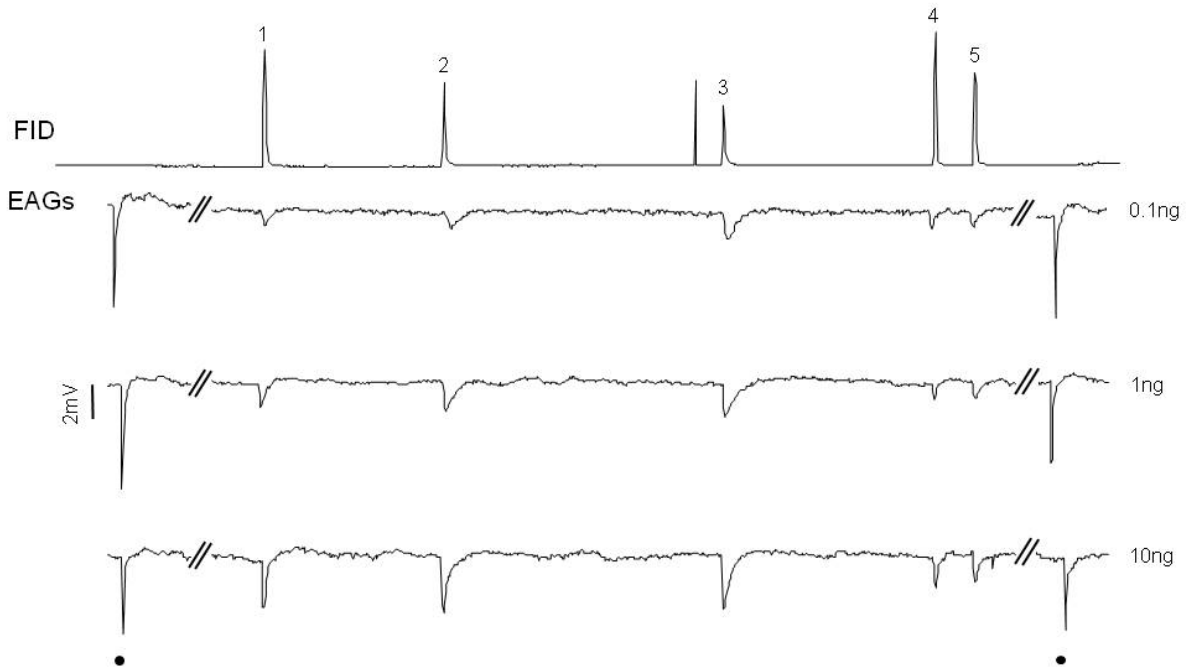

Figure 3.54: GC coupled EAG responses of *G. pallidipes* to different doses of a mixture composed of 1-octen-3-ol (1), p-cresol (2), 3n-propyl-phenol (3), β-caryophyllene (4) and geranylacetone (5). The response of the GC is the upper trace, and lower ones are EAG responses. At the beginning and at the end of GC-EAG recordings, reference stimulations (marked by black dotes) were made with puffs of air from a filter paper strip with 1µg 1-octen-3-ol in a stiumlus syringe.

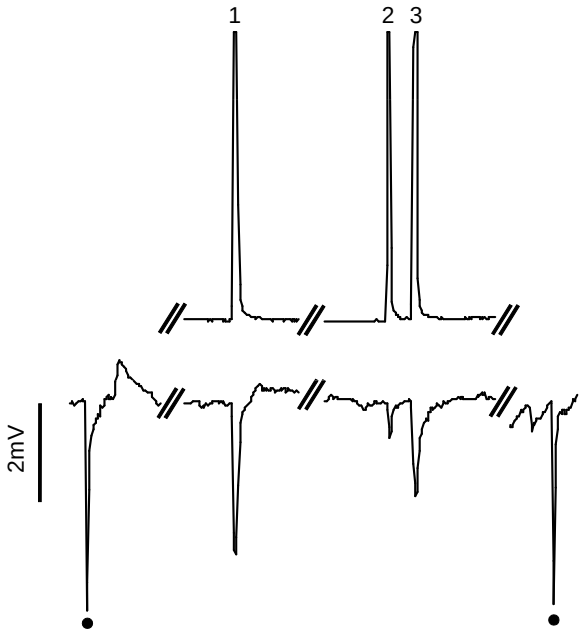

Figure 3.55: GC coupled EAG responses of *G. pallidipes* to a mixture composed of 33ng each of 1-octen-3-ol (1), nerylacetone (2) and geranylacetone (3). The response of the GC is the upper trace and the lower one is the EAG response. At the beginning and at the end of the GC-EAG recording reference stimulations (marked by black dotes) were made with puffs of air from a filter paper strip with 1µg 1-octen-3-ol in a stimulus syringe.

3.4. Discussion

3.4.1. Effect of breath and CO_2 on the recruitment of the target

I have demonstrated that flies exposed to the breath treatment were activated, exited the cage and flew up to the target in higher numbers compared to that of flies exposed to the CO_2 treatment. Moreover, the target on its own did not activate flies and it remained unattractive even for flies that flew out of the cage without any olfactory cues. This indicates that tsetse undertake investigations of a target in a precise context which was host seeking in my experiments and that a 2s pulse of breath is sufficient to solicit a visual response from the fly in such a context. So the response to visual stimulation is strongly influenced by olfactory cues as already established by Bursell (1990) and Warnes (1989). This is also consistent with the fact that baited targets or traps catch more flies than unbaited ones in the field (Green, 1993; Torr, 1989; Torr, 1990; Torr et al., 2011; Torr et al., 1995; Vale, 1974c; Vale, 1979; Vale, 1980; Vale and Hall, 1985b). Indeed, chemically- baited targets or traps recruit more flies as chemical stimulants activate resting flies and then elicit upwind flight to within the visual attraction range of devices. As demonstrated in *G. brevipalpis* by Gurba et al. (2012), *G. pallidipes* moves to a target surrounded by host odors in very directed flights at a high velocity and low curvature and then decelerates, making sharper turns as it approaches the target. The local search at the target is initiated by a decrease in velocity to permit sharper turns as proposed by Gibson et al. (1991). Although the relationship between velocity and curvature at a certain distance to the target is not altered by olfactory stimulation, flies stimulated with CO_2 had a lower average velocity and a higher average curvature than flies exposed to breath. This is consistent with Paynter and Brady (1996) who found that *G. morsitans* reduces speed when they are stimulated by CO_2. Warnes (1990a) showed that ox breath reduces flight speed while increasing doses of CO_2 reduces speed and increase tortuosity in *G. pallidipes*. The latter author suggested that velocity reduction with ox breath was due to its CO_2 content. I have found that CO_2 decreased velocity and increased curvature but I have also shown that flies stimulated with breath flew faster with a lower curvature than flies exposed to CO_2 at a level equal to that in breath. This

supports the view that breath contains VOCs that modify the response to CO_2 in terms of velocity and curvature.

3.4.2.Effect of breath and CO_2 on local search

Compared to CO_2, breath increased the distance covered and time spent flying around the target. These odour treatments modify the circling behaviour, named here local search, by changing the motivation of tsetse to search for the host: the more olfactory cues are pertinent the more tsetse increase the effort to find the host with the unrewarding target acting as a strong landmark here. The investigation of objects by circling them is a common feature in tsetse (Gibson et al., 1991; Green, 1986) and has led to the use of e-nets or adhesive tape devices besides targets or traps to evaluate the numbers of flies flying to the vicinity of visual objects (Mramba et al., 2013; Ndegwa and Mihok, 1999; Rayaisse et al., 2012; Torr et al., 2011). Host odours also affect the flight strategy during local search. The distribution pattern of blobs obtained with the continuous breath treatment is similar in shape to that obtained with the pulse of breath followed by CO_2 but tend to be extended with a bias in the upwind direction (wider distributed). This indicates that flies stimulated with breath extend into the limits of the wind tunnel in search of a host. The distribution pattern of blobs obtained with the pulse of breath showed a different shape than the two other treatments. Indeed flies responded to the visual stimulus alone with an investigation biased to the downwind side of the target. They also flew closer to the target with higher flight trajectories on the z-axis in the absence of odour whereas flies stimulated with the continuous breath treatment or with CO_2 flew on average lower on the z-axis. Tsetse fly upwind in a host odour plume and downwind outside the plume (Colvin et al., 1989; Gibson and Brady, 1988) and approach a visual target from below (Vale, 1982). Both visual stimuli and host odours are responsible for the behaviour. So tsetse are aware of the wind direction even near a strong visual stimulus and change the search strategy as a function of the presence or absence of olfactory cues. Taking into account that tsetse survive on a knife edge in the trade off between feeding and exposure to predation (Randolph et al., 1992) and that flight time in bursts last less than 5min (Brady, 1988; Bursell and Taylor, 1980; Williams, 1994) only pertinent olfactory cues could elicit such intense and wide

ranging local search. A similar phenomenon was described in *Anopheles gambiae*. In fact, the flight duration of this mosquito is increased when it is exposed to pertinent host cues such as heat and odours into a wind tunnel (Spitzen et al., 2013).

3.4.3.Breath and its constituents

Breath is a host effluent that is known to attract hematophageous arthropods such as tsetse (Bursell, 1990; Green, 1993; Warnes, 1989; Warnes, 1990a), mosquitoes (Gillies, 1980; Klun et al., 2013) and ticks (McMahon and Guerin, 2002). In the laboratory, human breath has already been described as an attractant for *G. brevipalpis* (Gurba et al., 2012) and for *G. pallidipes* (Joris, 2013). In the field, human breath was found attractive for *G. morsitans* and *G. pallidipes* (Vale, 1979). According to Vale (1979) CO_2 was probably the responsible agent for the attraction of tsetse to human breath. Indeed human breath was no longer attractive when it was filtered with soda lime, a mixture of Ca(OH), water, NaOH and KOH, that removes CO_2. It has been demonstrated in the laboratory that human breath was a better treatment than a similar dose of CO_2 alone to induce flight in *Stomoxys calcitrans* and to elicit upwind responses in this species (Warnes and Finlayson, 1985a; 1985b). Aspects of this phenomenon in tsetse were found by Gurba et al. (2012) who recorded that CO_2 activated *G. brevipalpis* but did not elicit upwind flights to the same extent as human breath. Moreover, Warnes and Finlayson (1985a) found that human breath filtered with sodalime and then mixed with CO_2 was not as effective to induce flight as untreated human breath, concluding that the filtration procedure does not just remove (or alter) CO_2 but also other compounds important for host detection and that compounds remaining after CO_2 removal can act synergistically with CO_2. Warnes and Finlayson (1985a) also argued that attraction is a complex behaviour and that each step must be considered to interpret the effect of compounds on the insects response. My results corroborate these findings and show further that CO_2 is not entirely responsible for the effect of human breath on tsetse. As no effect of the Tedlar bag manufacture products was found, it is reasonable to attribute the differences in responses to the breath and CO_2 treatments to compounds in human breath other than CO_2.

Human breath content has been widely investigated in order to develop diagnostic tools for detecting lung cancer or diabetes for example (Amann and Smith, 2005). Human breath is a complex mixture of CO_2 and about 200 different volatile organic compounds (VOCs) (Krotoszynski et al., 1977; Phillips et al., 1999). The origin of VOCs is divers arising from exchange between blood and air, as well as emissions from the walls of the respiratory system and from buccal and nasal bacteria. Odors from the upper part of the gastrointestinal tract could also contaminate breath through eructation or oesaphagus misclosing (Harraca et al., 2009a). Because of the tremendous area for exchange between air and blood in lungs, breath can be considered as a quasi-direct window into host metabolism that is exploited by tsetse flies to find a blood-meal. CO_2 is produced by the oxidation of sugar, lipids and proteins and is excreted in breath and is known to play an important role in the host seeking behaviour of hematophageous arthropods (see (Guerenstein and Hildebrand, 2008)) including tsetse. In the field, Vale and Hall (1985b) showed that catches of *G. pallidipes* and *G. morsitans* are positively correlated with the release rate of CO_2 used as bait. Torr (1990) also found that catches of *G. pallidipes* with targets are positively correlated with the dose of CO_2. In wind tunnel tests, CO_2 induces flight (activate) (Warnes, 1989) and elicits upwind anemotaxis in tsetse (Bursell, 1984a; Colvin et al., 1989; Evans and Gooding, 2002; Paynter and Brady, 1996).

Acetone

In addition to CO_2, human and bovine breath contains acetone (Phillips et al., 1999; Spinhirne et al., 2004) and my results have shown that the mixture of acetone and CO_2 increases activation and the number of flies that flew to the target compared to CO_2 alone. Moreover, I provide an indication that flies exposed to this mixture increased their efforts to find a host by flying around the target (Table 3.4). In mammals, acetone is mainly produced by the decarboxylation of acetoacetate during lipolysis or amino acid degradation (Kalapos, 2003). A part of the acetone is oxidized and this contributes to 1 to 2% of CO_2 production. It is also metabolized in glucose through acetyl-CoA and lactate. A part of acetone in blood is excreted in urine and in breath (Kalapos, 2003). The level of acetone in human breath is comparable to that found in bovine breath and can vary from 100ppb to just a couple of ppm (Amann and Smith, 2005; Elliott-Martin et

al., 1997; King et al., 2010; Mottram, 1997), a concentration range corresponding to that used in my wind tunnel experiment (0.36-18ppm). Torr (1990) and Vale and Hall (1985b) showed that *G. pallidipes* and *G. morsitans* catches are proportional to the dose of acetone alone released at a target or a trap, and Paynter and Brady (1993) demonstrated in the laboratory that acetone elicits upwind anemotaxis in *G. morsitans morsitans*. Acetone and CO_2 are always released together from the host suggesting that a mixture of CO_2 and acetone would be more efficient in catching flies than either alone. This was demonstrated by Torr (1990) and by Vale and Hall (1985b) who found a synergistic effect of acetone with CO_2 on catches in traps or on targets baited with a mixture of both. My results show that catches with this efficient mixture in the field are the result of the sum of the behaviours mentioned above. Indeed, this mixture increase the recruitment power of a visual target and it increased local search, *i. e.* the chances that a fly lands on a target or enters a trap is increased.

Table 3.4: Summary of experiment "Responses to synthetics" where fed and starved *G. pallidipes* were stimulated with mixtures of different compounds and 70ppm CO_2 except for EtOH* where the CO_2 level was 22ppm CO_2 above background. The upper part of the table summarizes the results obtained with tsetse stimulated with mixtures of CO_2 and pure compounds. Numbers in bold are significantly different from those obtained with CO_2 alone. Isoprene was diluted in acetone released at a dose corresponding to 0.36ppm and numbers in bold are significantly different from those obtained with the mixture of 0.36ppm acetone and CO_2. The breath treatment is compared with the effect of compounds with CO_2. The lower part of the table summarizes the results obtained with tsetse stimulated with mixtures of CO_2 and different doses of geranylacetone diluted in ethanol and numbers in bold are significantly different to those obtained with the ethanol-CO_2 mixture indicated by 0. Geranlyacetone was tested with fed and starved and teneral flies indicated as Geranyl1 and Geranyl2, respectively. Numbers in the center of columns are results pooled for males and females as no significant difference was found between the sexes: F female and M male. Act proportion of flies that were activated, exited the cage (Exit) and flew up to the target (Up).

Test	[ppm] [ppt]*	Act	Exit	Up	N	Distance [m]		Time [s]		Vel. [m/s]		N	
						F	M	F	M	F	M	F	M
CO2	80	39	31	25	153	7.0	9.2	5.0	7.2	1.3		21	18
Acetone	0.36	**57**	**51**	**49**	37	5.3	**16.6**	3.5	10.4	**1.6**		6	12
	1.81	**61**	**55**	**52**	44	6.1	9.0	4.2	6.3	1.5		11	12
	18.10	**61**	**61**	**53**	38	**11.8**	11.9	**7.8**	8.5	1.5		11	9
Isoprene	0.027	**86**	**78**	**73**	51	9.4	14.1	6.7	9.6	**1.4**		18	21
Heptane	0.091	**64**	44	40	50	10.1	**14.8**	6.9	10.0	1.4		7	13
	0.91	**75**	**66**	**63**	32	**11.0**	6.7	**8.0**	6.5	1.3		17	3
	9.1	**86**	**83**	**83**	35	**15.2**	11.5	**10.3**	8.6	1.4		19	10
EtOH	2.28	**69**	**60**	40	42	10.2		8.6		1.2		17	
EtOH*	2.28	**63**	**48**	35	40	7.3		5.7		1.3		14	
Hexane	1.03	40	34	26	47	7.3		5.9		1.2		12	
TBME		56	44	33	27	5.5		6.0		1.1		9	
Breath	80	96	93	90	98	18.2		12.3		1.4		88	
Geranyl[1]	0*	63	53	33	30	9.7		8.7		1.2		10	
	0.061*	65	50	45	20	8.9		6.7		1.3		9	
	6.06*	57	48	48	21	8.5		7.1		1.3		10	
	60.55*	82	64	64	11	5.4		4.1		1.5		7	
Geranyl[2]	0*	57	49	42	67	9.2	10.1	7.5	9.1	1.2	1.1	15	12
	0.00061*	56	51	51	45	**13.6**	11.6	10.5	10.0	1.3	1.1	10	13
	0.0061*	**79**	65	**63**	52	8.8	**14.8**	6.6	**13.0**	1.4	1.2	18	15
	0.061*	66	52	42	50	8.9	8.6	7.6	7.7	1.3	1.1	12	10
	0.61*	71	58	49	55	8.3	12.2	6.9	10.8	1.3	1.1	15	12
	6.06*	72	64	60	50	8.1	9.3	7.4	8.8	**1.1**	**1.1**	15	15

Isoprene

The addition of isoprene increased the recruitment to the visual target surrounded by the mixture of acetone and CO_2 (Table 3.4). Isoprene was tested at about 30ppb, a level similar to the 50-600ppb found in human breath (Karl et al., 2001; King et al., 2010). Isoprene is the major VOC in human breath (Gelmont et al., 1981; Krotoszynski

et al., 1977; Phillips et al., 1999) and originates from the mevalonic acid pathway of cholesterol and terpene synthesis (Deneris et al., 1984). Moreover, the level of isoprene in breath is closely related to that found in blood which is correlated with cholesterolgenesis (Karl et al., 2001). The bloodmeal is among others, a source of cholesterol, an essential sterol that insects are unable to synthetize themselves (Behmer and David Nes, 2003). However, isoprene is not produced just by mammals but also by plants (Sharkey et al., 2008). Isoprene alone is as such not a reliable host cue for hematophagous arthropods. Indeed no behavioural effect of isoprene alone was found for the the tick *Amblyoma variegatum* on a servosphere (McMahon and Guerin, 2002), a hematophageous arthropod that also responds to human breath. Tsetse can use isoprene in combination with CO_2 and acetone to locate a host for a bloodmeal. In another context, isoprene could also be used to locate a suitable habitat for shelter in the same fashion as beta-caryophylene that is released as a tsetse chemostimulant from plants (Syed and Guerin, 2004). Isoprene is also produced during the peroxidation of squalene (Stein and Mead, 1988), an ubiquituous vertebrate skin surface product.

Heptane

A mixture of heptane and CO_2 increased tsetse recruitment to the visual target and, as for acetone, local search at the target was also intensified (Table 3.4). Heptane is a VOC found in human effluents (Ellin et al., 1974), it is found in breath (Krotoszynski et al., 1977; Phillips et al., 2000a; Phillips et al., 1994; Phillips et al., 2000b; Phillips et al., 1999) and in skin emanations (Bernier et al., 2000). Heptane is also found in bovine breath (Spinhirne et al., 2004). Like the other alkanes it is produced by lipid peroxidation of polyunsaturated fatty acids (PUFAs) by reactive oxygen species (ROS), that damage cells membrane, a problem encountered in all aerobic organisms (Phillips et al., 2000a). According to Phillips et al. (1999), the emission of alkanes in the environment is the balance of the production and the clearance rate from the body. This balance is called the alveolar gradient and is estimated by subtracting the amount of alkanes in ambient air (inhaled air) and in exhaled air. In healthy people, the alveolar gradient for short chain hydrocarbons is negative indicating that the clearance by the organism is faster than production. This gradient approaches zero or becomes positive in older people as oxidative stress increases with the age (Droge and Schipper, 2007; Phillips et al.,

2000a; Phillips et al., 2000b). Contradictory data is found in studies by Phillips as a positive gradient was found for heptane in an earlier paper (Phillips et al., 1994) but a negative gradient in later papers (Phillips et al., 2000a; Phillips et al., 2000b; Phillips et al., 1999). This could be explained by the fact that Phillips experiments were conducted in Staten Island (NJ, USA), an urban and industrial site. It is known that urban sites are polluted with high concentrations of alcanes (Fraser et al., 1997) that could evidently influence alveolar gradients especially those that are close to 0. Moreover, the concentration of heptane in human breath is variable, situated between 0.12-4ppb (Krotoszynski et al., 1977; Poli et al., 2005), namely 758-23 times lower than the lowest concentration (91ppb) used in my experiments. Heptane is also found in bovine breath (Spinhirne et al., 2004) but no quantitative information is available. As mentioned above, heptane is found as a skin emanation resulting probably in a net increase of heptane surrounding the host. In my experimental conditions, females and males flew in the same manner in terms of velocity and curvature except that in the experiment “Pulse of breath” females flew significantly slower than males. I have no explanation for this.

Geranylacetone

I also found geranylacetone in human breath and this compound mixed with CO_2 increased the recruitment to the visual target of teneral flies and I have provided indications that it also increased the local search behaviour around the target (Table 3.4). The range of geranylacetone found in breath is about 0.035-0.35ppt independent of any pitfalls associated with the extraction method employed here such as breakthrough of the product on the adsorbent bed). Tsetse responded to an amount of 0.006ppt, a dose 5 times lower than that found in breath. Geranylacetone emanating from human skin is a chemostimulant for mosquitoes (Bernier et al., 2000; Dormont et al., 2013; Syed and Leal, 2009) and also a chemostimulant for *St. calcitrans* from bovine rumen (Jeanbourquin and Guerin, 2007). I have good reason to believe that bovine skin also releases geranylacetone as its production occurs from the degradation by ozone of squalene (Petrick and Dubowski, 2009; Wisthaler and Weschler, 2010), a fundamental lipid secreted onto the skin of mammals (Yoder et al., 1999). My results are in contrast with those of Vale et al. (2012) who demonstrated that geranylacetone added to the POCA bait reduced catches of *G. pallidipes* and *G. morsitans*. They

concluded that geranylacetone is a repellent but admitted that the dose used was far higher than the dose emitted by human skin. A proven example of differential response to different doses is that of tsetse to 1-octen-3-ol that is attractive at low doses and repellent at high doses (Vale and Hall, 1985a; Vale and Hall, 1985b). Moreover, it is known that the ratio between synthetic compounds in a blend is of major importance for their biological pertinence as insect attractants (Tasin et al., 2006). I showed that geranylacetone is attractive in the wind tunnel at low doses for teneral flies. Although no attractive effect was found for fed and starved females, I never observed a significant repellent effect at the doses tested (Table 3.4). The fact that fed and starved flies did not respond to geranylacetone in ethanol could be explained by the fact that responses to the mixture of ethanol and CO_2 was already too high, reducing the resolving power of the behavioural assay to a level to permit observation of the subtle effect of the addition of geranylacetone.

Other molecules

The addition to CO_2 of small molecules usually used as solvent such as ethanol or MtBE increased significantly the number of flies activated but not all flies activated flew to the target (Table 3.4). This means that on average the activated flies did not take off, flew downwind, or reached the ceiling before the target. These molecules are perceived by *G. pallidipes* but did not significantly affect the attractiveness of the target.

Male and female responses

In field and wind tunnel studies, no clear difference was found between males and females responses to host odours (Colvin et al., 1989; Harraca, 2008; Harraca et al., 2009a; Owaga et al., 1988b; Vale, 1974b; Vale and Hall, 1985a). I found significant differences between sexes in the velocity, time to activation and distance covered depending on treatments and feeding status. In fact, females were activated faster than males when exposed to human breath or to CO_2 alone, irrespective of whether they were fed or not before the test. This difference was not found with test compounds blends. Considering the average speed over the entire flight, teneral females clearly flew faster than teneral males when they were responding to human breath or to CO_2. This difference was also found for teneral flies exposed to geranylacetone. However,

both sexes of fed and starved flies responded with the same flight velocity to any treatment except in the experiment “The pulse of breath” where males flew faster than females when they approached the target. When flies were exposed to mixtures of CO_2 and different VOCs such as acetone and heptane, the highest doses of such VOCs produced a significant increase in distance flown by females compared to CO_2 alone whereas only the lowest doses produced significant results for males. When exposed to the geranylacetone treatment, teneral males flew a distance significantly higher than those exposed to the control to an amount of geranylacetone 10 times higher than for teneral females. I cannot give a satisfactory explanation for these findings.

3.4.4. Tsetse landing behaviour

The odour treatments did not affect the proportion of female *G. pallidipes* that landed on the blue sphere but did have an effect on males. In fact, for males the loss of the odour plume during the approach of the visual target reduced the power of the target to serve as landing stimulus. Bursell (1990) and Warnes (1989) demonstrated that host odours induce landing on targets. The target in my experiments was surrounded by CO_2 but at levels lower than those used by Bursell (1990) and no effect of CO_2 was detected on the landing behavior of females. In some studies in the field, POCA-baited targets caught less *G. pallidipes* males than females (Torr, 1989; Torr et al., 2011). Working on the assumption that males and females are equally attracted by host odours, my results could explain this difference in caches of males and females. As the olfactory baits are deployed near the ground, this implies that the target is not surrounded by odours in the field. Knowing that flies fly at about 30cm above the ground, they lose the odour plume when they approach the target leading to a reduction of 50% of males landing (according to my results).

Furthermore, the size of a visual target matters in eliciting landing in tsetse (Bursell, 1990). Using a black cloth flanking with an e-net, Torr et al. (2011) showed that the proportion of flies caught by the cloth targets decreases with size. This can explain why a smaller sphere induced less landing and that tsetse preferred to land on the big column compared to the narrower one. However, the size of an object may include more information than just how big its area is. Many factors intervene in the landing

behaviour of flies, rendering the situation much more complex. I showed that tsetse approach the sphere from below and so tsetse are exposed to a decrease in light intensity when they enter the shadow of the sphere. This could play a role in the landing behavior of tsetse as it has been shown that dimming the light intensity induces landing responses in *Musca domestica* and in *Lucilia sericata* (Braitenberg and Ferretti, 1966; Goodman, 1960). Moreover, it has been shown that approaching a black square on a white background or a white square on black background by a tethered *Calliphora erythrocephala* induces landing (Eckert and Hamdorf, 1980). The interface between black and white forms a contrast, a very important factor in the landing behavior of flies (Borst, 1990). When a black frame on a white background was presented to the tethered fly, it induced landing just as the squares did. The vertical component of the frame (moving in the horizontal plane) is the most relevant stimulus to elicit landing (Eckert and Hamdorf, 1980). However, vertical movements of horizontal strips are also important and elicit landing but only in the ascending direction by providing a descending illusion to the fly (Eckert, 1980). From the fly's point of view, the approaching frame produces a front to back movement over the visual field inducing landing and the inverse (back to front) inhibits landing (Borst, 1990). As a fly turns to the right for example, the right eye perceives a back to front movement whereas the left eye perceive the opposite movement. This, however, does not elicit landing, as both eyes need to perceive the same front to back movement to trigger landing behavior (Borst, 1990). The velocity of the movement produced by an approaching object in the visual field is a very important spatial and temporal component to elicit alighting. The front to back movement of objects must have an optimal velocity to induce efficiently landing (Borst, 1990; Eckert, 1980). With the assumption that a fly approaches targets of different size with the same speed, the borders of targets different in size travel over the visual field with different speeds. As an optimal velocity exists, an optimal size of targets should also exist. This could explain why *G. pallidipes* preferred to land on the big sphere when mounted on a thin column. The big sphere may provide important stimuli such as strong contrast, a reduction of light intensity and a front to back movement in every direction and at a suitable velocity to elicit landing. The thin column may not have been wide enough to produce a pair of stimuli travelling over the binocular visual field at

the required velocity. Indeed, when the bar was enlarged, tsetse preferred to land on it rather than on the sphere of the same size.

The 3 dimensional aspects of the spheres may also play a role as the number of flies that landed on objects with discs was half of that for objects with spheres. I have no explanation for this phenomenon. Intriguingly, flies tended to land on the outer ring of discs placed horizontally. A similar phenomenon was described in *Lucilia cuprina* that landed on a disc close to its edge when the disc was recognized by the fly as an object distinct from background (Kimmerle et al., 1996). Kimmerle et al. (1996) indeed showed that relative motion induced by Lucilia's own movement (that lacks stereopsis) serves as a cue to detect an object and land on it.

To assess more precisely the role of spheres as landing stimuli, further experiments are needed. In my experiments, the objects were surrounded by breath that may interfere with the response to visual information of the objects. Indeed, it has been demonstrated that CO_2 induces landing in tsetse (Bursell, 1990; Warnes, 1989). Although the CO_2 doses used by Bursell (1990) were far higher than ours, I cannot ignore its presence and that no olfactory stimuli interfere with the visual stimuli. The 2s pulse of breath could be used instead just to active the flies to fly to the visual object.

3.5. Conclusions

I have developed a new approach to determine in a wind tunnel the effect of host odour cues on crucial sequential behavioural steps such activation, flight to a target and local search in tsetse. This has allowed us to show that *G. pallidipes* can investigate an object smaller than a $1m^2$ target. Further, with the experiments on the landing behaviour I could show that *G. pallidipes* land on small objects such as blue spheres. So my setup provides a useful tool to improve targets as a landing stimulus for flies. I could also provide strong indications that a mixture of CO_2 and VOCs such as acetone, isoprene, heptane and geranylacetone partially explain the strong effect of breath on oriented flight responses of *G. pallidipes* and its local search behaviours at a visual target. These compounds merit further testing in the field to improve the performance of visual baits in the field.

4. Water vapour and heat combine to elicit biting and biting persistence in tsetse

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4.1. Introduction

Tsetse flies are obligate haematophagous insects that pierce the skin of the host with the haustellum to lacerate capillaries and suck blood. Tsetse are diurnal and their host-seeking behaviour is closely correlated to the activity of their hosts and predators (Gibson and Torr, 1999). Biting behaviour is the last step in host-seeking behaviour and represents a critical risk for tsetse. Indeed, upon landing on the host flies are exposed to predators and to the host's defence such as grooming and tail flicking (Randolph et al., 1992). Tsetse are k-strategists and are among the dipterans with the longest life span (Carey, 2001), that can extend up to several months in the field (Snow and Tarimo, 1985). Tsetse such as *G. pallidipes* feed every three to four days (Randolph et al., 1991; Turner, 1987). The trade-off between feeding and risk avoidance could explain the flies' strategy of infrequent host visits but feeding to repletion when they do (Randolph et al., 1992). As blood is not directly available to tsetse on landing, cues emanating from the skin stimulate tsetse to bite where they can readily find blood and thereby minimising the time spent on the host and so reduce risk. The heat of mammalian skin elicits biting in tsetse (Dethier, 1954) and in other ectoparasite arthropods (Aboul-Nasr and Erakey, 1967; Fresquet and Lazzari, 2011; Kröber and Guerin, 2007; Lall, 1969; Moskalyk and Friend, 1994; Sutcliffe and McIver, 1975). But heat is not the unique factor in eliciting biting as it is a multi-modal sensory response, *i.e.* diverse stimuli can affect this behaviour. Van Naters et al. (1998) showed that the combination of a chemical cue, such as uric acid, and heat increased the time spent probing by *G. fuscipes fuscipes*. Gatehouse (1967) also found that the probing

response of the stable fly *Stomoxys calcitrans* was multi-modal as an increase in humidity with the addition of ammonia induced more flies to probe than increases in humidity alone. Later, Gatehouse (1970a; Gatehouse, 1970b) illustrated the importance of hygroreception in the biting responses of this species by showing that increases in humidity were as efficient at inducing the biting response as heat alone or the combination of humidity plus heat. The importance of humidity in host-seeking behaviour in tsetse was underlined by experiments conducted in a wind tunnel by Evans and Gooding (2002). They showed that an increase in both moisture and air temperature is better at eliciting upwind flight to a source of CO_2 in *G. morsitans morsitans* than heated air alone. *Aedes aegypti* is also more attracted to combined heat and humidity increases than to heat alone in an olfactometer (Eiras and Jepson, 1994). Heat and moisture emanating from the host (McLean, 1963; Sokolov, 1982) are clearly important host cues for haematophageous insects.

To my knowledge, the interaction of heat and humidity has not been investigated in the biting behaviour of tsetse. To study this I built an experimental setup that allows us to quantify the biting response of *G. pallidipes* to an increase in temperature accompanied by increasing humidity or to increasing humidity accompanied by a temperature increase of a fraction of a degree. I demonstrate how combined temperature and humidity increases serve to influence the biting response, response latency, biting persistence and the dynamics of this fundamental behaviour in tsetse. In addition, I report on neurones with hygroreceptive properties present in basiconic sensilla on the maxillary palps of *G. pallidipes.*

4.2. Materials and Methods

4.2.1. Insects

G. pallidipes Austen, supplied by the International Atomic Energy Agency (IAEA) Siebersdorf Laboratories, Austria. The *G. pallidipes* colony originated from flies collected near Tororo, Uganda in 1975 that were reared successively at the University of Amsterdam, NL, the University of Bristol, UK, and at the IAEA-Siebersdorf Laboratories since 1986. Imagos were kept in 2 climate chambers offset by 2 hours with 10h light at 26°C, 85% relative humidity (RH) and 14h dark at 22°C, 85% RH. Sexes were separated at emergence. The behaviour of 3-day post-emergence unfed flies was observed during their two daily activity peaks in the first 1h 30 min and last 2 hours of the photophase. The response of each fly was only tested once.

4.2.2. Experimental set-up to measure tsetse biting

The biting responses of individual *G. pallidipes* to rapid changes in temperature and/or RH were measured by moving a fly between two vertical airflows in a cage mounted on the side of an index card drawer used as a sliding mechanism (Figure 4.1). Cages were made of 2 Plexiglas® cylinders that fitted one inside the other with one end covered with grey mosquito netting (polyethylene, 1mm mesh, bottom) and a nylon mesh (800µm, Sefar AG, Heiden, St-Gallen, Switzerland, top; Figure 4.1). The cage was suspended with the mosquito netting 12mm above the airflow (Figure 4.1). All flies were first exposed to an acclimatisation airflow that provided initial conditions at 24.5°C ± 0.1, 7.3% ± 0.8 RH (corresponding to a water partial pressure of 2.24hPa), similar across all experiments (see below for measuring devices). This charcoal-filtered air flowed at 40-44L/min through flow meters (Solartron Mobrey, Chanhassen, Minnesota, USA) and through two 11.5m long copper coils (8mm i.d.) immersed in a water-bath (LAUDA T, Lauda Dr. R. Wobser GmbH & Co, Lauda-Königshofen, Baden-Würtenberg, Germany; Figure 4.1). The copper coil outlets were joined in an insulated silicon tube to a 41mm diameter glass funnel with a nylon screen (40µm mesh, Sefar) to equalize air speed at its outlet (Figure 4.1). The funnel was mounted by a 35mm i.d. aluminium tube (40 o.d., 65mm long, Figure 4.1). The test airflow was produced by the apparatus described in

Taneja and Guerin (1995). Briefly, charcoal-cleaned air passed through two copper coils immersed in a water-bath. One flow was humidified in a saturation vessel filled with distilled water. Using flow controllers, the two flows were mixed at a defined ratio in a chamber whose outlet was connected via a water-jacketed Teflon® tube to a stainless-steel water-jacketed tube (35mm i.d., 255mm long; Figure 4.1). The desired RH was obtained by adjusting the flow rates of the humidified and dry flows at an air speed of 0.7m/s (Thermo-air anemometer, Schiltknecht Messtchnik, Gossau, Zürich, Switzerland). Airflow and ambient air temperatures were measured with 2 thermistor probes (Sable Systems, Las Vegas, Nevada, USA). RH of the test airflow was continuously recorded with a hygrometer (HMP50-60, Vaisala, Helsinki, Finland) and the same probe was used to measure that of the acclimatisation airflow each day before starting experiments. Thermistor probes and the hygrometer were connected to an interface (UI-2, Sable Systems) and signals were recorded with Expdata (version: 1.2.1, Sable Systems). An anemometer (Thermo-air anemometer, above) was placed 10-20mm above the centre of the cage to record the passage of the test airflow through it (Figure 4.1). Ambient temperature and RH in the experimental room were measured at 22.3°C ± 0.7 and 48.3% ± 2.2 during experiments.

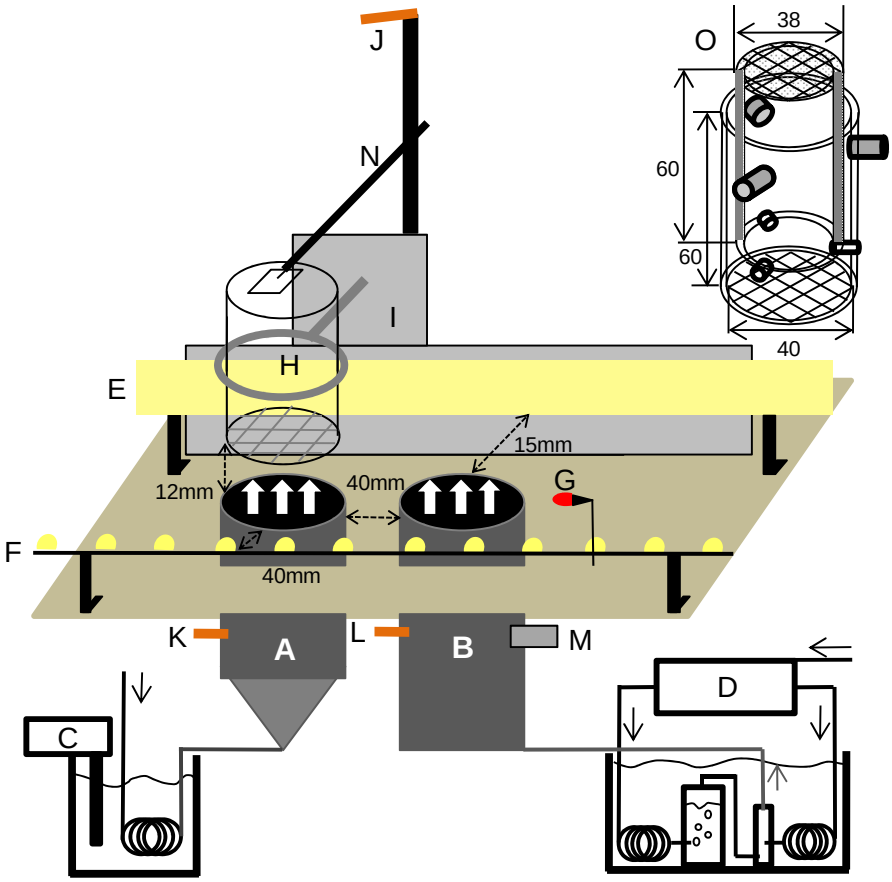

Figure 4.1: Experimental setup used to record the biting responses of *G. pallidipes* (not to scale). **A** acclimatisation airflow (the grey triangle symbolizes the funnel), **B** test airflow, **C** water-bath to control the temperature of the acclimatisation airflow, **D** apparatus to control the relative humidity and the temperature of the test airflow, **E** chain of power LEDs covered with opaque Plexiglas as background light, **F** chain of power LEDs to illuminate the underside of the cage, **G** red LED switched on by contact when the cage is in place on the test flow, **H** suspended cage, **I** reflective paper (grey colour), **J**, **K**, **L** thermistor probes measuring the ambient, acclimatisation and test airflow temperatures, the hygrometer **M** measuring the relative humidity of the test airflow and the anemometer **N**, **O** drawing of the insect cage in mm with a nylon mesh at the top of the inner tube and mosquito netting at the bottom of the outer tube. **H**, **I**, **J** and **N** were mounted on a sliding mechanism that allowed movement of the insect cage from the acclimatisation airflow to the test airflow.

Filming the biting response

The biting behaviour of *G. pallidipes* was recorded with two cameras (Full HD mod, Casio Exilim EX-ZR100, Shibuya, Tokyo, Japan) oriented to film the fly haustellum crossing the mosquito netting (Figure 4.1). Background was provided by retroreflective paper (Scotchlite 680-CR, 3M, Rüschlikon, Zürich, Switzerland; Figure 4.1) and by a string of LEDs (Power LED warm white, 30 spaced equidistant at15mm; Luniscontrol Gmbh, Lanzenhäusern, Bern, Switzerland) covered with an opaque sheet of Plexiglas® to equalize light intensity (Figure 4.1). Additional light was used to illuminate the lower

part of the cage with a string of similarly mounted LEDs tilted at an angle of 45° on the same side of the cameras. The fly holding cage closed a contact that caused a red LED to light up (visible on the video) as a record of the arrival of the cage over the test airflow (Figure 4.1). Light intensity was 1440lux at the cage lower screen.

Flies, in batches of 10, were placed individually in cages and held in the dark of the room where experiments were conducted. Timing was co-ordinated using a stopwatch. At time 0s, records of temperature, RH and wind speed was launched. At 5s the first camera began filming and at 15s the cage was moved into the acclimatisation airflow for 120s. At 125s, the second camera started to film and at 135s the cage was moved into the test airflow to expose the fly for 120s to the test conditions listed in Table 4.1.

Table 4.1: Temperature, RH and corresponding partial pressure of water vapour differences in the air streams.Values are means ± SD for the physical parameters; number of flies of each sex tested with each treatment is also provided. Water vapour partial pressure was calculated for treatments by multiplying the RH by the saturation pressure (Buck, 1981) at an atmospheric pressure of 951hPa.

Temperature increase [°C]	RH increase [%]	Partial pressure of water vapor increase [hPa]	Number of flies tested		
			Male	Female	Total
0.2 ± 0.2	1.1 ± 0.5	0.4 ± 0.2	20	21	41
	10.8 ± 1.1	3.4 ± 0.3	19	20	39
	60.6 ± 0.4	18.0 ± 0.2	30	20	50
	75.4 ± 0.7	23.5 ± 0.5	20	20	40
3.0 ± 0.2	1.4 ± 0.04	1.0 ± 0.02	0	10	10
	11.1 ± 0.3	4.5 ± 0.1	0	10	10
	60.0 ± 0.4	22.4 ± 0.2	0	10	10
13.1 ± 0.1	0.2 ± 0.2	2.7 ± 0.2	14	20	34
	10.5 ± 0.4	9.4 ± 0.3	20	28	48
	34.5 ± 0.6	24.7 ± 0.3	20	20	40
	60.0 ± 0.6	41.8 ± 0.5	20	20	40
	72.4 ± 1.3	49.8 ± 0.8	21	18	39

Analysis of the biting responses

To quantify biting, a bite was defined as the act of passing the haustellum through the netting. No substrate was supplied beneath the mosquito netting, and flies were only exposed to the temperature and humidity stimuli provided in the air streams. The time the red LED switched on and each time the haustellum crossed the netting and was withdrawn, was recorded with the video analysis programme Kinovea (version: 0.8.14) .

For statistical analysis, the program R (version: 2.15.0) was used and the level of significance was set at 0.05.

Proportions of responding flies were analyzed using a generalized linear model (GLM) with a logit link function. Latency was measured as the time between the moment the cage arrived in the test flow (red light on) and the onset of the first bite. To determine if a treatment increased or reduced latency, Cox proportional hazards regression was used with the R package *survival* to allow accounting for flies that did not respond as censored data.

As flies bit successively, the number of bites was log transformed and analysed by a factorial ANOVA, following a test for homogeneity of variance with a Bartlett test. Multi comparisons between treatments were made with Tukey's Honest Significant Difference test (Tukey HSD). Data on time spent biting, average time per bite and average time between successive bites were analysed with a GLM with a reciprocal link function (Gamma family). Where necessary, multi comparison tests were done with a GLHT function depending on the model used (below) with a Tukey matrix of contrast (R-package: multcomp). As the number of responding flies exposed to an increase of 0.2% RH and 13.1°C was low (5 over 34 individuals tested), these data were pooled with data obtained for an increment of 10.5% RH at the same temperature increase. No significant difference in deviance was observed by pooling these two categories (ANOVA). Each step in reducing the Cox model and GLM was controlled by a deviance analysis following a X^2 distribution (ANOVA). Temperature, RH, partial pressure and saturation pressure of water for experiments were analysed with Expdata (version: 1.2.1, Sable Systems) and are shown in Table 4.1.

4.2.3.Electron microscopy

Sample preparations for scanning and transmission electron microscopy were made following protocols described in Kessler et al. (2013). For scanning electron microscopy, excised heads of *G. pallidipes* were fixed in 70% ethanol, dehydrated gradually in acetone and desiccated by critical point drying using CO_2 (Bal-Tec CPD 030, Balzers, Liechtenstein). For transmission electron microscopy, whole heads of female and male *G. pallidipes* were immersed in Karnovsky fixative (pH 7.4) at a sucrose concentration

of 4% and 10% to test if swelling of outer dendrites was not due to a hypotonic effect. The palps of 12 female and two male *G. pallidipes* were serially cut in cross and longitudinal section. Thin sections (100 nm) were collected every 2 µm over 20 µm. For four females, thin sagittal sections of the basal parts of sensilla across the axis of the palp (from the ventral to the dorsal side) were examined over 4 µm along the lateral external palpal groove (Figure 4.2). To determine the arrangement of dendrites, eight basiconic sensilla of four flies were cut serially in 100nm thick sections and every section was examined from half way along the sensillum to the ciliary roots.

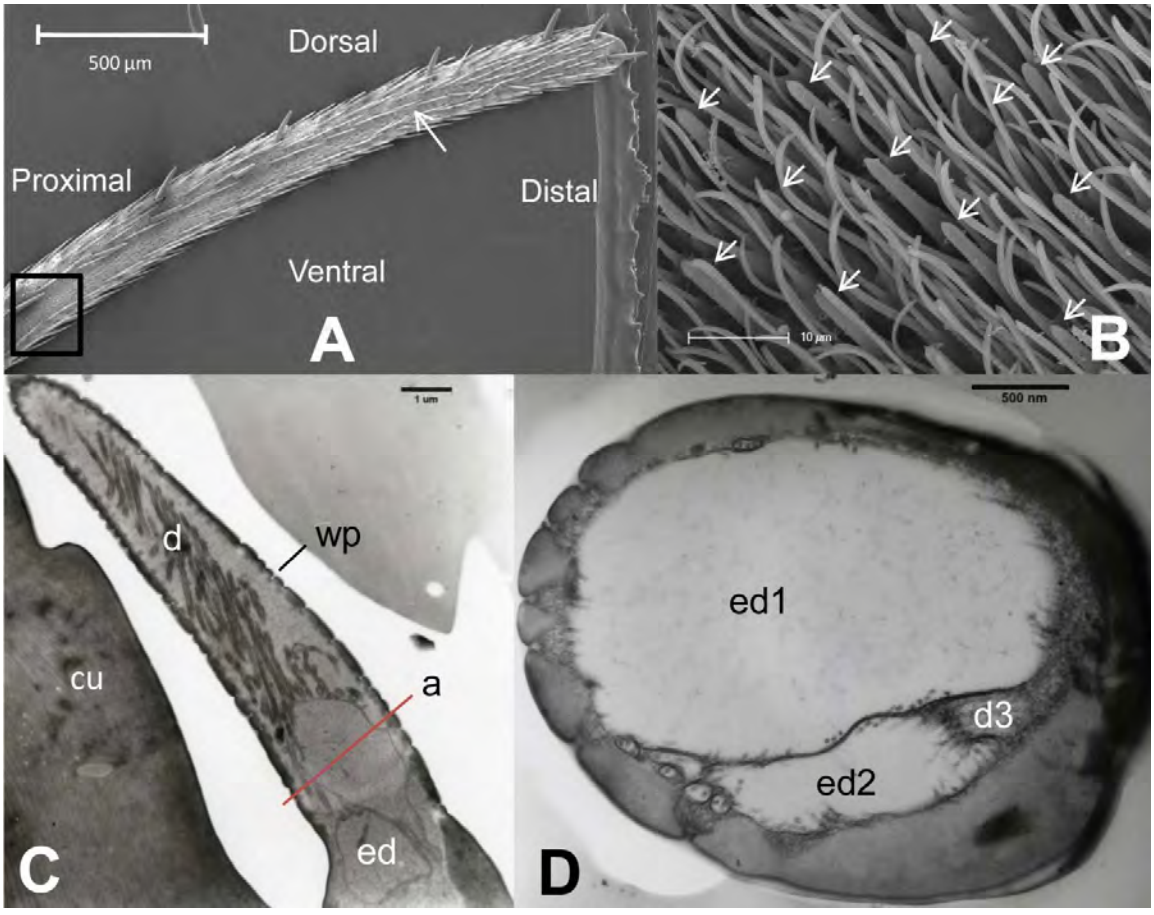

Figure 4.2: Micrographs showing the location and the fine structure of basiconic sensilla on *G. pallidipes* palps. **A** Image of the right palp with basoconic sensilla along the lateral groove delimited between the inside of the box proximally and the white arrow distally. **B** Magnified proximal zone (box in A) showing numerous basiconic sensilla (arrows) surrounded by microtrichae. **C** Longitudinal section of a sensillum with a thin porous wall (wp), ramified dendrites (d), two enlarged dendrites (ed) at the base and cuticle (cu). **D** Cross section at level a in C showing two enlarged dendrites (ed1 and ed2) and the third unbranched dendrite (d3). The scale bar is 500, 10, 1µm and 500nm for A, B, C, D, respectively.

4.2.4.Electrophysiological recording from palp sensilla

A modified version of the set up used by Taneja and Guerin (1997) was used to record action potentials from receptor cells within basiconic sensilla on the palps of *G. pallidipes*. After anesthesia with CO_2, the fly was immobilized in a disposable micropipette tip (Kartell Spa, Noviglio, Lombardia, Italy) cut at the tip and held horizontally with the head protruding at the narrow end. The disengaged haustellum was attached to the tip with sticky tape. A V-shaped silver reference electrode was placed between the protruding head and the end of the micropipette tip. Mouthparts, except the maxillary palps, and eyes were embedded in dental cement (Ketac™ Cem radiopaque, 3M ESPE, Seefeld, Bayern, Germany) to prevent movement of the head. A 0.1mm diam. tungsten recording electrode sharpened by electro polishing in saturated KNO_2 solution was placed randomly in the proximal 2mm of the lateral external palp groove (Figure 4.2) and connected, in succession, to a high impedance preamplifier (x10; Syntech, Kirchzarten, Baden-Württemberg, Germany), an AC/DC amplifier (UN-03, Syntech) and the intelligent data acquisition controller (IDAC) analog-digital converter (USB-IDAC box, Syntech) to a computer. Spikes were recorded with Autospike (Syntech). Spikes were considered when the amplitude was greater than 1.9 - 2 times the standard deviation of the entire signal amplitude. The number of spikes counted for the first 2s of stimulation was divided by 2 to estimate the spike frequency in Hz. Statistical analyses of spike frequencies were performed by ANOVA following a test for homogeneity of variance with a Bartlett test and multi comparisons between treatments were made with Tukey's Honest Significant Difference test.

To test the responses of neurones to changes in RH, palps were exposed to five RH drops from 100% (-0.02% ± 0.3; -26.4% ± 1.7; -44.1% ± 4.0; -69.4% ± 4.8; -91.1% ± 3.4), obtained by mixing moist and dry air at different proportions with a stimulator. For this, two airflows were mixed by manually adjusting two flow controllers (C05K510418, Norgren, Lichfield, Staffordshire, United Kingdom) for a desired moist to dry air ratio. The flow controllers mounted on solenoid valves (RPE 3105 NC 230V/AC, RPE S.r.l., 22070 Carbonate, Lombardia – CO – Como, Italy) were arranged in six pairs providing six channels triggered by an external electronic control that allowed the manual

selection of the desired RH shift. Moist and dry airflows were produced by splitting charcoal-filtered air at 0.4 bar into two channels isolated from each other with non-return valves (T51P0008, Norgren). The moist channel was first humidified by bubbling it through a 2L gas-wash bottle filled with distilled water immersed in a water-bath at 35°C. This humidified air was cooled to room temperature (25°C) by passing it through a copper coil before bubbling it through a second glass bottle (1L) filled with distilled water immersed in a second water-bath at room temperature. The dry channel air passed through copper tubes immersed in the same water-baths as the moist airflow. Stimulation with RH drops was made by simultaneously closing the first water-saturated channel and opening one of the five other channels for 8s. At the output, RH of the airflow was measured with a hygrometer (HMP50-60, above). The air speed at the output of the stimulator was measured with an anemometer (Thermo-air anemometer, above) and set at 1m/s. Temperature of the output airflow was measured daily with a thermistor (PT-6 Physitemp, Science products GmbH, Hofheim, Hessen, Germany) at 25.4°C ± 0.2 for the water-saturated flow and 25.0°C ± 0.4 for the dry flow (8.9% RH).

4.3. Results

4.3.1. The biting response

Biting responses to a temperature increment of 13.1°C and varying RH increments

An increase of 13.1°C brought the air stream temperature near that of the host. Under these conditions the proportion of flies that bit at least once increased significantly (GLM, $P<0.001$) with increasing RH increments to reach 94.9% for an increment of 72.3% (Figure 4.3A). Latency of flies exposed to RH increments of 10.5%, 34.5%, 60% and 72.4% was, respectively, 2.6, 5.1, 12.8, 24.3 times shorter than flies exposed to the minimal RH increment of 0.2% (Figure 4.3B). The unique difference found between males and females was in latency as males responded 1.7 times faster than females (significant intercept of the Cox regression for the sex variable, $P<0.01$). The number of bites increased significantly (ANOVA, $F_{3,113}=8.689$, $P<0.001$) with increasing RH increments (Figure 4.4). Increments above 34.5% RH significantly affected the number of bites but increments below 34.5% RH did not, *i.e.* flies exposed to the RH increase of 72.4% bit 26 times (detransformed mean), 3.3 times more than flies exposed to a RH increase of 34.5% (detransformed mean of 8 times, Figure 4.4).

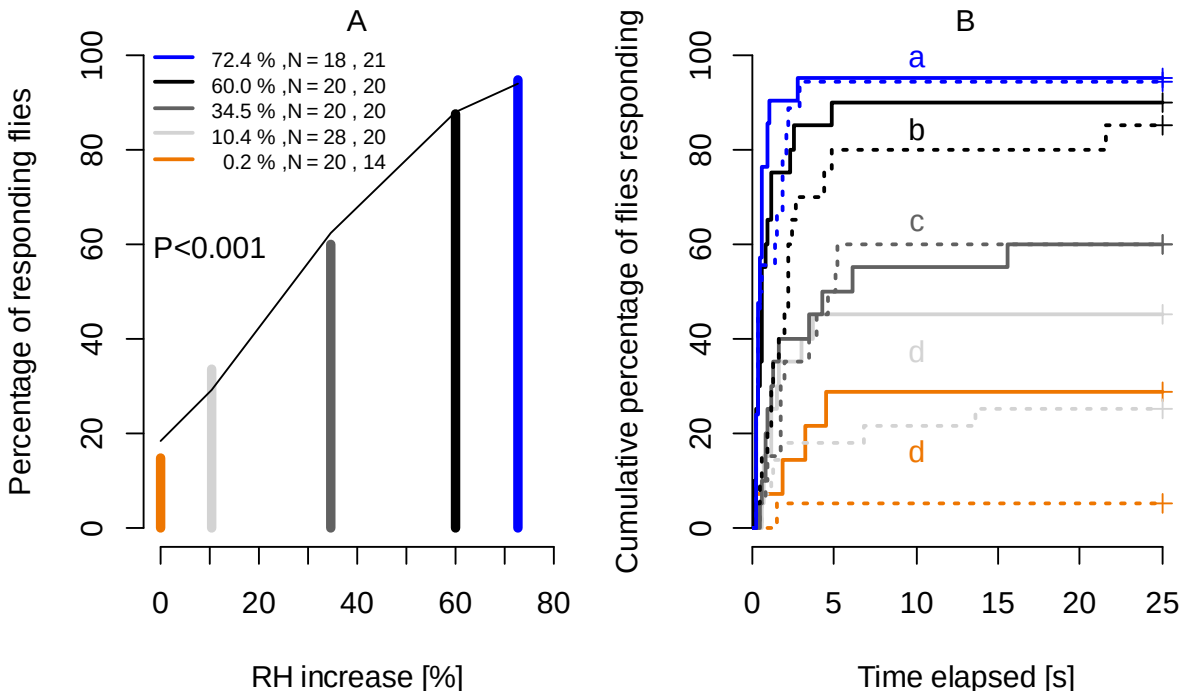

Figure 4.3: Proportion of flies biting and response latency for increases in RH at a 13.1°C air temperature increase. **A** Proportion of flies that bite at least once. Line shows the predicted values from the GLM model. **B** Proportion of flies biting as a function of the response latency. Recording time was 120s but only the first 25s is presented as latency did not exceed this. Curves with different letters are significantly different. Solid lines are for males and dotted lines are for females.

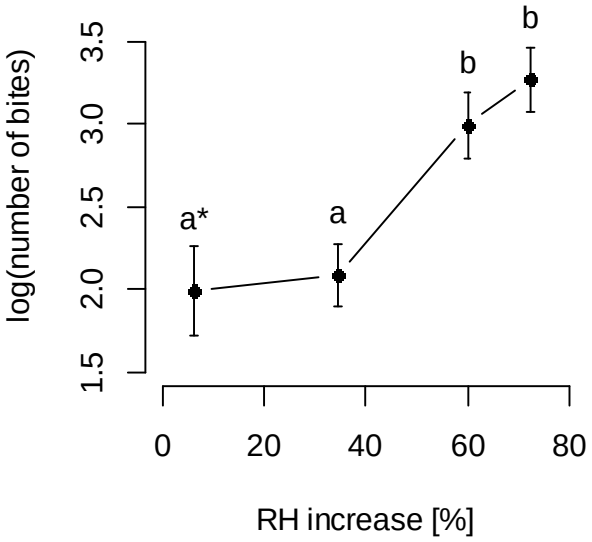

Figure 4.4: Number of bites per fly at increasing RH increments with a 13.1°C air temperature increase. Log transformed mean number of bites (± SEM) with different letters are significantly different following the Tukey HSD post-hoc test. As the number of flies responding to an increment in RH of 0.19% was very low (5 over 34 individuals tested) this data was pooled with responses obtained for an increment of 10.45% (asterisk).

The time spent biting was positively correlated with increasing RH (Figure 4.5A). Only the highest RH increments significantly increased the biting time, compared to the lowest increase. The ratio between the time spent biting by flies exposed to RH increments of 72.4% and 34.5% is 2.1, lower than the ratio obtained for the number of bites for these two treatments (see above). This indicates a change in either average time between successive bites or in the average time of a bite between the two RH levels. In fact the average time per bite was significantly shorter for flies exposed to 72.4% than flies exposed to 34.5% RH (Figure 4.5B). No significant difference was found between interbite intervals as a function of humidity (medians of 0.43s, 0.39s, 0.35s, 0.4s for RH increments of 6.2%, 34.5%, 60%, 72.4%, respectively; GLM, P=0.6675). The highest mean frequency of biting at 8.45 per 5s was recorded for the highest RH increment (Figure 4.6).

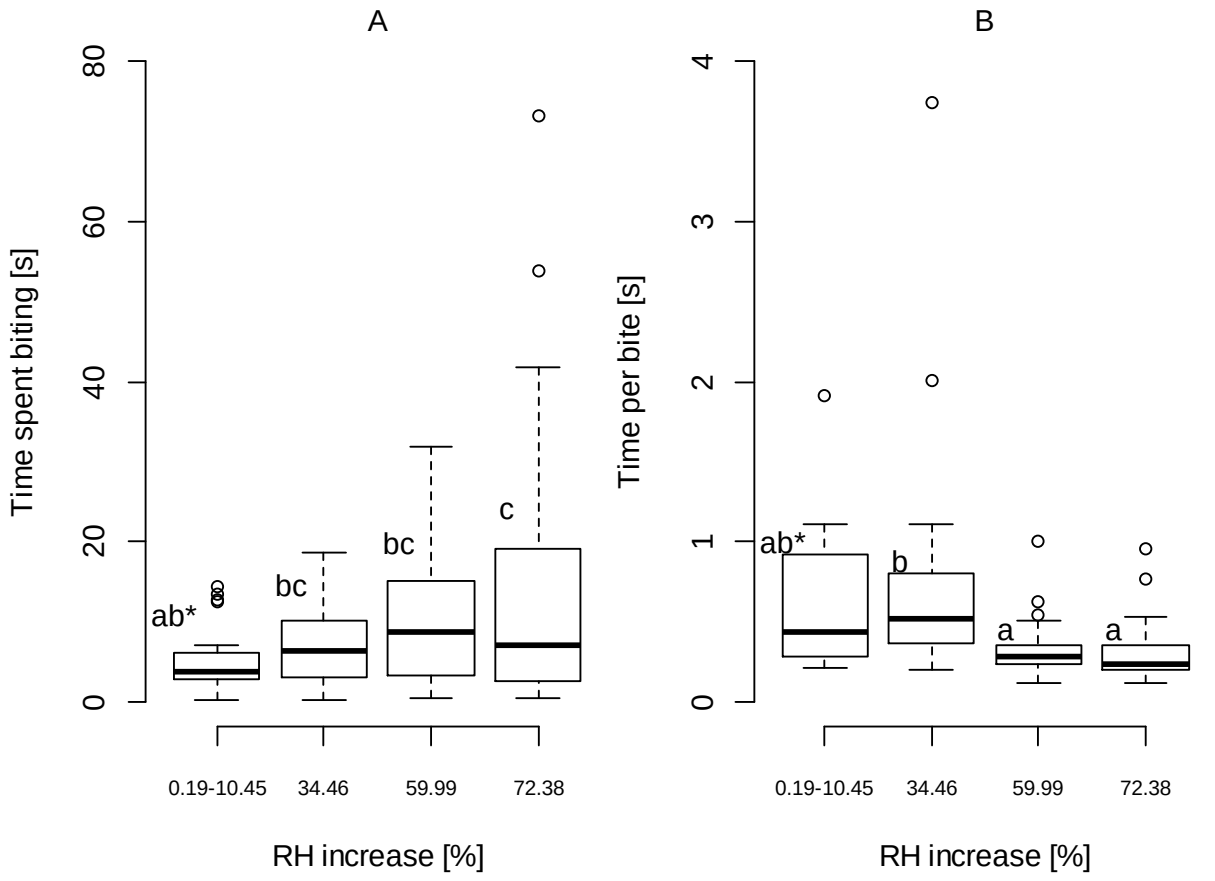

Figure 4.5: Time spent biting (A) and average time per bite (B) at increasing RH. The temperature increment was 13.1°C. Boxplots with different letters are significantly different according to a post-hoc test following a GLM with a reciprocal link function (Gamma distribution) with a Tukey contrast matrix. For time spent biting at increments of 0.2% and 10.5% RH see legend to Figure 4.4. In B, one point is not shown (at 34.5%, 10.2s) as the y-axis was limited to 4s for purposes of readability.

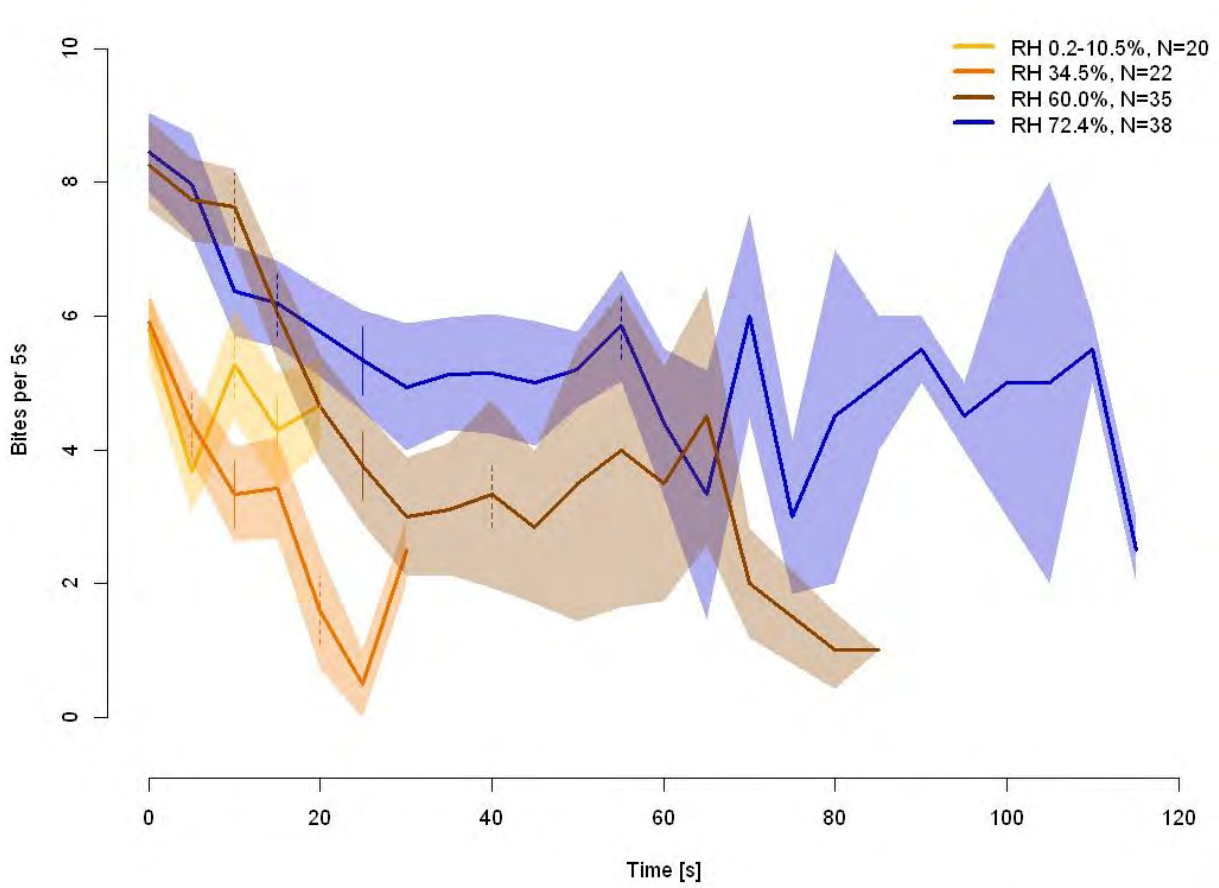

Figure 4.6: Biting frequency dynamics over time for RH increments with a 13.1°C air temperature increase. The frequency was calculated for each RH increment (different colours) as the number of bites per 5s and presented as the average biting frequency (bold lines) ± SEM (shaded areas). Time 0 is the onset of the first bite. All flies did not bite for 120s so medians (vertical solid lines) for the time spent biting by flies are drawn for each RH increment; vertical dashed lines to the left of the median represent the first quartile and to the right the third quartile.

All parameters used above to describe the biting behaviour of *G. pallidipes* do not take into account the fact that the biting response occurs over time. It appears that flies did not bite with the regular frequency of a standing wave (Figure 4.6). In the first 5 seconds the frequency was at its maximum: 5.8, 5.9, 8.3, 8.4 bites per 5s, respectively, at RH increments of 0.2-10.5%, 34.5%, 60% and 72.4% and then biting frequency decreased. This drop in biting frequency was related to RH and was highest for the 34.5% RH increase and lowest for the 72.3% RH increase. This resulted in biting termination at 30s for the 22 responding flies at the 34.5% RH increase, at 85s for 35 responding flies at the 60% RH increase, and one fly was still biting at 120s of the 38 responding flies at the 72.4% RH increase (Figure 4.6).

Biting responses to temperature increments of 0.2°C and 3°C and varying RH increments

When the temperature shift was minimized to 0.2°C, RH increments of any size did not significantly (GLM, P=0.092) increase the proportion of flies biting: of the tested flies (Table 4.1), 0, 0, 10 and 2.5% responded to the RH increments of 1.1, 10.8, 60.6, 75.4%, respectively. Furthermore, when females were exposed to an increase of 3°C accompanying RH increases of 1.4, 11 and 60% (Table 4.1) only one of 10 females responded to the 11% RH increase and the other RH increases failed to elicit biting.

Biting responses to minimal RH increments

When RH increases were minimized to less than 1.5%, only a temperature increase of 13.1°C succeeded in eliciting biting: 14.7% of the 34 flies tested responded (Figure 4.3A). However, this comparison needs to be qualified: RH increases at different temperatures do not produce the same increase of water vapour pressure in air (Table 4.1). An increase of 13.1°C and 0.2% RH results in water vapour pressure of 2.7 hPa, roughly the same order as produced by an increase of 0.2°C with a 10.8% RH increase (3.4 hPa) or by a 3°C increase with a 11.1% RH increase (4.5 hPa). Nevertheless, with similar minimal water vapour increases of between 2.7 to 4.5 hPa none of the 39 flies responded to an increase of 0.2°C and only one female out of 10 responded to a 3°C increase.

Whereas RH increases accompanying minimal temperature increases or temperature increases accompanying minimal RH increases succeeded in inducing biting in no more than 14.7% of flies, the proportion of responding flies reached nearly 95% when both temperature and humidity were increased by 13.1°C and 72.4% RH (Figure 4.3A). This indicates a strong synergism between RH and temperature.

4.3.2.Neuroanatomy of palp basiconic sensilla

On the lateral side of each palp at approximately 800µm from the tip, a ventro-lateral field of wall-pore sensilla extends over a distance of 1200µm and 30µm in width (Figure 4.2A & B). These hairs are wall-pore single-walled (wpsw) basiconic sensilla (Figure 4.2C). They are short (10-15 µm in length) with a diameter of 1.6 µm in the middle and 2.6 µm at the base (Figure 4.2C). The cuticular apparatus is a thin single wall (100 nm

thick) pierced by numerous pores (30-40 nm in diameter) with pore-tubules extending into the lumen shaft. The lymphatic space (ls) of the sensillum contains numerous dendritic branches (Figure 4.2C). The ls contains few or more dendritic branches (up to 72) and can be filled with filaments which make it electron dense.

At the base of the sensillum three sensory cells are associated with three enveloping cells (thecogen, tricogen and tormogen). From the thecogen cell a short sheath emerges surrounding the outer dendritic segments. To describe the fine structure of the outer dendritic segments and their branching pattern, all of the 56 sensilla examined (18 flies) presented two swollen dendritic segments at the base of the sensilla (Figure 4.2C & D). The swelling of the outer dendrites is not due to an uptake of water driven by a hypotonic fixing solution, as the same swelling was also found using a hypertonic fixative.

4.3.3.Electrophysiology responses of dry cells

Neuronal responses to RH decreases were recorded from 17 wpsw basiconic sensilla of four male and one female *G. pallidipes*. In all of the 17 sensilla tested, the spike frequency of neurones increased by at least a factor of 1.5 when RH was decreased. Spike frequency increased significantly with RH drops (ANOVA, $F_{4,81}$=12.86, P<0.001; Figure 4.7A). The spike frequency reached a plateau between a drop of 44% and 69% RH and decreased significantly for a drop of 91% RH (Figure 4.7A). The response of neurones to RH drops of 44% showed a phasic-tonic response pattern (Figure 4.7B): the spike frequency increased from 30Hz to 67Hz in the first second of stimulation and then decreased to 45Hz but neurones did not completely adapt to the 8s RH drop (Figure 4.7B). After stimulation, when the RH of the airflow returned to the 100% RH level, the spike frequency decreased to the pre-stimulation level (Figure 4.7B). An example of the responses recorded from a sensillum housing a dry cell to RH drops of 0.1%, 27.4% and 41.4% is provided (Figure 4.8). During preliminary experiments, only four sensilla of 60 tested presented a neurone that responded to an increase in RH (Figure 4.9).

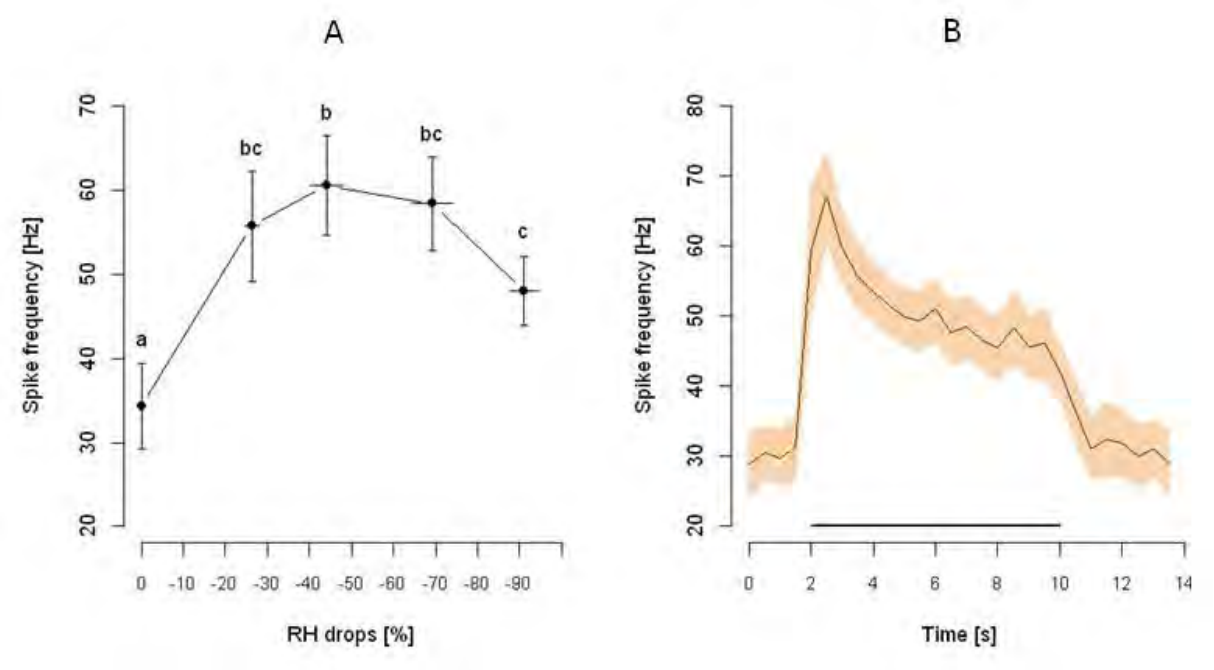

Figure 4.7: Neuronal responses in *G. pallidipes* palp basiconic sensilla to RH decreases in the air. **A** Mean neuronal responses (± SEM) recorded from 17 basiconic sensilla on 1 female and 4 male *G. pallidipes* to RH decreases (from 100%). Spike frequencies with different letters are significantly different. **B** Mean responses of neurones in the same 17 sensilla (± SEM, shaded area) to a RH decrease of -44.1% for 8s (bold black bar). Responses of the neurones to an increase of RH were also recorded as the end of stimulations when the RH increased. The spike frequency is the integration of the number of spike over 500ms bins.

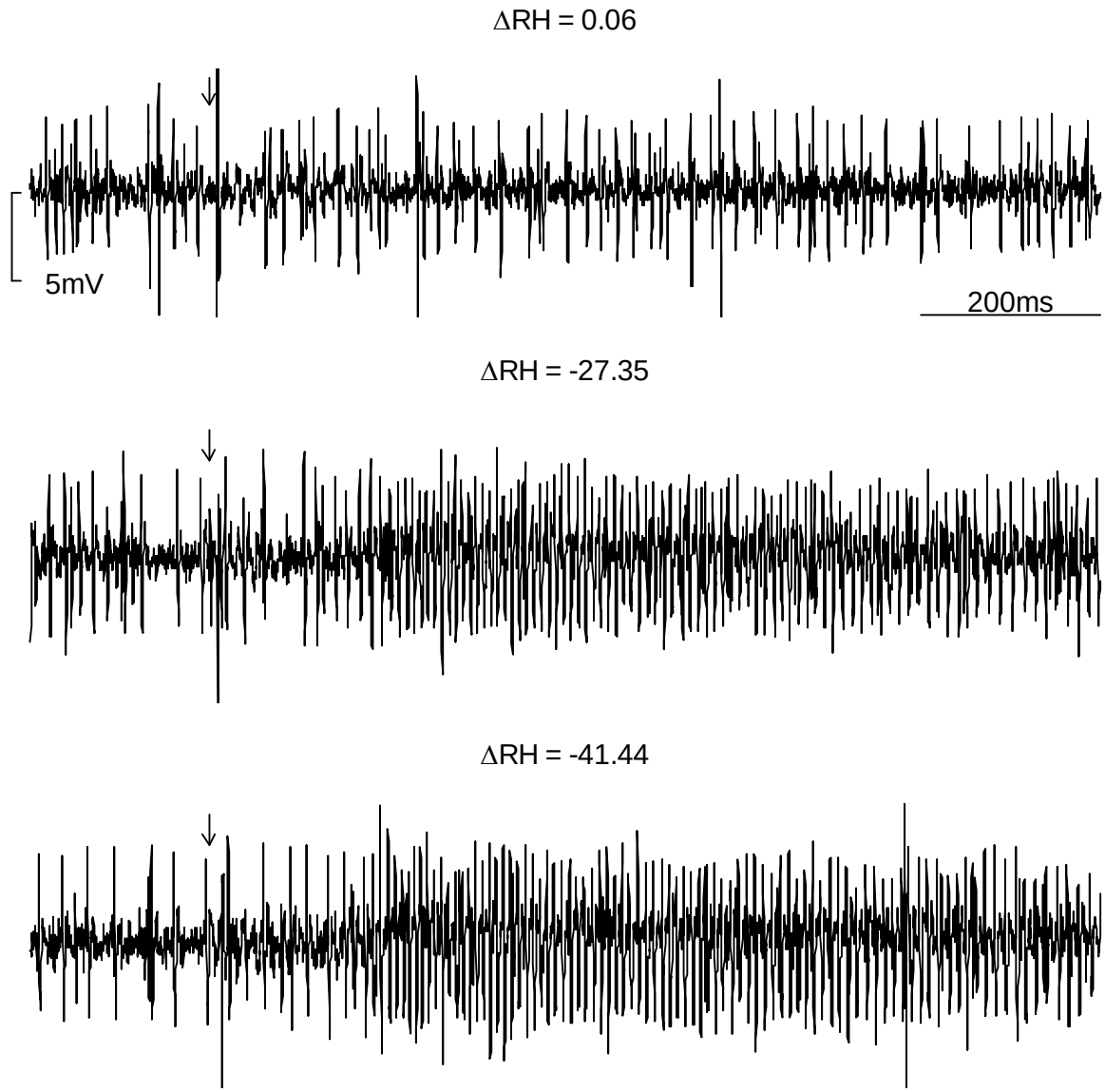

Figure 4.8: Neuronal responses in a *G. pallidipes* palp basiconic sensillum to RH decreases in the air. Arrows indicate the beginning of stimulation.

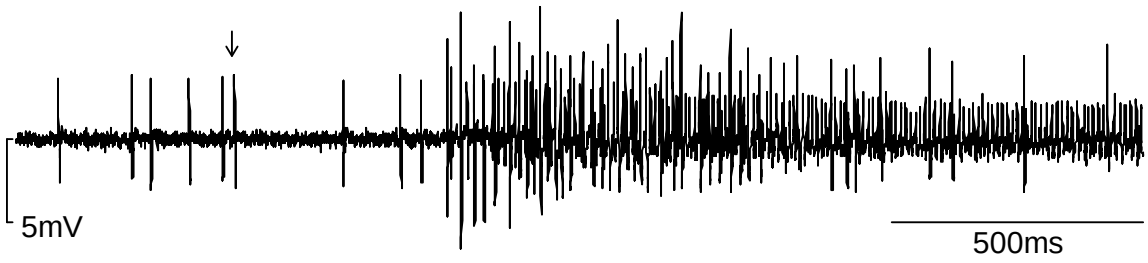

Figure 4.9: Neuronal response in a *G. pallidipes* palp basiconic sensillum to a RH increase from about 8% to more than 90% during preliminary experiments. The arrow indicates the approximate onset of stimulation.

4.4. Discussion

My results show that combined RH and temperature increases act synergistically to induce the biting response in tsetse. Moreover, at a temperature increment of 13.1°C, as RH increased, the flies responded more quickly and in higher numbers. The persistence of biting behaviour measured by the number of bites and the time spent biting also increased with increasing RH. The dynamics of the biting response correlated over time with the amount of water in warm air, with a biting frequency that was higher at the beginning of the 2min exposure and then fell off rapidly, but flies adapted more slowly at higher RH increments.

Latency and the proportion of flies responding are, respectively, negatively and positively correlated to RH increases at the 13.1°C air temperature increment. This indicates a very close link between the integration of thermoreception and hygroreception to initiate biting behaviour in tsetse. *G. pallidipes* and other savannah species exploit mainly mammals to obtain a blood meal (Moloo et al., 1993; Moloo et al., 1980). These hosts lose heat and water through their skin (Sokolov, 1982), creating a humidity gradient between the skin and the environment (Allen et al., 1970; Ingram et al., 1963; McLean, 1963), equipping these animals with a means of regulating body temperature through evaporation. Indeed, water droplets are formed by aprocrine glands during sweating (Ferguson and Dowling, 1955) indicating that the humidity is close to saturation in the air near the skin (>90% at 37°C, 57hPa). Taking into account the environmental conditions prevailing during the dry season in Africa (<40% at 30°C, 17hPa) (Gibson and Torr, 1999), differences in water partial pressure of more than 40hPa could occur between a vertebrate host and its environment during the dry season and in the range of 38hPa during the wet season (60% at 25°C, 19hPa). This corresponds to the range of water vapour partial pressure differences tested here. The combination of heat and water vapour assist tsetse to find hosts as demonstrated by Evans and Gooding (2002) and I show here how temperature and humidity combined serve to elicit the biting response at the feeding site. My findings corroborate those of Khan and Maibach (1971) who found that heat must be combined with humidity to elicit biting in another dipteran, *Ae. aegypti*. Previous studies have demonstrated that heat

elicits a biting response in tsetse flies described by Dethier (1954) and later by Brady (1973) and this has been amply exploited in the development of artificial feeding systems using membranes over heated blood for mass production of these vectors of disease (Langley and Maly, 1969). My results suggest that it is the change in RH accompanied by an increase in temperature that induces the biting response in flies coming from cooler and dryer conditions. I did not investigate the biting response of tsetse to RH increases at a constant temperature of 37°C for example or to temperature increases for flies maintained at high RH conditions. An increase in one factor, heat for example, may be sufficient to induce biting when a fly is already in a very humid environment or vice versa. Increasing the temperature increases the metabolic rate in tsetse (Terblanche and Chown, 2007) but in the absence of an increase in humidity a temperature increment alone is insufficient to induce the biting response. The water balance state of a fly could influence its eagerness to bite. My flies were maintained under constant and high RH conditions before experiments. The role of water vapour can also differ according to the degree of haematophagy in a species: tsetse hardly responded to RH increments alone in this study whereas an increase in humidity alone is sufficient to elicit probing in the stable fly that is both haematophagous and a nectar feeder (Gatehouse, 1970a; Gatehouse, 1970b) and in the nectar feeding hawkmoth (Von Arx et al., 2012).

As well as affecting response latency and the number of flies responding, increasing water vapour accompanied by an increase in temperature also increased the persistence of the biting response in *G. pallidipes*. This indicates that heat and humidity combined constitute an adequate stimulus to induce flies to persist in biting, a behaviour that represents a critical risk for tsetse (Randolph et al., 1992). Van Naters et al. (1998) found that two sensory modalities presented together, heat and uric acid, increased the biting persistence of *G. fuscipes fuscipes* compared to flies stimulated by heat alone. This interaction between sensory systems increased the time spent probing but not the numbers of bites. In my conditions the interaction between thermoreception and hygroreception increased the number of bites and correspondingly the time spent biting. Moreover, for a temperature increment of 13.1°C the average time per bite decreased with increasing RH above 34.4%. This suggests that the more adequate the stimulus,

the quicker the fly withdraws its haustellum to try again when haustellum extension fails to reach the skin under optimum stimulus conditions, i.e. the haustellum is quickly brought back to initiate a new biting attempt. Since the average time between two consecutive bites remains unchanged, this suggests that flies repeat a stereotypic positioning behaviour between biting attempts that assists them to reach the skin through the host's fur. The frequency of biting events was correlated with the amount of water in warm air but was not stable in time, being higher in the first 10s of the response, before falling rapidly thereafter. However, flies adapted more slowly at the higher dose of water vapour in warm air.

Neurones responded with a significant increase in spike frequency in all wpsw basiconic sensilla exposed to a decrease in RH. When these sensilla were also exposed to an increase of 9°C in dry air, no significant response in spike frequency was observed (data not shown). The overall responses represent complex spike patterns and my recordings did not permit to differentiate between the responses of the three neurones within the basiconic wpsw sensilla on the palps. However, I can conclude from my recordings that these sensilla contain one or more dry cells. Wet cells also occur in basiconic sensilla on the palps, but were very rare as only four were recorded in preliminary recordings from more than 60 sensilla. Tsetse are thus able to perceive RH changes with dry and wet cells increasing their spike firing frequency as humidity decreases or increases. To my knowledge, this is the first report of hygroreceptor cells in tsetse. Bursell (1957) previously demonstrated that several tsetse species including *G. pallidipes* are more active in dry air than in moist air and that antennal removal did not modify the response, indicating that hygroreceptor cells responsible for the observed behaviour occur elsewhere on the fly. Evans and Gooding (2002) showed that in the presence of CO_2, moist and heated air is more efficient at eliciting upwind flight in tsetse than heated air alone. Temperature and humidity conditions affect water loss in tsetse flies (Kleynhans and Terblanche, 2011), so hygroreceptor cells could evidently serve to guide flies in the selection of resting sites where they would be subject to lower rates of water loss, as proposed by Chown et al. (2011) and demonstrated in mosquitoes (Kessler and Guerin, 2008).

The ultra-structure of the basiconic wpsw sensilla housing the dry cells on the palps of *G. pallidipes* is very similar to typical olfactory wpsw sensilla of insects (Shanbhag et al., 1999). I recorded responses to the host-related volatiles 1-octen-3-ol, n-valeric acid and dimethyl-trisulphide from cells in these sensilla (data not shown), confirming earlier findings by Lewis (1972) of responses by these cells to carboxylic acids. The fact that basiconic sensilla of insects can contain both hygro- and chemoreceptor cells and has already been documented (cf. (1985)), but to my knowledge this has never been reported before for testse. All basiconic sensilla examined present 2 neurones with swollen dendrites that occupy all the lymphatic space at the base of the sensillum, separating the sensillar lymph into proximal and distal selections. The swelling remained unaffected by a hypertonic fixative solution. The transduction mechanism for the coding of differences in RH is unknown, but it appears clear that pressure exerted on the membrane of hygroreceptor cells could play a key role (Tichy and Kallina, 2010). I propose that as RH levels drop, water loss from the wall pore sensillum could cause the inflated dendrite to be deformed as a result of a pressure difference exerted on either side of the dendritic swelling.

4.5. Conclusions

My results show that temperature acts synergistically with water vapour increments in air in inducing the biting response in tsetse and that palp sensilla housing hygroreceptor cells are involved in the perception of differences in RH. As such, the tsetse biting response exploits the homoeothermic requirements of hosts where evaporative water loss is a crucial means of lowering body temperature. The findings also underline the perception of a substance as essential to life as water in the biting behaviour of obligate haematophagous insects like tsetse where host animals are the only source of both nutrients and imbibed water.

The experimental design described here that permits quantification of the biting response of tsetse to heat and humidity constitutes a means to study inhibition of this crucial response in trypanosome transmission. Disruption by chemicals for example of either thermoreception or hygroreception is likely to be sufficient in considerably reducing the biting response. The set up also provides a means to investigate the effect of trypanosome infection on tsetse biting responses.

5. General discussion and conclusion

Attraction of an insect to an odour source involves a complex sequence of behavioural steps (Kennedy, 1977). Past research in the laboratory has shown that olfactory stimulation activates resting tsetse flies (Warnes, 1992), elicits anemotaxis (Bursell, 1987; Colvin et al., 1989; Paynter and Brady, 1993), influences flight speed and tortuosity (Warnes, 1989; Warnes, 1990a) and induces landing in combination with a visual stimulus (Bursell, 1990; Warnes, 1989). All these papers focussed on a precise behavioural step, namely activation, anemotaxis, ortho and klinokinesis, and landing. Due to their high velocity (more than 3m/s in my wind tunnel), tsetse are difficult to observe during flight in a wind tunnel. The temporal and spacial resolution is very low inside a wind tunnel of 2.5m where the upwind end can be reached in less than 1s. To this difficulty can be added the relatively short flight duration and activity period of tsetse (Bursell and Taylor, 1980; Williams, 1994) and their poor ability to find a source of olfactory cues (Bursell, 1984b). I overcome these problems by presenting tsetse with a strong visual stimulus, a blue sphere surrounded by olfactory cues. This kind of combined stimulation provides the opportunity to record, with the help of a 3D tracking system, behaviours never recorded before in sequence for tsetse. The effect of different olfactory cues on behavioural steps such as activation, cage exit, flight to the target and local search could be quantified (see appendix B.1). Moreover, with this approach I could turn to my advantage the fact that tsetse fly in short bursts of activity as mentioned above. Taking into account that tsetse live on a knife edge with low energy reserves and high predation pressure (Bursell and Taylor, 1980; Rogers and Randolph, 1990), flying is dangerous and thus only pertinent host cues can succeed to elicit the consistent flying behaviours described here.

In this context, human breath is much more pertinent than its principle component CO_2. I have demonstrated that the discrepancy in tsetse responses to human breath and to CO_2 is at least partially due to some VOCs that cattle share with humans. In the past, the analysis of bovine breath proved fruitful. Acetone and 1-octen-3-ol, two components of bovine breath are important host cues and are used as chemical baits in trapping

devices. I show how breath has not released its last secrets in this regard. The examination of the end-products of lipid metabolism, released in breath, led to the discovery of new chemostimulants such as isoprene, heptane and geranylacetone, linking tsetse to the physiology of the host. These findings underline the idea that breath provides tsetse with a window into host metabolism that can be used to exploit hosts. For example, isoprene accompanied by other host metabolites could be used by the fly as a signal of cholesterol, a fundamental substance that insects cannot produce on their own. More than just playing a role as host olfactory cues, compounds such as short-chain alkanes could guide tsetse in the physiological assessment of the host leading to a preference in the choice between individuals of the same species. Indeed, the concentration of heptane, as for the other short-chain alkanes, varies in host breath as a function of oxidative stress level. A way to evaluate this hypothesis could be to investigate possible correlations between the behaviour of tsetse and the level of the lipid peroxidation that occurs in a host. Tsetse could be exposed to breath samples of individual with different degrees of lipid peroxidation that can be monitored by quantifying the lipid hydroperoxide content in blood (Frei et al., 1988) and alkanes in breath (Guilbaud et al., 1994; Phillips et al., 2000b). Other VOCs such as methylated alkanes for example could be also involved in this phenomenon. It would be also interesting to test the oxidative stress hypothesis with the vector of malaria, *Anopheles gambiae*. In fact, some evidence has been provided that malaria-infected hosts are more attractive than uninfected individuals (Day et al., 1983; Day and Edman, 1983; Lacroix et al., 2005; Mukabana, 2002) and that malaria infection is associated with lipid peroxidation (Becker et al., 2004).

The behavioural responses of *G. pallidipes* to heptane plus CO_2 are remarkable as they are similar to those for acetone plus CO_2, if not even superior and approach the responses recorded to breath. Taking into account the results obtained by Harraca (2008) with *G. brevipalpis* responding to heptane combined with POC, heptane should be assessed in the field for its capacity to replace acetone in the POCA blend of tsetse attractants. The other alkanes such as pentane, octane and nonane did not produce any significant effect except at the highest dose of nonane that decreased the activation rate. However, they are perceived by the antenna. So these alkanes could affect

behaviours other than activation and anemotaxis and they should be tested in the presence of the blue sphere to evaluate their effect on local search behaviour. The role of geranylacetone remains unclear as I obtained significant results only with teneral flies. The control, a mixture of ethanol and CO_2 could be implicated as fed and starved flies responded more to it than teneral flies did. So to evaluate the role of geranylacetone, it should be tested in a solvent that is not perceived by tsetse or in an biological active solvent with a role that is known, as for isoprene. Isoprene was diluted in acetone, a chemostimulant found in breath and known to attract tsetse flies. As isoprene is released in breath, tsetse are exposed to both when they enter a plume that contains host breath. Isoprene is emitted by vegetation and it has reported as a chemostimulant for the parasitic wasp *Diadegma semiclausum* (Loivamäki et al., 2008) and *Manduca sexta* larvae (Laothawornkitkul et al., 2008). To my knowledge, this is the first time that isoprene is reported to intervene in the behaviour of a hematophageous insect. At the dose tested, this substance increased the recruitment power of the target but had no effect on local search behaviour. Tests to evaluate the responses as a function of doses to this compound are necessary to evaluate its effect on local search and its role in the host seeking behaviour of tsetse.

Furthermore, I have shown that a 2s pulse of breath was sufficient to elicit all behaviours observed in the wind tunnel. However, the 2s pulse of breath followed by CO_2 did not increase by much the local search behaviour around the blue sphere compared to the 2s pulse of breath. The 2s pulse of breath followed by CO_2 is in fact the continuous breath treatment robbed of VOCs after 2s. This suggests that tsetse change their search strategy by dropping some steps of the host seeking behaviour when the odour plume is lost but also when the odour plume is reduced qualitatively. This phenomenon is crucial to assess the importance of semiochemicals during flight. In these experiments, the treatments induced similar proportions of flies to be activated but had different effects on the behaviour of tsetse during flights. So, semiochemicals may not deliver the same message to the fly at different steps of the host seeking behaviour. A resting fly may not consider host odours in the same manner as a fly that is already in flight because the two flies do not have the same choices. The resting fly has the choice between flying or remaining inactive whereas the fly in flight, can consider changing

direction or stop flying. This implies different trade-offs between a potential reward and the physiological status of the fly. So the experimental paradigm of removing a relevant semiochemical from a host effluent during a particular behaviour of tsetse could be considered as an experimental approach to reveal the contribution of particular products to the host seeking behaviour. In fact, subtracting a semiochemical from a blend that is made up of numerous different compounds has the advantage to detect more easily key compounds that, when presented alone may only deliver limited information to the fly (Byers, 1992). My wind tunnel set up provides a first step in this direction.

Overall, I demonstrated in my wind tunnel that *G. pallidipes* flight can be influenced by a small object such as a 16cm diameter blue sphere. Such an object elicits crucial behaviours such as directed flights, local search behaviour and finally landings. This is supported by the work of Mramba et al. (2013) who found in the field that 0.5m^2 targets smaller than the standard 1m^2 one caught more flies per unity area of fabric surface compared to the bigger target. I used a sphere for aerodynamic purposes and to provide a constant shape independent of the fly position. Moreover, round shape instead of squares were demonstrated as the most attractive for *G. pallidipes* in the field (Torr, 1989). I made a further experiment, where tsetse were stimulated with breath as the olfactory stimulus and with different sizes of blue spheres placed on a black stem. Objects were ranked as a function of decreasing proportions of flies that landed on them, giving rise to the following ranking: big sphere, medium sphere, discs, column and the complex multi-sphere object. Although results from this experiment are difficult to interpret, I demonstrated that I can modulate the landing behaviour of *G. pallidipes* by the shape and size of objects in a wind tunnel and that spheres are pertinent as a landing stimulus. The spatial and temporal resolution of my wind tunnel is adequate to study the landing behaviour of tsetse flies on such objects and my experimental setup could lead to a better understanding and consequently to a better exploitation of the landing behaviour of tsetse in control tools.

Having alighted on the host, the last step of the host seeking behaviour begins when the flies bite. This is a crucial phase in the life history of long living insects such as tsetse since during feeding they are exposed to predation and host defence. The trade-off

between feeding and risk avoidance could explain the adaptation where tsetse visit hosts infrequently but feed to repletion when they do so (Randolph et al., 1992). As blood is not directly available to tsetse upon landing, cues emanating from the skin facilitate tsetse to bite and to readily access blood to reduce risk. Heat and water vapour emanate from the skin of the host and I was interested to know how these two emanations could elicit the biting response. I built a new experimental set-up that allowed us to assess the contribution of air temperature and humidity increments as quasi-independent variables to the biting response of tsetse. I demonstrated that tsetse use combination of both heat and humidity, exploiting in this way the cooling capacity of mammals. The obligate hematophagy of tsetse implies that these flies are stimulated by both factors (temperature and humidity) in most cases. The situation can become more complex when the ambient temperature is higher than the temperature of host skin, a situation that can arise in savannah. Huyton and Brady (1975) demonstrated that feeding in *G. morsitans morsitans* is inhibited by a light intensity of 25’000lux, suggesting that tsetse feed under shaded (proposed by) conditions where the temperature difference between ambient air and the host will be highest. Moreover, *G. pallidipes* is active in the morning and evening, i.e. at conditions where the ambient temperatures are mostly cooler than on the host.

Tsetse responded hardly to humidity alone, contrary to what was found by Gatehouse (1970a; Gatehouse, 1970b) with *St. calcitrans*. He showed that water vapour alone was sufficient to elicit probing responses in *St. calcitrans,* a muscid that feeds on both blood and nectar, and that heat coupled with water vapour elicited a probing response no higher than to heat alone or to water vapour alone. Recently Von Arx et al. (2012) demonstrated that the amount of water vapor emanating from flowers is related to the nectar content of flowers. These authors also demonstrated that an increase in humidity is a reliable cue of nectar reward as it attracts the hawkmoth, a nectar feeder, and is sufficient to elicit probing on artificial flowers. This suggests that the role of water vapour in probing can differ according to the degree of hematophagy. For obligate hematophagous insects such as tsetse humidity has to be combined with heat to be pertinent as a potential reward whereas humidity alone is sufficient for nectar feeders or for organisms that feed on both nectar and blood such as *St. calcitrans*. To summarize,

humidity is a reliable signal for nectar and humidity plus heat is a reliable one for blood. So it would be interesting to test heat, humidity and the combination of both with mosquitoes predicting that males, that only feed on nectar, will only respond to humidity whereas females, that feed on both blood and nectar, will respond to humidity alone and the combination of humidity and heat, according to their physiological demands. It has been demonstrated that humidity and heat combined to elicit biting in mosquitoes that needed blood (Khan and Maibach, 1971; Klun et al., 2013).

My results reveal a new role for water in the sensory ecology of tsetse. Tsetse use moisture emanating from the host parsimoniously, for host location (Evans and Gooding, 2002) and to successfully bite the host (Chappuis et al., 2013). However, humidity could be also an important factor in the choice of resting sites for tsetse, serving to lower water loss rate as described in mosquitoes (Kessler and Guerin, 2008). It would also be interesting to investigate whether humidity plays a role in the alighting response of tsetse. Tsetse are equipped to perceive humidity changes as I found hygroreceptors on their palps, a very interesting organ. In mosquitoes, palps are involved in olfaction and carry CO_2 receptor neurones (Grant et al., 1995). The role of palps in tsetse behaviours has been neglected. In addition to moisture, I recorded responses to the host-related volatiles 1-octen-3-ol, *n*-valeric acid and dimethyl-trisulphide from the same cells in sensilla housing the dry cells. This confirms the only previous evidence for olfactory sensilla on tsetse palps of Lewis (1972). Further experiments are needed to assess the role of these VOC-responsive neurones in the behavioural ecology of tsetse.

How tsetse exploit host physiology to satisfy the demands of their own physiology is simply fascinating. Blood is not directly available for tsetse but they exploit cues produced by host metabolism to find a blood meal. In fact metabolism produces heat and metabolites such as CO_2, acetone, 1-octen-3-ol, carboxylic acids, phenols and terpenes, intervenes in the host seeking behaviour of tsetse. The heat loss system of mammals serves the fly to bite and breath as a host effluent that contains semiochemicals serves the fly to locate the host by linking tsetse to host metabolism. In this context I found new compounds that could be used in visual- and odour-bait

strategy of tsetse population control. Field studies are required to prove the usefulness of these newly identified semiochemicals for large-scale control.

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Appendix A. Behavioural responses to pentane

N-pentane released by piezo nebulizer froze at the tip of the capillary blocking the pentane flow inside the capillary. Pentane was then released by passing 60-80ml/min CO_2 into a 100ml gas wash bottle filled with 15ml pentane. The release rate of pentane was about 161µg/min. The testing procedure is described in chapter 1. The responses of *G. pallidipes* to the mixture of n-pentane and CO_2 did not differ significantly from those to CO_2 alone (Figure A.1).

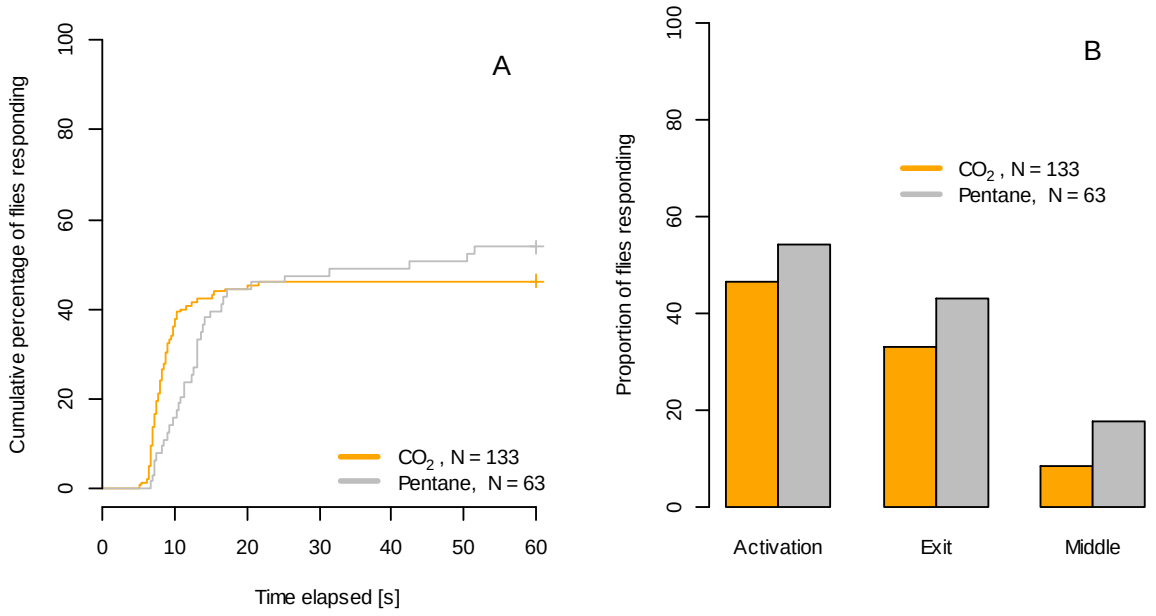

Figure A.1: **A** Cumulative percentage of activated *G. pallidipes* over a 60s period to CO_2 (~50ml/min) and to the mixture of *n*-pentane and CO_2 (indicated as pentane). Zero on the abscissa is the beginning of treatment release that needs about 5s to reach the insect at the downwind end of the wind tunnel. No significant difference was found between the two treatments at the 0.05 level of probability following a Cox proportional hazard model. **B** Proportion of flies that completed behavioural criteria "Activation" the fly moves in the cage, "Exit" the fly exits the cage, "Middle" the fly passes the midline of the wind tunnel (1.25m). No significant difference was found between the two treatments at the $p< 0.05$ (*) level of probability following a GLM with a logit link function.

Appendix B. Examples of flight tracks

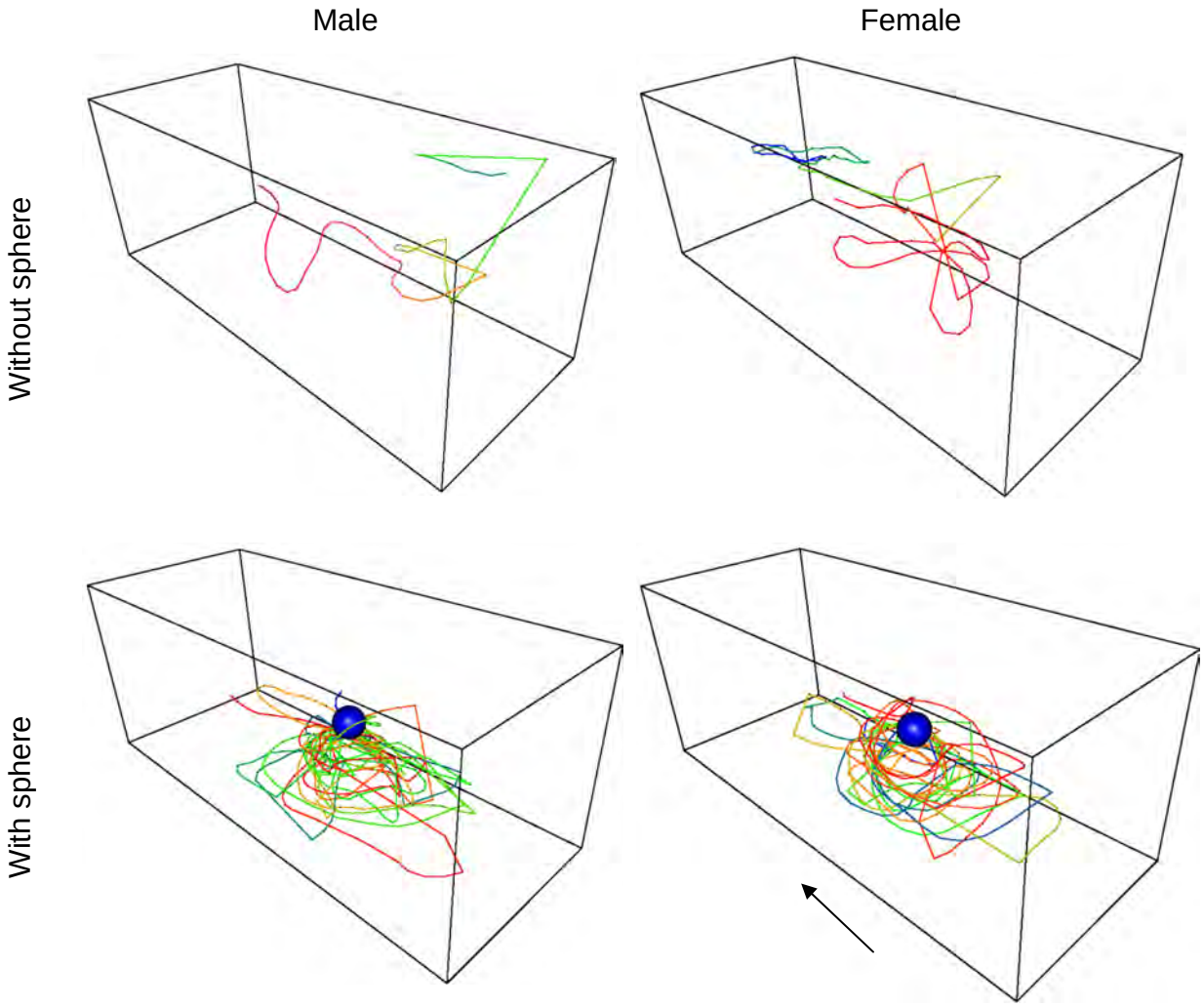

Figure B.1: Examples of flight tracks of single teneral *G. pallidipes* responding to exposure for 1min in breath in a wind tunnel in absence (Chapter 2) and presence of a blue sphere (Chapter 3). The longest flights were chosen for these representations. The flight tracks begin in red and end in blue. The arrow shows the wind direction. The boxes indicate the outline of the wind tunnel (for details see Materials and Methods in Chapter 2 and 3).

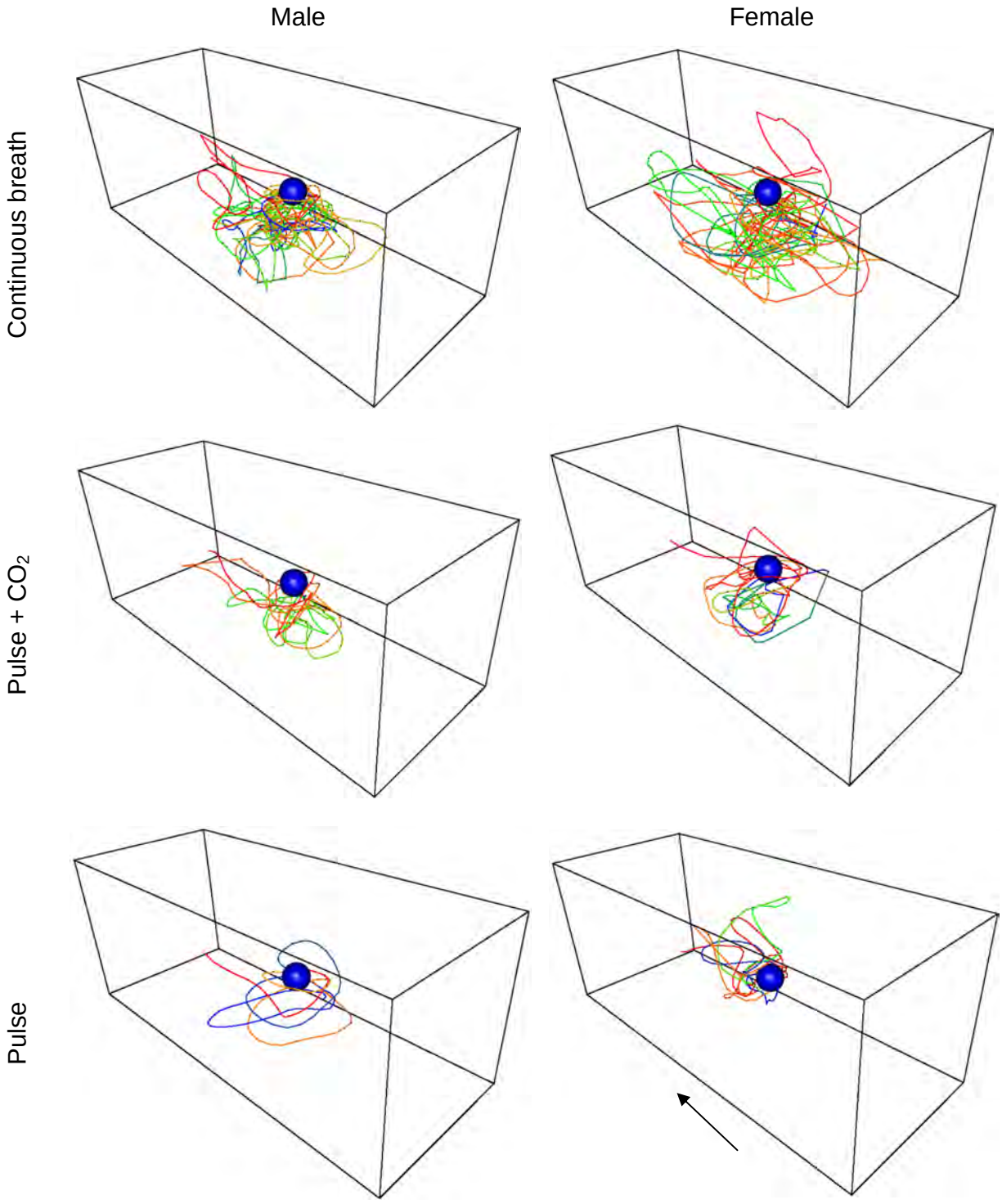

Figure B.2: Examples of flight tracks of single fed and starved *G. pallidipes* responding to the 2min exposure to breath, 2s pulse of breath followed by CO_2 and 2s pulse of breath. Only flights for which the distance covered approached the average distance for a treatment were selected. The flight tracks begin in red and end in blue. The arrow shows the wind direction. The boxes indicate the outline of the wind tunnel (for details see Materials and Methods in Chapter 3).