WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

Masking the attractiveness of human odours to mosquitoes:

A dual-port olfactometer study which investigated the putative allomonal effects of several selected human volatiles on *Anopheles gambiae s.s* host seeking behaviour.

No: 6.15
Name: Maaike van Agtmaal
Subject-code: Ent-30806
Reg.nr: 840520007080
Period: 13-02-2006 / 08-12-2006
1st Examinator: M.Dicke
2nd Examinator: W.Takken

Preface

Why are humans attractive to mosquitoes? And why am I more attractive to mosquitoes then my family members? I have been asking these questions since the final two years of high school. In that period I would like to have mosquitoes as subject of my final project, but due to practical reasons I had to choose another subject. I have never let these questions go and kept on wondering why there are differences between attractiveness between humans.
In this report I would like to present the results of my first thesis, which I have been working on for more than half a year. I have investigated the ability of human born sweat components to reduce or mask the attractiveness of human odours. I tested several selected compounds that might act as 'natural' repellent.
From this study I learned a lot, maybe even more than what I learned previous study years, without the help of people of the laboratory and discussions with my supervisors I could not have been carried out this study.
I still do not know the answer on my questions: Why are humans attractive to mosquitoes? And why am I more attractive to mosquitoes then my family members? With the results of my thesis I came a bit closer to the answers. I am very proud that I could contribute with solving a part of this puzzle.

Wageningen, december 2006
Maaike van Agtmaal

Index

Summary

Can specific human odour components mask, overrule or reduce the attractiveness of human odours? In this thesis several putative human born repellent compounds were tested in a dual port olfactometer to test the behavioural response of *Anopheles gambiae sensu stricto* mosquitoes.

Geranyl acetone, sulcatone, nonanal, and indole were selected to determine the behavioural effects of these compounds on the malaria mosquito *anopheles gambiae s.s.*

These compounds were tested in different concentrations in a dual-port olfactometer to determine the concentration effects of the putative repellent. In the setup a test compound was added to a human odour equivalent at one port and at the other port only a human odour equivalent was offered. Two of the test compounds, nonanal and sulcatone, were tested in a mixture. The first three series were accomplished with worn socks as human odour equivalent, the other series with skin washing samples.

Geranyl acetone, indole and the mixture did not show any significant behavioural effects. Sulcatone was repellent at two concentrations (1 and 5 ml/min and 5 and 50 ml/min resp.) in combination with both used human odour equivalents. Nonanal was also repellent at two concentrations (5 and 50 ml/min).

Differences in experimental setup might explain the differences with previous studies. Differences within results between both series with 6-methyl-5-hepten-2-one might be due to the used human odour equivalents, likely the composition and concentration of the odours were different. It would be nice to know the actual offered concentration of the test compounds. Not all total responses decreased with adding a test compound: the mode of action of the test compounds is unknown. This might explain this phenomenon. The mixture did not show any effect, there are evidences that compounds react different in a blend, probably other concentrations would show repellency. Testing a broader range of different concentrations would show more about the allomonal or maybe kairomonal actions of the tested compounds.

Future research should focus on further tests of the repellent compounds in semi-field or field experiments, which would say more of the natural mosquito behaviour.

This study provides evidences that human born repellent can reduce, mask or overrule the attractiveness of human odours. The results of this study might be used for developing a mosquito control system, such as a push and pull system, and might thus be used to reduce malaria transmission.

1 Introduction

1.1 Introduction

Mosquitoes and humans have had a close parasite-host relationship since ages. The mosquito is an important vector of several pathogens, like yellow fever, filariasis, dengue and the most serious parasitic disease in the world, malaria (Gibson and Torr 1999). Several mosquito species can act as vector for the protozoa *Plasmodium*, the one celled organism that causes malaria. Between 300 and 600 million people worldwide suffer from this disease (estimated by (Snow and others 2005)) and each year over one million people, especially young children and pregnant woman, die as result of a malaria infection (Korenromp 2005).

Before a mosquito can bite her victim, she first has to find a host. The most important sense organ in host location is the 'nose' of the mosquito which is located in the antennae of the insect. Host seeking and feeding behaviour are much affected by odour cues (Takken and Knols 1999). The different species of *Culicidae* differ in their response to human odour. Some react very strongly to human skin emanations and some do not react or only slightly. That has to do with host preferences, some mosquito species are generalists and some are specialists, some species are zoophilic and others highly anthropophilic (Gibson and Torr 1999; Lehane 2005). The mosquito species *Anopheles gambiae sensu stricto* (hereafter termed *An. gambiae)* s the main malaria vector due to its endophageous and endophilic behaviour and its preference for a human blood donor (Lehane 2005) and is therefore responsible for most of the malaria transmission in sub-saharan Africa (Takken and Knols 1999).

It is difficult to control malaria, as a result of drug resistance, insecticide resistance and many other factors like the economic situation in the area where malaria is common. Recent research focuses on host-seeking behaviour. It has been shown that host seeking behaviour of *An. gambiae* is odour mediated (Takken 1991; Takken and Knols 1999). *An. gambiae* is highly anthropophilic and reacts therefore strongly to human emanations. Some emanations, for example human sweat have been shown to be attractive (Braks and others 1997; Costantini and others 2001; Takken and Knols 1999). Chemicals that show attraction are termed kairomones and compounds that act as repellent are termed allomones. Which compounds that are present in human odour act as attractant for mosquitoes (i.e. show a kairomonal effect) and which act as repellent (i.e. show an allomonal effect) is not completely known yet.

1.2 Biology of the malaria mosquito Anopheles gambiae s. s.

1.2.1 Anopheles life cycle

From egg to adult

Female mosquitoes lay 50-500 boat shaped eggs each time they oviposit and deposit them on water (Clements 1992). The female drops them individually on the water surface where the eggs float on. Depending on the temperature it takes 2 days to a week to develop to a larva (Clements 1992) and thereafter the larva will hatch, induced by a fall in oxygen concentration (Clements 1992). The released mosquito larvae are legless and have a clear head region. The larvae lay horizontally, directly under the water surface. They are filter-feeding on small organic particles and microorganisms. Respiration takes place trough the spiracles, these lay dorsally in the abdomen and have direct contact with the air.
Mosquito larvae pass through four instars (L1-L4), from the first larval stage after emerge called L1 until the fourth called L4. The cranium and the respiratory siphon, which are heavily sclerotisized increase in size immediately after each ecdyzis (Clements 1999) and the different larval stages can be distinguished by the size of the cranium. The growth rate of the larva depends on temperature, nutrition and larval density (Lehane 2005).
The first three occasions that the growing larva moults, the larva appears almost the same as before apart from size. During the period of the fourth moult the imaginal discs will develop and grow very rapidly and change the larva in an almost adult shape; the organism that leaves the fourth larval skin is the pupa. This pupa still remains in the water, floating at the water surface. The metamorphosis takes one or two days depending on the temperature and after this period the mosquito will emerge (Clements 1999).
After completion of the metamorphosis the eclosion starts with air entering the pupa. When the new emerged mosquito slowly raises its abdomen horizontal the pupal cutilcle will split. The young adult wrestles its thorax through the split and the body of the adult slowly rises. Movements of the abdomen and the legs free the adult. Usually the mosquito remains a while on the water site (Clements 1992).

1.2.2 Adult: Mating, food and life cycle completion.

The night of emergence and the first full night of adult life are demonstrated to be crucial for obtaining food, in this phase sugar is preferred (Foster and Takken 2004). Mating generally occurs during twilight between 3-5 days of adult life. Males form swarms and virgin females are fertilized when they fly through or close to such a swarm. Females are recognized by their sound (Takken and Knols 1999). Females can store sperm and therefore they do not need to mate after each time they oviposit, but only once (Clements 1999).

Both female and male mosquitoes need plant sugars as energy source. For males plant juices are the only food resource but also females will die in absence of nectar or other plant sugars. Females are not obligatory nectar feeders, they can also ingest blood. Vertebrate blood is usually meant for the developing of the eggs, but can also be used as energy source (Clements 1992; Foster and Takken 2004).

For completion of their lifecycle and for development of the eggs blood proteins are essential and thus finding a suitable host is crucial. Mediated by host emitted odours the female mosquito is attracted to a potential blood donor. After the blood meal the female rests, while the eggs mature. After maturation of the eggs the mosquito has to find a suitable oviposition site, usually a pool. Cues to find this site are volatile semiochemicals (Allan and Kline 1995; Du and Millar 1999). In her search for a suitable site she reacts to different stimuli; odour plumes, warmth, and CO2 concentrations (Clements 1999). At the found site she drops her eggs individually on the water surface. After oviposition, the female mosquito is again susceptible to host odours. She can bite and oviposit several times in her lifespan of about a month (Clements 1999).

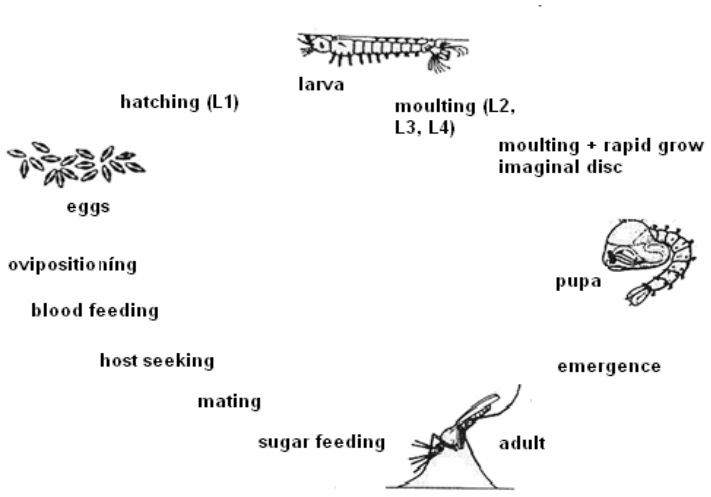

Figure 1 Anopheles life cycle (source: (http://www.objectivistcenter.org/images/graphics/mosquitolifecycle.gif)

1.2.3 The *Anopheles gambiae* complex

The species *Anopheles gambiae sensu stricto* is part of a species complex, in which the species are morphological identical but different in host preferences. The name of the whole complex is *Anopheles gambiae sensu lato*. This complex contains seven sibling species, four of them use freshwater as oviposition sites. The different species differ in importance as

malaria vector; *An. gambiae* s.s. is a very sufficient plasmodium transmitter due to its longevity, its preference to rest inside houses and to bite inside and its preference for human blood. Other species are less important because of their highly zoophilic behaviour or preference to bite outside (Lehane 2005).

1.3 Human–mosquito interactions: how do An. gambiae females select their hosts?

1.3.1 Introduction

For completion of their life cycles female mosquitoes need a blood meal (with a few exceptions). There are several factors that can influence the host seeking behaviour of these insects. Important cues affecting this behaviour are temperature, humidity and visual objects (Pates and others 2001). But the major cue is odour. With their preference for human hosts (Pates and others 2001) it is expected that host location is mediated by human odour blends (Smallegange and others 2003; Takken 1991). In a choice situation between human and cow odour *An. gambiae s.s.* will prefer the human odour (Pates and others 2001). The variation in attractiveness between individual people is due to differences in emanated odour, influenced by gender, age, sex and diet but also other physical and chemical characteristics, like body colour, body moisture, body temperature and body mass might influence attractiveness (Mukabana and others 2004; Qiu 2005; Smallegange and others 2003; Takken and Knols 1999). The odour mix that emanates from a human host is very complex, consisting of at least 350 different compounds (Bernier and others 2002).

Differences in attractiveness of individual humans is a tool to identify compounds that can act as kairomones to female mosquitoes, or maybe act as repellents (Bernier and others 2002). Most of the released human odours are sweat born. But also breath, and especially carbon dioxide (CO2), can be a strong activator. In *Aedes aegypti* CO2 can influence the behaviour of the mosquito and makes it more susceptible to human skin odours (Dekker and others 2005; Qiu 2005). Comparing sweat samples from different volunteers and measuring the attraction in a bioassay can help identify key components of host selection. Gas chromatography and mass spectrometry, used in these studies, are important tools for identifying and selection of key compounds in mosquito host interaction (Qiu and others 2004; Smallegange and others 2003). GC (gas chromatochraphy) and MC (mass spectrometry) data can give evidence for kairomonal and allomonal compounds of human origin. These techniques can help to identify individual compounds that might play a role in

host seeking behaviour . Comparing head spaces of highly attractive people with less attractive people can give evidence for attractive or repellent compounds.

Comparing samples of freshly collected sweat and incubated sweat (for 1 or 2 days) showed clear preference for the incubated samples (Braks and Takken 1999). Therefore the authors hypothesize that continuous bacterial actions on human skin secretions result in volatiles that act as kairomones (Braks and Takken 1999). This indicates that differences in attraction between individual persons can be due to microflora differences. Not only differences in microflora could influence the attraction of individual people, differences in ammonia emission between individual humans might influence attractiveness (Braks and others 2001). Differences in L-lactic acid and CO2 emission may also be the reason for differences in mosquito attraction between individuals (Dekker and others 2002; Dekker and others 2005)

1.3.2 Chemoreception

1.3.3 Introduction

The main sense organ in host selection of (nocturnal) mosquitoes is their ‘nose’. Chemoreception takes place at the sensillae, containing the receptor neurons. These are located at the head region of the mosquito, mostly distributed at the antennae but also located at the maxillary palps. Antennae of female *An. gambiae* contain four types of thin walled sensilla; sensilla trichodea, sensilla basiconica (or grooved peg sensilla), sensilla coeloconica and sensilla ampullacae (Clements 1999). Sensilla coeloconica and sensilla ampullacae have a theromoreception function whereas sensilla trichodea and the grooved peg sensillae have an olfactory function. Sensilla basiconica or grooved peg sensilla respond to CO2 (Clements 1999) and to odours (Qiu 2005). *An. gambiae* mosquitoes are possibly able to distinguish small changes in CO2 concentrations (Grant and others 1995). Sensilla trichodea, which can be distinguished in different subtypes, respond to a spectrum of olfactory cues (Qiu 2005).

Morphology

A sensillum contains one or more neurons surrounded by three auxillaire cells, the surrounding glia, epidermis and cuticle (Clements 1999; Qiu 2005). The wall of a olfactory sensilla contains pores through which odour molecules are transported into the sensillum lymph (Clements 1999). The lymph of the sensory hair contains water soluble proteins that bind odorants and transport them to receptor molecules in the dendritic membrane.

Signal transduction.

Olfactory signal transduction occurs in the olfactory receptor neurons, mediated by a distinctive family of seven transmembrane proteins containing odorant receptors that are coupled to G proteins that lay on the cytoplasmic site of the plasma membrane. When an odour binds to its receptor, a conformational change takes place that facilitate binding and activation of the G protein. The G protein, which is activated, releases one of its subunits and this release effects a downstream reaction of effector enzymes. These enzymes produce one or more second messengers. When a certain level of second messengers is reached, ion channels open. Than the membrane depolarized and action potentials are generated (Clements 1999; Qiu 2005; Zwiebel and Takken 2004).

Host selection occurs through physical and chemical cues. Most important factors are volatile chemicals, originated by human emanation. Odourants, released by humans, can be detected by mosquitoes through their chemosensors located in the antennae and mouthparts. Odorant targets go through pores in the sensilla and bind to receptors, finally leading to an action potential and a behavioural response of the mosquito.

1.4 Compounds

In different studies human sweat samples have been collected for chemical and behavioural analysis (e.g. (Bernier and others 2000) for identification of olfactory stimuli. There are several odours identified to play a role in the host selection of *An. gambiae s.s.* Major roles are demonstrated for CO2 (Dekker and others 2005; Qiu 2005), carboxylic acids (Knols and Jong 1996); (Bosch and others 2000); (Smallegange and others 2005) ammonia (Braks and others 2001; Braks and Takken 1999; Smallegange and others 2005) and lactic acid (Braks and others 2001; Dekker and others 2002; Smallegange and others 2005). The role of each of these components is different. Some show attraction on their own, like ammonia (Braks and others 2001; Smallegange and others 2005) and in several mosquito species CO2, but others only in the presence of other compounds, like lactic acid (Smallegange and others 2005). Recently a synergistic effect was found of a blend of ammonia, carboxylic acids and lactic acid in a bioassay with *An. gambiae s.s.* (Smallegange and others 2005). A synergistic effect means that the value of the combination is greater than the sum of the individual values.

1.4.1 CO2

CO2 is present in the breath of all vertebrates and can be detected by mosquitoes from a large distance (Mboera and others 1997). In field studies CO2 causes an increase in the number of trapped mosquitoes (Mboera and others 1997). In the anthrophilic *An. gambiae s.s*. CO2 is believed not to play a very big role in host recognition because this mosquito is

specialized on human blood hosts and therefore this more general cue is probably less important than in more generalist species (Mboera and others 1997). In a field study for several natural occuring species CO2 alone trapped less mosquitoes, except for *Aedes spp*, compared with a reference odour blend of ammonia, L-lactic acid, and CO2 (Qiu 2005). (Constantini and others) (1996) tested the attractiveness of CO2 in an odour-baited entry trap and the results in a choice experiment between human odour and CO2 (at level of human emission) showed that the number of *An. gambiae s.l.* in the CO2 trap was half the number of these mosquito species trapped with human odour (Costantini and others 1996; Qiu 2005). So CO2 is an important cue in mosquito host seeking behaviour but not the major cue for *An. gambiae* s.l. females.

1.4.2 Carboxylic acids

Volatile carboxylic acids, identified in Limburger cheese, have been shown to be attractive at low concentrations (Knols and Jong 1996; Knols and Meijerink 1997). In *An. gambiae* a strong landing response to 2-oxopentanoic acid has been shown (Healy and Copland 2000). (Bosch and others 2000) found also an attractive response in *Aedes aegypti* towards fatty acids. The relative amount of attraction depends on the chain length of the carboxylic acid and of the specific combination in the mixture. Also concentration is important, repellency is detected in attractive chemicals that are offered at higher concentrations (Bosch and others 2000; Qiu 2005).

1.4.3 Ammonia

In a study that compared freshly collected and incubated sweat, incubated sweat was the most attractive in a bioassay with *An. gambiae*. One of the compounds that differed in concentration in the two samples was ammonia (Braks and others 2001) which was increased in the incubated sample. Ammonia was also demonstrated to be attractive on its own (Braks and others 2001; Smallegange and others 2005). A strong synergistic effect has been shown in a bioassay with *An. gambiae s.s.*: The combination of the compounds ammonia, lactic acid and carboxylic acids was more attractive than ammonia on its own (Smallegange and others 2005). In *Ae aegypti* ammonia was attractive in combination with L-lactic acid and increased the kairomone effect of the latter compound (Geier and others 1999) but was not attractive on its own (Geier and others 1999).

1.4.4 Lactic acid

Lactic acid is a specific compound present in human emanation. Humans have unique high levels of this compound and therefore lactic acid can contribute to intraspecific and

interspecific selection by several mosquito species (Braks and others 2001). Lactic acid can act as an important kairomone on its own in *Ae. aegypti* (Geier and others 1999) and also as a very important enhancer in this species. In the presence of lactic acid several other chemicals, a fraction of skin samples, showed attractiveness (Geier and others 1999). In *An. gambiae s.s.* selective removal of lactic acid from sweat did not affect the attractiveness of the sample (Braks and others 2001) and also does not act as an attractant on its own (Smallegange and others 2005). However a blend of lactic acid with carboxylic acids and ammonia is attractive (Smallegange and others 2005).

1.5 Allomones and kairomones; chemicals that show repellency respectively attraction in Anopheles gambiae s.s.

Different researches tried to find specific components of human emanations that act as key compounds in mosquito host seeking (Bernier and others 2000; Smallegange and others 2003). Goal is to develop a trap that is more attractive than human odour that will catch the malaria vector and prevents thus Plasmodium transmission (Qiu 2005; Smallegange and others 2005). Besides the fact that several kairomones are identified (see paragraph ‘Compounds’) and several others are expected to be attractive (but not tested in a bioassay yet), other chemicals of human origin are found to show repellency (Costantini and others 2001; Knols and Meijerink 1997; Smallegange and others 2005) or are expected to show an allomonal effect (Bernier and others 2002).

In the previous section several factors that can influence the attraction of individual humans by female mosquitoes are discussed. The presence of one or more repellent factors could be an explanation of differences in attraction of people (Mukabana and others 2004). Differences in mosquito preferences for different persons could be explained by the presence of one or more repellent factors. This is observed by Mukabana and others (2004). In this study constituents with repellent properties have been found in human breath (Mukabana and others 2004).

Identifying components in less attractive individuals that differ from highly attractive persons, in concentration or composition and testing these in bioassays and/or field studies can lead to a better inside in anopheles host seeking behaviour and preferences. This can be used in developing odour traps (kairomones) in combination with mosquito ‘banning’ systems (allomones) in a push and pull system.

.

1.5.1 Compounds that influence attractiveness/repellency

An odour component can play different roles in female mosquito host seeking behaviour. The role that a given chemical plays is very much dose dependent, a specific compound can react as kairomone or as allomone, dependent on concentration. Very diluted carboxylic acids blend acted as an attractant for female *Anopheles gambiae*but the same blend showed repellency when undiluted (Knols and Meijerink 1997). This is also reported in (Du and Millar 1999): a blend with possible oviposition stimulants was attractive to *Culex tarsalis* and enhanced oviposition in this species but at the highest concentration tested in this assay the blend showed to be repellent.
Other remarkable effects are described in other studies. Indole on its own showed to be an allomone at the second lowest concentration described in (Qiu 2005). This compound also showed repellency in a blend at a concentration that was not repellent in an assay with only indole (Qiu 2005). Ammonia could suppress the repellency of a carboxylic acid blend that showed an allomonal effect without this component (Smallegange and others 2005).
Since the mosquito is expected not to react upon a single key odour but several odours, more like an odour spectrum, the relative context of each compound is important, although mosquitoes could show a reaction to a single odour (Qiu 2005). A single odorant, tested on its own, can cause a behavioural response, for example attraction at a certain concentration but can cause a different reaction in a blend (Smallegange and others 2005). This is also one of the results of Osterkamp and others (1999): two tick species, *Boophilus microplus* and *Ixodus ricinus,* reacted on substances that were presented in high concentrations but in a mixture this reaction could be seen at lower concentrations.

1.6 Research questions and goals

The main malaria vector *An. gambiae* is highly anthropophilic and specialized upon its human host. Human emanations and in special human odours play a major role in mosquito host seeking behaviour. Female mosquitoes are attracted by odour plumes that leave bedrooms and houses. Some people are frequently more bitten than others indicating that the female mosquitoes are able to distinguish differences between individual people, can make choices and have a preference for certain persons. This preference can be caused by the abundance of one or more attractive factors or can be caused by the absence of one or more repellent compounds in human emanations or due to concentration differences of certain components.
The possibility of the abundance of repellent compounds in human emanations is very interesting. Less attractive people could have some repellent compounds in their odour

spectrum that overrule the attractiveness of other present compounds and are therefore less frequently bitten by mosquitoes.

1.6.1 Questions and goals

The goal in this research is to investigate putative human born repellents on their ability to mask or overrule the actractiveness of human odours to *An. gambiae s.s.*

Which known and putative allomones decrease or overrule the attractiveness of human odour?

This question is split in two research questions;

1. **What is the effect of concentration on repellency of the selected compounds?**
2. **What is the effect of these compounds in a blend?**

1.6.2 Hypothesis

Less attractive people could have some repellent compounds in their odour spectrum that overrule the attractiveness of other present compounds and are therefore less frequently bitten by mosquitoes. The concentration is very important, possibly only the right dose could cause this reaction. Also the abundance of different possibly repellent components could be important. Maybe the repellent effect can be influenced when a combination of odours is present. The expectation is that a combination of repellent odours will give a stronger reaction.

1.6.3 Hypothesis for the research questions

1. Malaria mosquitoes are highly sensitive to odours and are able to notice very low concentrations. For example *An. gambiae* mosquitoes are possibly able to distinguish small changes in CO2 concentrations (Grant and others 1995). The expectation therefore is that the offered concentration is very important. The offered concentration can determine the behavioural response of the host seeking female mosquito. Previous studies (Du and Millar 1999) show that the role that a given chemical plays is very much dose dependent, a specific compound can react as kairomone or as allomone, dependent on concentration.
2. Mosquito females could react differently to odour components in a blend. The combination of different odours could cause a different reaction in a bioassay than the

specific odours on their own (Osterkamp and others 1999; Smallegange and others 2005). This indicates that the effect of different selected compounds in a blend could be different. The expectation is that a mixture of different components could cause a stronger repellent reaction.

1.6.4 Selection of test compounds

Candidate compounds

There are several human emanation compounds known as either kairomone or allomone, and from several others it is prospected that they might play a role in mosquito host selection. (Bernier and others 2002) analyzed human skin emanations of attractive and less attractive individuals for *Aedes aegypti* females. Compounds that are substantially or slightly more abundant in less attractive hosts were proposed to be candidate allomones. In Qiu (2005) several compounds are described that were tested for their attractiveness. But for some of them in stead of attraction repellency was reported. These compounds are also candidate test compounds in this study. The candidate test compounds are listed in table 1.

Table 1 List of human emanations that are known or prospected to play a role in *Anopheles gambiae s.s*. host selection. Compounds substantially (first column) and slightly (second colmn) increased in less attractive hosts for *Ae. aegypti* are presented in the first and second column (Bernier and others 2002). In the third column compounds that showed repellency in a bioassay with *An. gambiae* (Qiu 2005) are listed.

Substantially more abundant in more attractive host (Bernier and others 2002).	**Slightly more abundant in more attractive host (Bernier and others 2002).**	**Previous reported repellent components in a bioassay ((Qiu 2005))**
dodecanoic acid	decanoic acid	4-ethylphenol
Cholesterol	heptanal	indole
Methylpentanol	nonanal	3-methyl-1-butanol
Heptane	2,4-nonadienal	geranyl acetone
Methyliodide	2-nonene	6-methyl-5-hepten-2-one
1,3-butanediamine	methylundecene	
14-methylpentadecanoic acid, methyl ester	pentacosane	

Further selection

Further selection of these components was based on more indications that a specific component could possibly show to have a behavioural effect in a bioassay. The selection criteria were:

- The ability for *An. gambiae* to smell this component.
- Test results from previous studies. Results from tests with this component with *Anopheles spp, Aedes spp* or other mosquito or insect species
- The compound has to be reported to be present in human skin emanations
- The compound has to be commercially available

The selection of different compounds that might show repellency and could be tested in a dual port olfactometer depended on different criteria. The aim of this research is to test several putative human born repellent components; this means that all of the test compounds have to be human emanations. Several studies have tried to identify components present in head spaces of humans (Bernier and others 2000; Curran and others 2005; Meijerink and others 2000).

Some components are excluded from further selection because of the lack of literature and further information about this compound. Not for all components results from electroantennographic responses were measured. And some chemicals are excluded due to practical reasons; the compounds are not commercially available.

Indole

Indole has been shown to be present only in incubated sweat samples (Meijerink and others 2000) and the authors suggested that this compound could act as a kairomone for *An. gambiae* due to its strong electroantennogram (EAG) response. (Qiu 2005) analyzed the function of olfactory neurons by testing responses from different single sensilla and showed also a response to indole. In a dual port olfactometer test, contrary to what was expected, indole, when tested individually, acted as a repellent at the second lowest concentration tested. And a higher concentration, which was not repellent when tested individually, indole was also repellent when tested in combination with ammonia and lactic acid (Allan 1995). This latter is an indication that this compound could cause a different behavioural response when offered in a blend. This is reported previously by (Barrozo and others 2004; Du and Millar 1999) In these studies the same phenomenon, namely a different reaction of compounds when they were offered in a blend. In the highest concentration (Du and Millar 1999) tested (10 microgram/liter) to identify oviposition stimulants for *Culex quinquefasciatis* and *Culex tarsalis* indole was also found to be repellent, whereas in a blend and in lower concentrations it showed to be an oviposition stimulus. The antennae of *Cx. tarsalis* and *Cx.*

quinquefasciatus females responded to indole in combination with 2-undecanone in a gas-chromatography electroantennogram set-up (GC-EAG) (Du and Millar 1999). In this study also dose-responses were measured with EAG and the response to indole decreased at the higher doses (100 and 1000ng). A blend of naphthalene, 2-undecanone, 2-tridecanone, dimethyltrisulfide, nonanal, 3-methylindole, p-cresol, phenol, 4-ethylphenol and indole at the highest concentration has been shown to be repellent (10 microgram/Liter) for *Cx. quinquefasciatus* and *Cx. tarsalis* (Du and Millar 1999).
In a study of oviposition mediators (Allan and Kline 1995) oviposition rates were measured for several synthetic compounds. Oviposition responses to indole were significantly lower than expected (Millar and others 1992) at 100 microgram/liter for *Ae. albopictus* (Allan and Kline 1995).

Geranyl acetone

Geranyl acetone is present in both fresh collected and incubated sweat (Meijerink and others 2000) in a relative high amount. This could suggest that it can induce a behavioural response in *An. gambiae.* (Qiu) (2005) found a response of olfactory neurons in this mosquito species. In a bioassay (Qiu 2005) geranyl acetone was tested and showed an allomonal effect at the second lowest concentration (5ml/min) in combination with ammonia and lactic acid (Qiu 2005). The odour geranyl acetone was tested in an electrophysiology set-up, sensilla of *An. gambiae s.s.* females responded to this compound (Qiu 2005).

4-Ethylphenol

4- ethyl phenol is reported to be a oviposition cue (Du and Millar 1999; Takken 1999). The antennae of *Cx. tarsalis* and *Cx. quinquefasciatus* females respond to 4-ethylphenol in a gas-chromatography electroantennogram set-up (GC-EAG) (Du and Millar 1999). In this study also dose-responses were measured with EAG and the response to 4-ethylphenol decreased at the higher doses (100 and 1000ng) (Du and Millar 1999). In a blend with naphthalene, 2-undecanone, 2-tridecanone, dimethyltrisulfide, 3-methylindole, p-cresol, phenol, indole and nonanal at the highest concentration this blend which also contained ethylphenol has been shown to be repellent (10 microgram/liter) (Du and Millar 1999) for *Cx. tarsalis.*
In the study of (Allan and Kline 1995) the response of *Ae. aegypti* and *Ae. albopictus* towards synthetic oviposition mediators were measured. Oviposition responses to 4-ethylphenol were significantly lower than expected (Millar and others 1992) at 0.01 microgram/liter for *Ae. albopictus* and at 0.01microgram/liter and 100 microgram/liter for *Ae. aegyppti*. In the latter species 4-ethylphenol has also been reported to act as oviposition attractant at another (1 microgram/liter) concentration (Allan and Kline 1995).

In a dual port olfactometer test with *An. gambiae s.s.* 4-ethylphenol in combination with ammonia and lactic acid acted as a repellent at a flow rate of 5 ml/min. And also strong electroantennogram responses are reported to 4-ethylphenol for this mosquito species (Qiu, 2005).

Nonanal

In a blend with naphthalene, 2-undecanone, 2-tridecanone, dimethyltrisulfide and nonanal at the highest concentration this has been shown to be repellent (10 microgram/liter) for *Cx. quinquefasciatus* (Du and Millar 1999) and the same for *Cx. tarsalis* were this blend is completed with 3-methylindole, p-cresol, phenol, 4-ethylphenol and indole . Antennae from *Cx. tarsalis* females respond strongly to nonanal and also the antennae of female *Cx. quinquefasciatus* respond to this compound in an electroantennography method coupled with gas chromatography, but less strong (Du and Millar 1999). Dose-response EAG showed an increase in response at higher doses. In a bioassay with *Cx. quinquefasciatus* nonanal was attractive at 0.01 microgram/liter.

Blood sucking bugs *Triatoma infestans* can also detect nonanal. Electrophysiological tests showed responses of the grooved peg sensilla of these insects to nonanal. In a behavioural study the effect of this component was also measured. Nonanal increased the walking speed of the bug, an effect that stopped after removing the stimulant (Guerenstein and Guerin 2001).

(Curran and others 2005) reported that nonanal was present in all the samples of human sweat headspace that were tested. Bernier and others (2000) compared human sweat volatiles and tested also the attractiveness of all the volunteers for *Ae. aegypti*. Nonanal is reported to be slightly more abundant in less attractive hosts and this compound also slightly decreased at a less attractive day (Bernier and others 2000).

6-methyl-5-hepten-2-one

The antennae of *An. gambiae s.s.* females can detect 6-methyl-5-hepten-2-one (Meijerink and others 2000; Qiu 2005). This component of human sweat volatiles (Bernier and others 2000; Bernier and others 2002; Curran and others 2005; Meijerink and others 2000) was present in relatively high amounts in headspace samples of both incubated and fresh sweat (Meijerink and others 2000). Not all subjects tested had this component in their headspace samples (Curran and others 2005).

In the study of Bernier (2002) components that were detected in more and less attractive persons were compared. On a more attractive day for *Ae. aegypti* 6-methyl-5-hepten-2-one has been found to be slightly decreased. Remarkably 6-methyl-5-hepten-2-one was slightly more abundant in attractive people (Bernier and others 2000; Bernier and others 2002). This

could, in contradiction with the previous finding, be an indication that this odour could act as a kairomone. However in a bioassay with *An. gambiae s.s.* females no such effect was seen. At a flow rate of 50 ml/min this component (in combination with ammonia and lactic acid) has been shown to be repellent (Qiu 2005). In the study of Birkett and others (2004) 6-methyl-5-hepten-2-one was tested on cattle fliesin different experimental set-ups, using gas chromatography-electrophysiology, coupled gas chromatograpy-mass spectrometry, electrophysiology, laboratory behaviour and field studies. In a wind tunnel experiment the upwind flight behaviour was tested, 6-methyl-5-hepten-2-one increased upwind flight but in the field situation results gave tendency towards a repellent effect of 6-methyl-5-hepten-2-one and fly loads were reduced by this component. In identification study of volatile emissions from *Platyphus mutates*, a coleopteran species 6-methyl-5-hepten-2-one is reported to act as attractant for females of this species and this component is part of a sex pheromone (Audino 2005)6-Methyl-5-hepten-2-one is also reported to be part of a larval aggregation pheromone of the codling moth, *Cydia pomonella*, in the study of (Jumean 2004a; Jumean 2004b). This mixture of different volatile compounds (which contains for example 6-methyl-5-hepten-2-one, nonanal and geranyl acetone) attract the parasitoid species *Mastrus ridibundus.*

Nonane

Nonane is reported to be a human volatile (Bernier 2002). This is also reported in Curran (2005). In this study nonane was found in two of the test persons. This compound was also tested on basiconic sensilla of fifth-instar *T. infestans* nymphs but no response was obtained to nonane (Guerenstein and Guerin 2001).

Hexanoic acid

Hexanoic acid is a component of human sweat (Bernier and others 2000; Meijerink and others 2000). Electroantennogram responses to this volatile odour are demonstrated in a study with *An. gambiae s.s.* females (Qiu 2005). A dual port olfactometer study showed a repellent reaction (at the flow rate of 0.5 ml/min) of this compound, tested in combination with L-lactic acid and ammonia (Qiu 2005). When hexanoic acid was applied on a sandblasted glass slide in stead in a glass bottle attraction was seen at a dose of 1 ng (Qiu 2005).

Dodecanoic acid

The amount of dodecanoic acid is substantially increased in less attractive humans (Bernier 2002). In this study sweat samples of different people were compared and their attractiveness for *Ae aegypty* was compared too (Bernier and others 2000). Females of the mosquito species *An. gambiae s.s.* are able to smell dodecanoic acid (Qiu 2005). In a

bioassay with this species behavioural responses to this carboxylic acid neither reported attraction nor repellency (Qiu 2005) for this component, which is present in human sweat (Bernier and others 2000).

Heptanal

This component is slightly increased in samples that are reported to be less attractive to *Ae. aegypti (*Bernier and others 2000). And is also reported to be present in human sweat (Bernier and others 2000; Curran and others 2005). An EAG study did not show any response in *An. gambiae s.s.* females to this odour (Qiu 2005).

1.7 Relevance

Developing simple control mechanism for the malaria mosquito species *Anopheles gambiae s.s*. that can be used to reduce malaria transmission is of major importance in the sub-Saharan part of Africa. The economic situation in these countries requires simple and cheap control methods. Because females of *An. gambiae* are the main vectors of malaria in humans, which is a direct mortality cause for more than one million people each year (Korenromp 2005), it is important to find methods for control that are sufficient. This study may help to develop a push and pull system. Previous studies in laboratory and field tested the possibility of odour traps and the results from these gave evidences for future improvements of the system (Qiu 2005). As work continues, the development of odour baited traps maybe in combination of a supplier of repellents that prevents the entrance of mosquito females in houses could be refined.

2 Materials & methods

2.1 Description mosquito rearing

The *An. gambiae s.s* mosquitoes which are reared in the Laboratory of Entomology of Wageningen University (The Netherlands) since 1988 originate from Suakoko in Liberia (courtesy of Prof. M. Coluzzi, Rome). The mosquitoes used in the experiments are 5-8 days old females. These mosquitoes are not fed with blood yet. But they have mated already, which generally occur during dusk in day 3-5 in adult life (see introduction, biology of

Anopheles). In this study a large amount of *Anopheles gambiae s.s*. mosquitoes are used in experiments.
Mosquitoes are reared under a continuous high relative humidity (80%) a temperature of 27 ℃ and a day-night rhythm of L:D 12:12h, similar wi th African countries south of the Sahara in the rain season (Aggelen; Spitzen and Takken 2005)
An. gambiae s.s. mosquitoes are nocturnal, with a peak in activity and biting in the last hours before sunset (Gillies and Coetzee 1987). In the mosquito rearing there is a night /day rhythm of 12:12h L:D. One hour before the TL lights will switch on a dim light is switched on, increasing its luminosity over time. Just before the lights will switch on the dim light is working at full power (F. van Aggelen, personal communication). The dim light imitate the dusk and dawn periods, which are needed for mating.
In the experiments field conditions are imitated as far as possible and the experiments have to be carried out during peak activity of the mosquito. During this time the mosquito females are most susceptible for odour cues. Due to practical reasons therefore the light and dark regime in the climate room is changed, and is set from 06:00-18:00 towards 00:30-12.30 so that the experiments can carried out during daytime (Spitzen and Takken 2005).

The mosquitoes used for rearing are blood fed twice a week. Three volunteers alternate offer their underarm to adult *An. gambiae s.s* for 10 minutes. A day after the feeding a wet filter paper, folded in a cone, is offered to the females. The filter paper is set down in a round plastic holder filled with tap water. The folded filter paper acts as oviposition site for the females and the eggs can thus collected simply.
After collection the eggs are carefully placed in elongated larval trays in which they can emerge. After emergence of the eggs the larvae are daily fed with Tetramin® fishfood. After the fourth moult and the change of the larva to pupa, the floating pupa will be collected in plastic cups with tap water en and will be placed in adult cages (30x30x30) for emergence, this occurs daily.
The newly emerged mosquitoes have free access to a 6% glucose solution on filter paper (Spitzen and Takken 2005); F. van Aggelen, personal communication)
A healthy mosquito culture is of major importance of an *An. gambiae s.s*. behaviour study.

2.2 Description of the olfactometer

The dual-port olfactometer (Figure 1) contains a Plexiglas flight chamber of 160 x 0.66 x 0.43 m described in (Knols and others 1994). The flight chamber has two ports (diameter 4 cm, 28

cm apart) behind these (and partly through these ports) trapping devices can be connected. These trapping devices that fit in the ports can be connected via tubes with an air stream system. This stream flows through the trapping devices into the flight chamber. For attraction of female *An. gambiae s.s*. mosquitoes the air stream requires special conditions. Before the air stream enters the flight chamber it first passes an active charcoal filter, to filter out all of the possible odours. At the opposite site of both ports the flight chamber contains an opening in which a release cage exactly fits. At the start of an experiment a release cage has to be put in this opening and the mosquitoes can be released by opening the cage. The mosquitoes will fly into the flight chamber, upwind the air stream, and are maybe trapped during the experiment in one of the devices.
The highly anthropophilic *An. gambiae* especially shows host seeking behaviour during the last four hours of the night (Gillies and others 1987). Therefore all experiments have to be done in darkness, with dimmed spotlights above the flight chamber as artificial moonlight.

The test compounds were offered to the mosquitoes in one of the two ports. The main airstream has to be split up, one part flows directly to the trapping devices, the other part first through the glass bottle with the test odour and is than connected to a trapping device. The test odour therefore flows only through one side of the dual-port olfactometer. If the glass bottle is connected to the main air stream the dual-port olfactometer is prepared for testing a specific concentration of test compound.

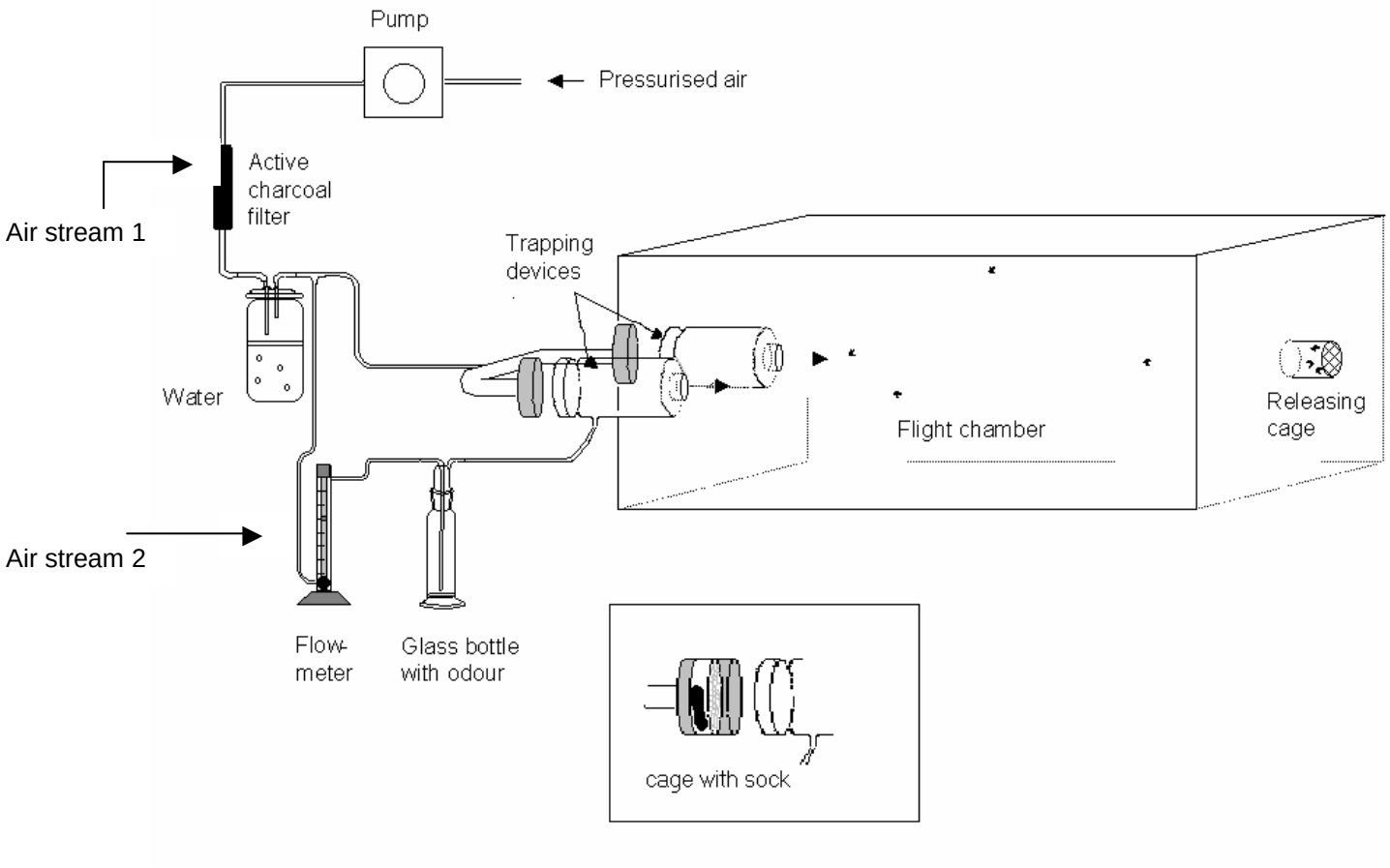

Figure 2 Dual-port olfactometer (after Qiu, 2005)

2.3 Used odour compounds

In this study different human sweat components were selected for testing their possible repellent effects on host seeking behaviour of female mosquitoes. Geranyl acetone, 6-methyl-5-hepten-2-one, nonanal and indole were tested in a bioassay in this study. The specificities for these components are listed below in table 3.
Which test compounds finally were chosen was due to the selection criteria with of major importance the amount of evidences of a repellent behavioural response. Which compounds were chosen to test, which were rejected and why is described in this paragraph.

2.3.1 Rejected compounds

Many more than the selected compounds were putative repellents. The choices to select or not could be a bit arbitrary. Most of the compounds that are more abundant in a less attractive host for *Ae aegypti* (Bernier 2000) are interesting compounds in view of the present study and could possibly show repellent effects for *An. gambiae* females. But for not all the candidate compounds (Bernier and others 2002; Qiu 2005) that were putative repellents

more previous test results were known. That is why many putative compounds were rejected. Further search was concentrated on indole, geranyl acetone, 4-ethylphenol, nonanal, 6-methyl-5-hepten-2-one, nonane, hexanoic acid, dodecanoic acid and heptanal. 4-ethylphenol seems not to be present in human sweat and is therefore not tested in this study. No behavioural responses of female mosquitoes in a dual port olfactometer experiment were seen for dodecanoic acid. Heptanal did not induce any EAG responses (Qiu 2005) and is therefore not selected to test. Less is known in literature about nonane. This compound and also hexanoic acid (Qiu 2005) were putative repellents but were rejected because of the lack of time.

2.3.2 Chosen compounds

Geranyl acetone, 6-methyl-5-hepten-2-one, nonanal and indole were finally selected to test for their behavioural effect in an olfactometer study. Firstly, two compounds that were tested in a similar experiment previously and did show repellence in that study (Qiu 2005) were selected. Both geranyl acetone and 6-methyl-5-hepten-2-one were chosen because of previous bioassay results and their usefulness to test the used method as well. The other chosen test compounds were selected later. Indole was chosen because of the remarkable results in the study of Qiu (2005). Nonanal was not tested in a comparable setup with *Anopheles gambiae s.s.* before but from the study of Bernier and others (2000) strong evidences for a repellent effect of this compound raised.

2.3.3 Concentrations

For three of the four selected compounds previous results were known (Qiu 2005). For comparability of the test results the same concentrations as in the study of (Qiu 2005) were chosen. Nonanal, the only test compound without previous reported repellent data were tested in the same concentrations. Different concentrations of each of the test compounds were tested. Which compound was tested in which concentration is listed in table 3 and 4. For preparing the tests with the pure compound 3 ml of liquid compounds was pipetted into a 250ml glass bottle. This glass bottle is thereafter connected to one of the trapping devices with tubes. After weighing 0.384g of the only non liquid compound, indole, was put in a 250 ml glass bottle. All tests with odour components contained at one port human odour equivalent in combination with the test odour and the other port contained only the human odour equivalent.

Table 2 List of test odours with brand, purity and amount

Test compounds	purity	brand	amount
geranylacetone	≥98%	Fluka	3ml
6methyl-5-hepten-2-one	99%	Sigma	3ml
nonanal	95%	Aldrich	3ml
indole	≥99%	Sigma	0.384g
nylon socks		Hema	

Table 3 Concentrations in which each compound was tested

compound	**tested concentration (ml/min)**				
	0.5	1	5	50	100
geranyl acetone		X	X	X	X
6-methyl-5-hepten-2-one		X	X	X	X
nonanal		X	X	X	X
indole	X	X	X	X	X
blend of 6-methyl-5-hepten-2-one and nonanal			X	X	

2.4 Human odour equivalents

2.4.1 Preparation

- **Worn socks**

In this study the aim is trying to mask human odours. In the experimental setup of the first three test series worn socks are the human odour equivalent. Human skin emanations can be collected from a foot by wearing nylon socks. The nylon socks that were used as attractant were worn for at least 12 hours by the experimenter. They were stored in a clean glass bottle at -20ºC when they were not used in experiments.

Worn socks were strongly attractive to *An. gambiae* mosquitoes in previous studies (Qiu 2005; Pates and others 2004). Before putative repellents could be tested in combination with worn socks, first had to be determined if these socks are really attractive to *An. gambiae s.s.* and could be used in following experiments.

- **Skin washing samples**

The skin extract was supplied by Y.T. Qiu. The extraction is made as described below: Previous research showed that human skin washing with ethanol were reliable mosquito attractants (Pates 2002). Therefore human skin washing sample was collected, which can be used in the behavioural bioassay as a standard attractive odour source, and for GC-EAD analysis to identify the attractive components. Cotton wool pads with ethanol were used to rub both hands, under the arm pits and foot of a human subject for two minutes (female, 39 years old). Twenty five pieces of the cotton wool pads containing human skin emanations were packed in a glass column (1 m long) and eluted with 550 ml absolute ethanol. The ethanol elution was concentrated from 190 ml to 27 ml (SW1E or sample 4) using a rotating vacuum evaporator, 55℃ and 625 mBar. (Y.T. (Qiu), personal communication).

2.4.2 Offering

- **Worn socks**

For testing the attractiveness of worn socks these socks are placed in a trapping device. For each test series a new pair of socks was used. These socks are placed in the connection tube that connects the main air stream to the trapping devices, and not into the trapping device itself, to prevent contaminating with test odour. To prevent contamination of the connection tube the socks are placed in a Perspex/fine mazed cage. The airflow goes first through the sock and afterward the test compound is mixed with that odour (Figure 3).

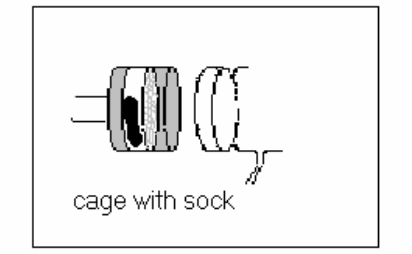

Figure 3 The worn sock is placed in a cage and put in the contaminating tube to prevent contaminating with test odour.

- **Skin washing samples**

The third, fourth and last test series are done with human skin washing sample as human odour equivalent in stead of worn socks. Evidences for contaminating with test odours and

other disadvantages were reasons to switch the offered human odour equivalent (see also conclusions and discussion, chapter 4). Before each experiment starts 100 micro liter of ice stored diluted (0.01%) human skin washing sample is pipetted onto a sand blasted glass slide. After evaporation of the ethanol the slides are placed in the trapping devices at both ports. After closing the devices the main air stream and the air stream that flows through the glass bottle with the test odour are connected to the trapping devices.

2.5 Experimental procedure

2.5.1 Selection of mosquitoes

12-18 Hours before the experiments started, 5-8 days old females were selected randomly from the cages, with using a sucking tube. These females are not fed with blood. For each experiment 30 females are hold in release cages with access to cotton wool moistened with tap water. The release cages remain till the start of the experiments in the rearing room. At the start of the experiment the release cages with mosquitoes are transported to the olfactometer room in a box, to prevent perceiving of (day) light by female mosquitoes.

2.5.2 Experimental procedure

All the experiments with *An. gambia s.s*. took place between 8.30 and 12.30.
An experiment in the dual-port olfactometer needs some preparations before an experiment can start. A timer that regulate the start time of the humidifiers and heaters has to be adjusted.
At the start of the experiments two clean trapping devices have to be placed behind both ports. The speed of the air stream has to be measured and should be between 20-22 cm/s, the same wind speed is obtained for both ports. The air stream flows through a charcoal filter to clean the air stream. The clean air stream flows through a bottle of warmed water to humidify the air and to warm the stream. The outflow has a relative humidity of at least 80% and a temperature of approximately 28℃.
The air stream splits up after passing through the water bottle. One part flows directly to the pvc connection tubes that can be connected to the trapping devices (air stream 1), the other air stream has to pass through a flow meter. Afterwards this stream flows through as glass bottle which could contain one of the test compounds (except for the control experiments with human odour equivalent against clean air or ethanol). The air stream (air stream 2) with test compound can be connected to a special connection site on the trapping device. Inside this device the air stream with test odour is mixed with the main air stream.

At the start of each experiment one of the release cages is taken out of the transport box and set in the opening of the gauze of the dual-port olfactometer. The cage is opened afterwards and the mosquitoes are released in the wind tunnel. The experimenter leaves the wind tunnel room and the females can fly in the flight chamber for 15 minutes. After this period the release cage is closed and the trapping devices are removed. The mosquitoes which are trapped in a trapping device have to be anaesthetized with 100% CO2 and counted. Afterwards the flight chamber has to be disposed of remaining mosquitoes with a vacuum cleaner.
Each test series on a particular day is started with an experiment without any added odours. After the clean air experiment the first experiment with a treatment is started. For each different test series details are described below.
Each series of experiments is repeated at least 5 times on different days. The tests with specific test compounds are randomized between different days and different times and also the left and right port were alternated. This prevents day effects, time effects and positional effects.
The used human odour equivalents were tested against clean air to measure the response of the mosquitoes. The worn socks were tested between 10.30 and 11.00, the skin washing extract between 11.30 and 12.15.

Control experiments

Before putative repellent odours could be tested first the symmetry of this olfactometer had to be tested. Several types of tests were carried out in the dual-port olfactometer to determine the relative attractiveness of the worn socks. They are tested against clean air, against a clean sock and against a sock worn by another person. In addition, socks worn at different days were tested against each other to determine possible day effects.

Experiments with different test compounds

For the experiments with different test compounds the glass bottle with the test compound has to be connected with the trapping device with tubes at a special connection site. The flow meter has to be adjusted at the proper flow rate. Which compound were tested and in which concentration is listed in table 4
The experiments with the mixture required an adaptation of the experimental setup. In this setup a second split up of the air stream, after the flow meter, was needed. After the split air flows through two bottles with different test compounds and are combined again before they enter the trapping device (see Figure 4).

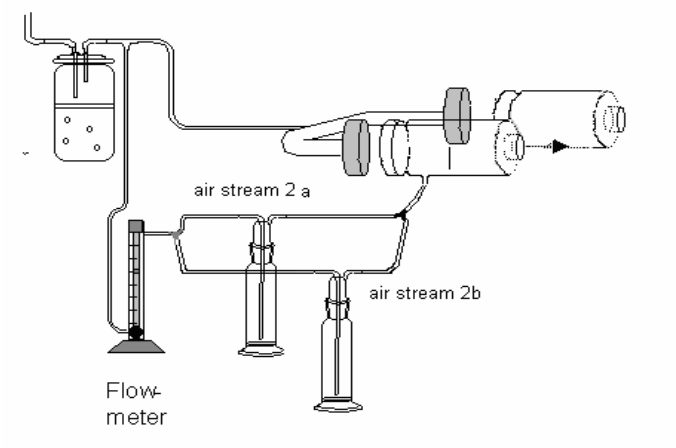

Figure 4 Adapted experimental setup for the test series with mixture.

2.5.3 Avoiding contaminating with odours

Avoiding contamination with odours

To avoid contaminating with (human) odours the experimenter has to wear vinyl gloves inside the olfactometer room. This to prevent touching glassware, the olfactometer and other materials and prevent also effects of other compounds. The experimenter has to leave the olfactometer room during the experiments; this is also to prevent effects of the abundance of a human and the presence of human odours in the room of the trapping of mosquitoes.
All the materials have to be clean and some have to be replaced each test series because of possible contaminating. After each test series the tubes that connect the glass bottle to the trapping device have to be replaced.
For each test series a new pair of socks was used. The pair of socks could be contaminated with the test compound. The socks were placed in front of the trapping device with the connection to the test compounds to prevent contamination as much as possible. In this way, the airflow went first through the sock and afterward the test compound was mixed with that odour.

2.5.4 Cleaning

Before each test series the olfactometer is cleaned with warm water with perfume-free soap. Afterwards the tunnel is washed again with water. Third and fourth step of the cleaning process is removing all the polair and apolair residues with hexane and ethanol. Now the tunnel is ready for a new test series.
After each test day the used trapping devices are washed with perfume free soap in a washing machine.

The sand blasted slides have to be hand washed. First with warm water and perfume free soap, after that step the slides have to be rinsed with enough warm water. The last steps for cleaning the slides is rinsing with ethanol (90%) and a night in the oven, by 180 degrees. The glass bottles will get the same treatment as the sand blasted glass slides

2.6 Statistical analysis

The expected distribution of number of mosquitoes that was trapped in both trapping devices, the control (no odour) device and the device with the test odour was 1:1 in each experiment. The total response was measured as the sum of trapped mosquitoes as fraction of the number of mosquitoes that entered the flight chamber. A chi-square test was used to test whether the distribution of all two choice tests of one test series differ from the expected 1:1 ratio. Differences were considered to be significant at $P<0.05$. The effect of different test compounds on the total response was investigated with a Generalised Linear Model in each test series. A univariable test was done for all of the test series. Total responses were considered to be significant different from each other at $P<0.05$. Further investigations were done with a post-hoc test (Tukeys) when significant differences were found.

3 Results

3.1 Response to different treatments

3.1.1 Control experiments

The first series of experiments were test with clean air coming from both ports, in these tests there was a slight difference between both ports, but both ports did not differ significantly from each other and this demonstrated that the dual-port olfactometer was symmetrical. When clean air was tested against a worn sock the worn sock attracted significantly (P<0.001) more mosquitoes than clean air. This test was done to test the responsiveness of the female *An. gambiae s. s.* and was done as control. Socks worn at different days (8th of March and 13th of March) were tested against each other to see if there were big day effects, but there were no significant effects (P:0.26). A worn sock was also tested against a clean sock, also for measuring responsiveness of the mosquitoes. A worn sock was significant more attractive than a clean sock (P<0.001). A worn sock was tested against another worn sock for testing the symmetry of the tunnel with a stimulus, both socks did not differ significantly from each other (See figure 5).

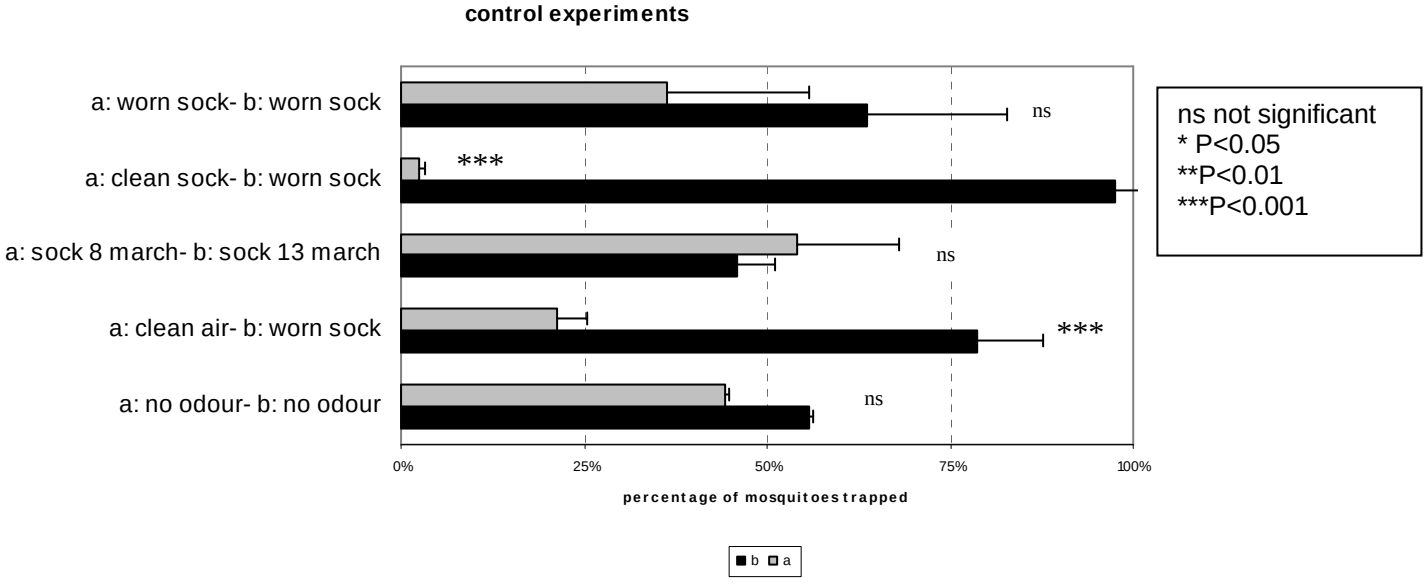

Figure 5 Series with control experiments. The standard error of mean is showed for each treatment. Different series were accomplished: no odour no odour (not significantly different), clean air worn socks (clean sock highly (P> 0.001) significant), sock 8 march against sock 13 march (no differences between both days), worn socks against clean socks (worn socks highly (P>0.001) attractive to mosquitoes). Worn socks are tested against worn socks (no significant differences).

3.1.2 Geranyl acetone

Clean air was tested against clean air to test the symmetry of the olfactometer. Both ports did not differ significantly from each other. A worn sock was tested against clean air to measure the response of the mosquitoes. No significant differences between the treatment and this control were seen (P>0.37). Geranyl acetone in the concentrations 1, 5, 50 and 100 ml/min combined with a worn sock was tested against a control containing only a worn sock. None of the four concentrations showed a significant difference (P<0.05) with the control (P: 0.10; 1.0; 0.21; 0.05) (Figure 6).

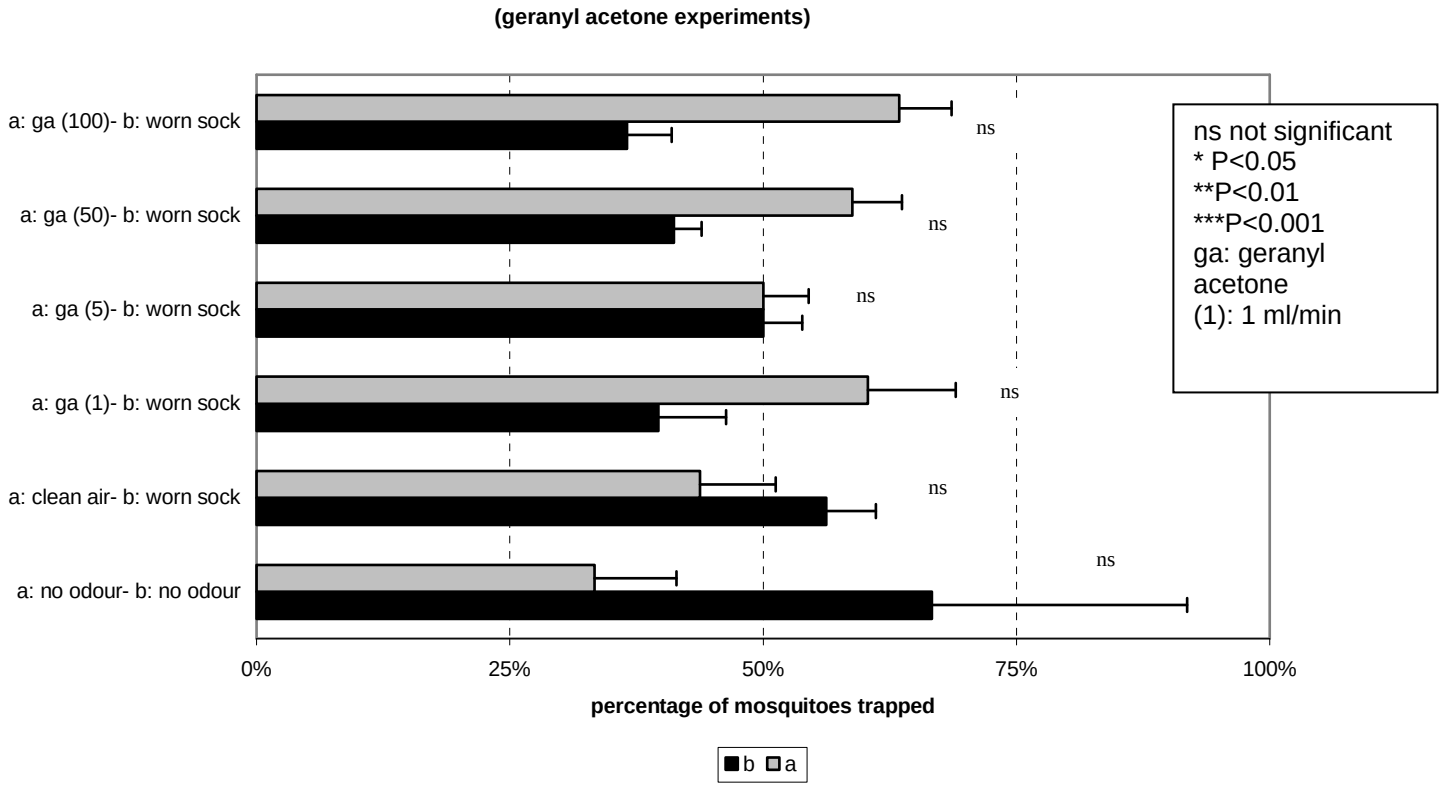

Figure 6 Responses of mosquitoes to different concentrations of geranyl acetone combined with a worn sock tested against a worn sock. The total percentage of mosquitoes that flew into either port is shown. Also the standard errors of mean are shown for each treatment. None of the experiments gave significant (P<0.05) differentces.

3.1.3 6-methyl-5-hepten-2-one

6-methyl-5-hepten-2-one with worn socks

The treatment clean air flowing through both ports for testing the symmetry did not give significant results; only one mosquito was trapped in this treatment. In the test with at one side a worn sock and at the other side clean air the worn sock attracted significantly more mosquitoes (P<0.001). The tests with a worn sock and 6-methyl-5-hepten-2-one at 1 ml/min in one of the ports and in the other port only a worn sock did show a significant difference between both treatments. The combination of the worn sock and the test compound attracted less mosquitoes than the worn sock only (P<0.048). And the concentration of 5 ml/min 6-methyl-5-hepten-2-one showed the same result (P<0.35). The other two concentrations tested in this test series (50 and 100 ml/min) did not show significant effects (P: 0.37; 0.66). (See Figure 7).

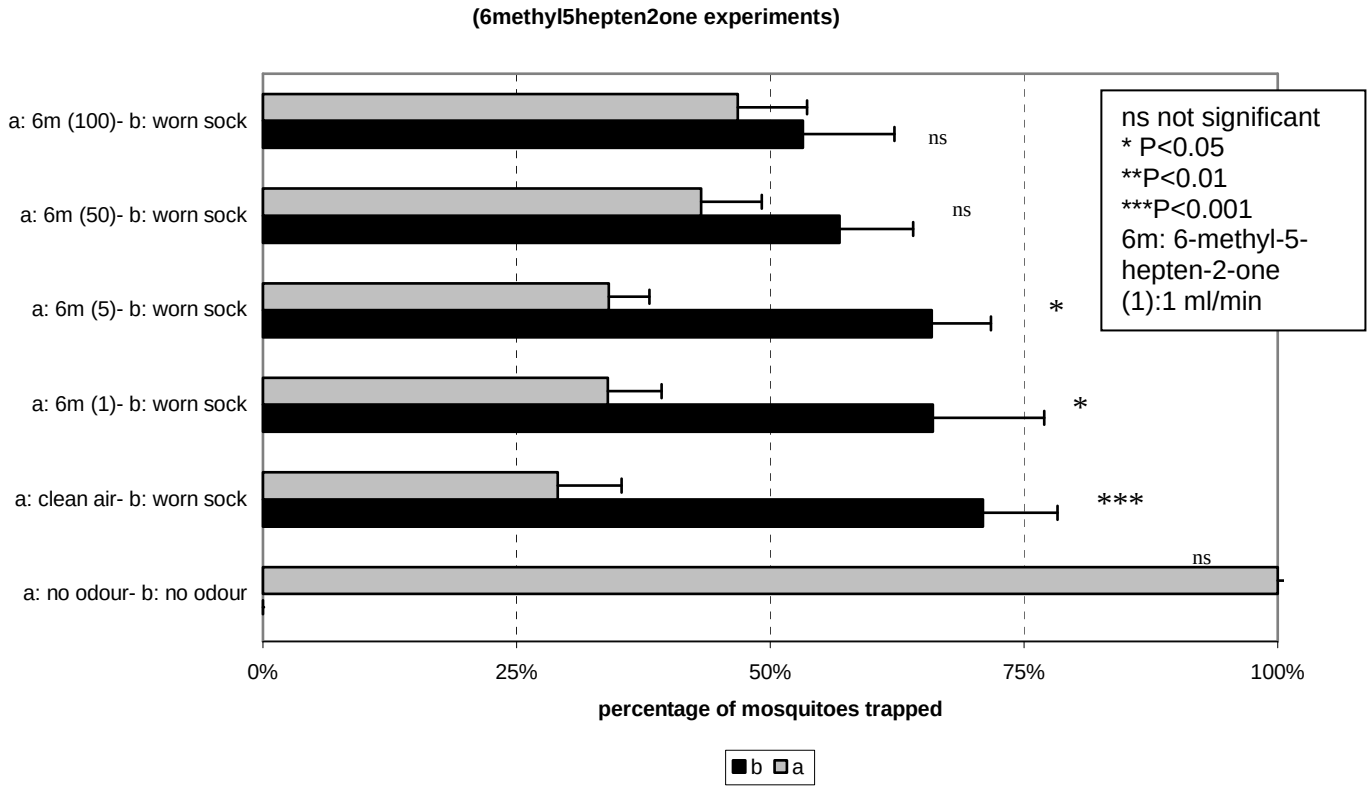

Figure 7 Responses of mosquitoes to different concentrations of 6-methyl-5-hepten-2-one + a worn sock tested against a worn sock. The total percentage of mosquitoes that flew into either port is shown. Also the standard errors of mean are showed for each treatment.6-Methyl-5-hepten-2-one attract significant less mosquitoes in concentrations of 1 and 5 ml/min (P=0.047, P=0.035 resp.). Worn socks attract more mosquitoes then clean air (P<0.001).

6-methyl-5-hepten-2-one with skin sample

No mosquitoes were trapped in the control experiments with clean air at both sides. In the experiment for testing the responsiveness was placed at one side a glass slide with ethanol

and at the other side a glass slide with skin sample. No significant differences between the numbers of mosquitoes trapped at both sides were seen. The test series 6-methyl-5-hepten-2-one in combination with a glass slide with skin sample tested against only a glass slide with skin sample did not showed significant differences at the concentration 1 ml/min. For both the concentrations 5 ml/min and 50 ml/min there the differences were significant (P<0.05 for both). The concentration 100 ml/min did not differ significantly (Figure 8).

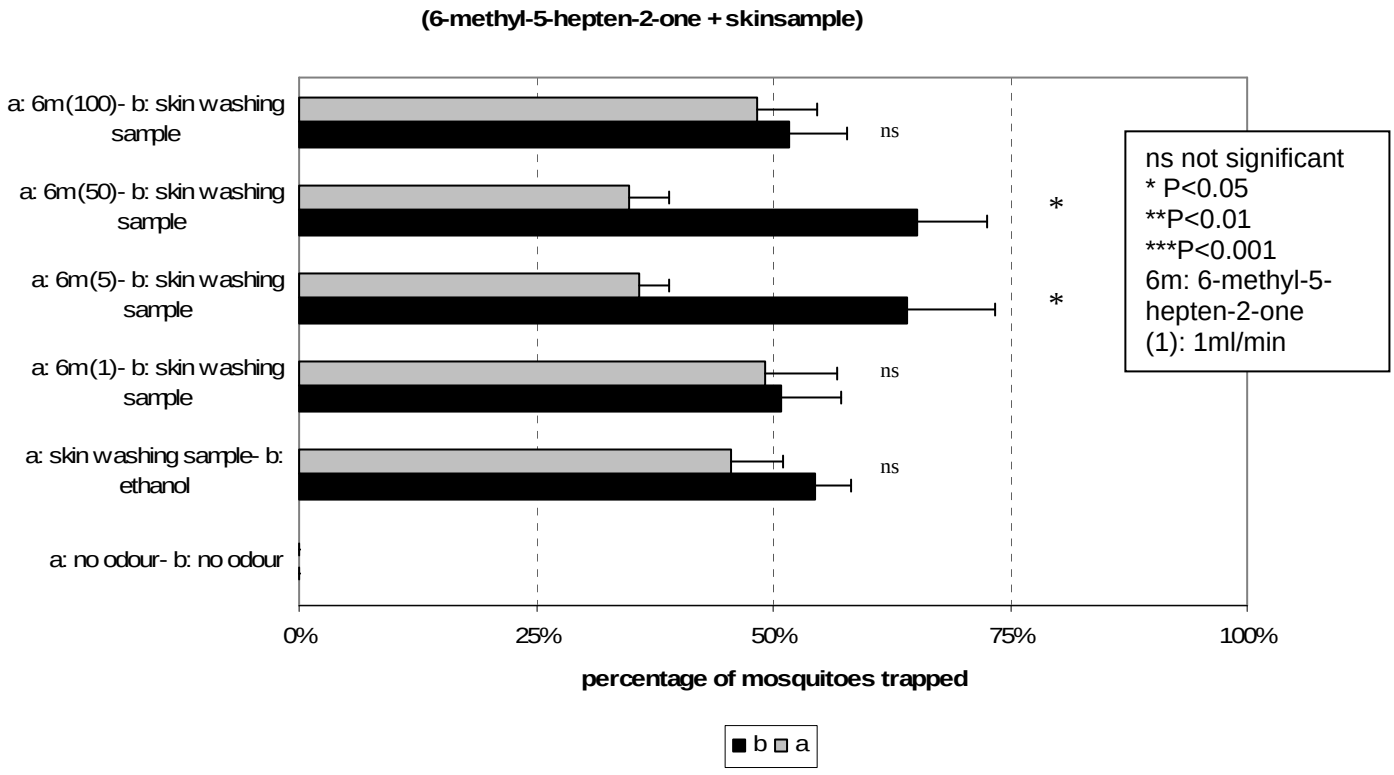

Figure 8 Responses of mosquitoes to different concentrations of 6-merthyl-5-hepten-2-one in combination with a worn sock tested against a worn sock. The total percentage of mosquitoes that flew into either port is shown. The error bars show the standard error of mean for each treatment. Significant less mosquitoes then expected flew into the ports with 6-methyl-5-hepten-2-one in the concentration of 5 and 50 ml/min (P=0.039, P=0.014resp.).

3.1.4 Nonanal test series

In the experiments with clean air flowing through both ports no significant differences between the two ports of the olfactometer has been shown. This indicates that the wind tunnel is symmetrical. The test with skin sample against ethanol showed significant attraction for the skin sample trap (P<0.001).Two flow rates (5 and 50 ml/min) did significantly trap less

mosquitoes than the control with only skin sample (P<0.05 for both concentrations). The other two tested flow rate did not show significance (P:0.26; 0.05935) for nonanal with flow rate 1 and 100 ml/min respectively (See Figure 9).

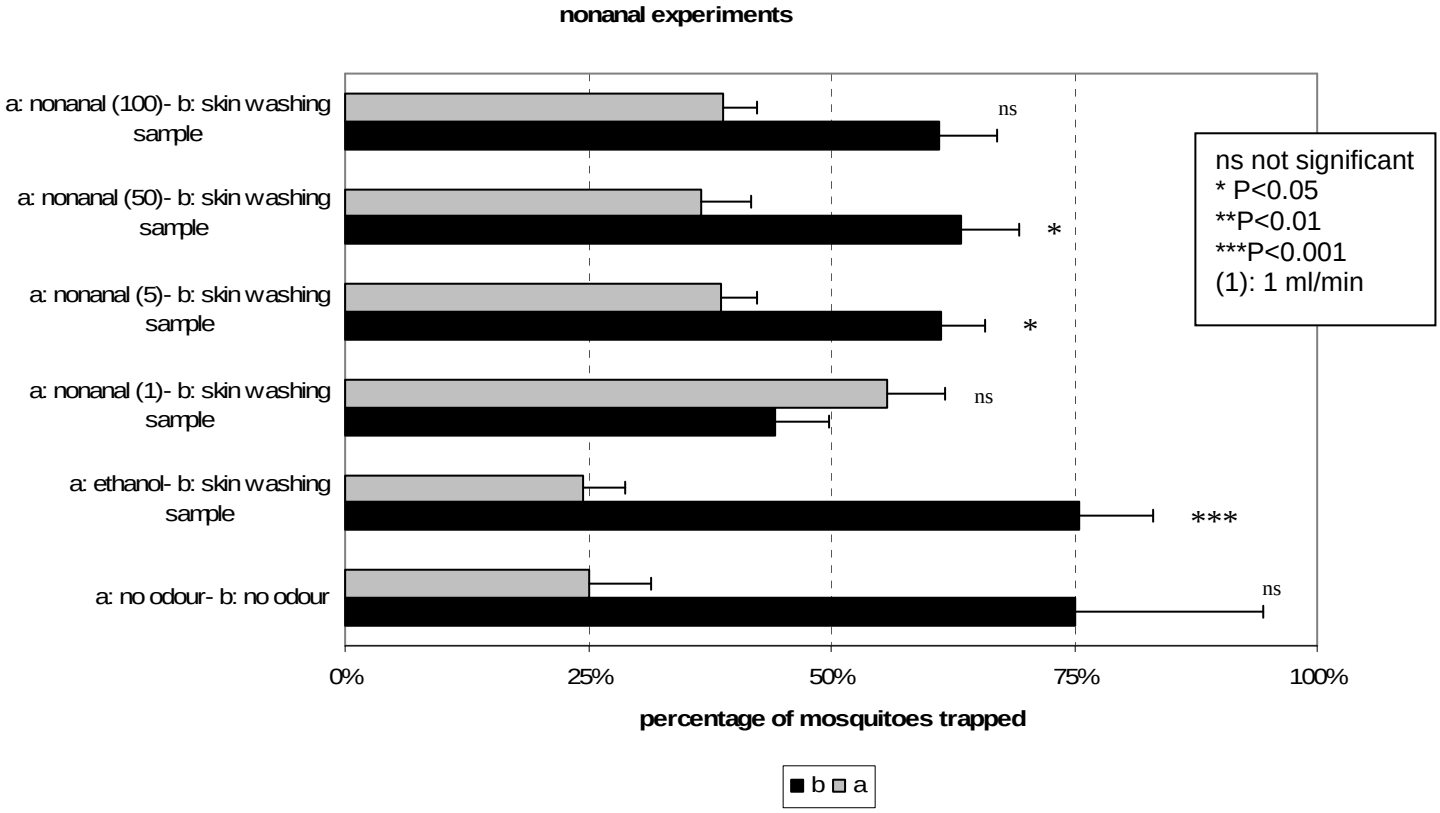

Figure 9 Responses of mosquitoes to different concentrations of nonanal and skin washing sample tested against a skin washing sample alone. The total percentage of mosquitoes that flew into either port is shown. The error bars show the standard error of mean for each treatment. Ethanol is tested against skin sample as control experiment. Nonanal attract significant less mosquitoes at 5 and 50 ml/min (P=0.05, P=0.024). Ethanol attact less mosquitoes then the skin washing sample (P<0.001).

3.1.5 Indole test series

There were no differences that were significant in the treatment with an odourless (clean air) air stream coming through both ports. The experiment which measured the responsiveness of the female mosquitoes was done with at one side an odour trap that contain a glass slide with skin sample and a port that contain a odour trap with inside this trap a glass slide with ethanol. Significantly more mosquitoes were trapped with the skin sample trap (P>0.05). There were also 5 different flow rates tested (0.5, 1, 5, 50, 100 ml/min) in combination with a skin sample glass slide with as control only a glass slide with skin sample. None of the tested flow rates were significantly different from the control (Figure 10).

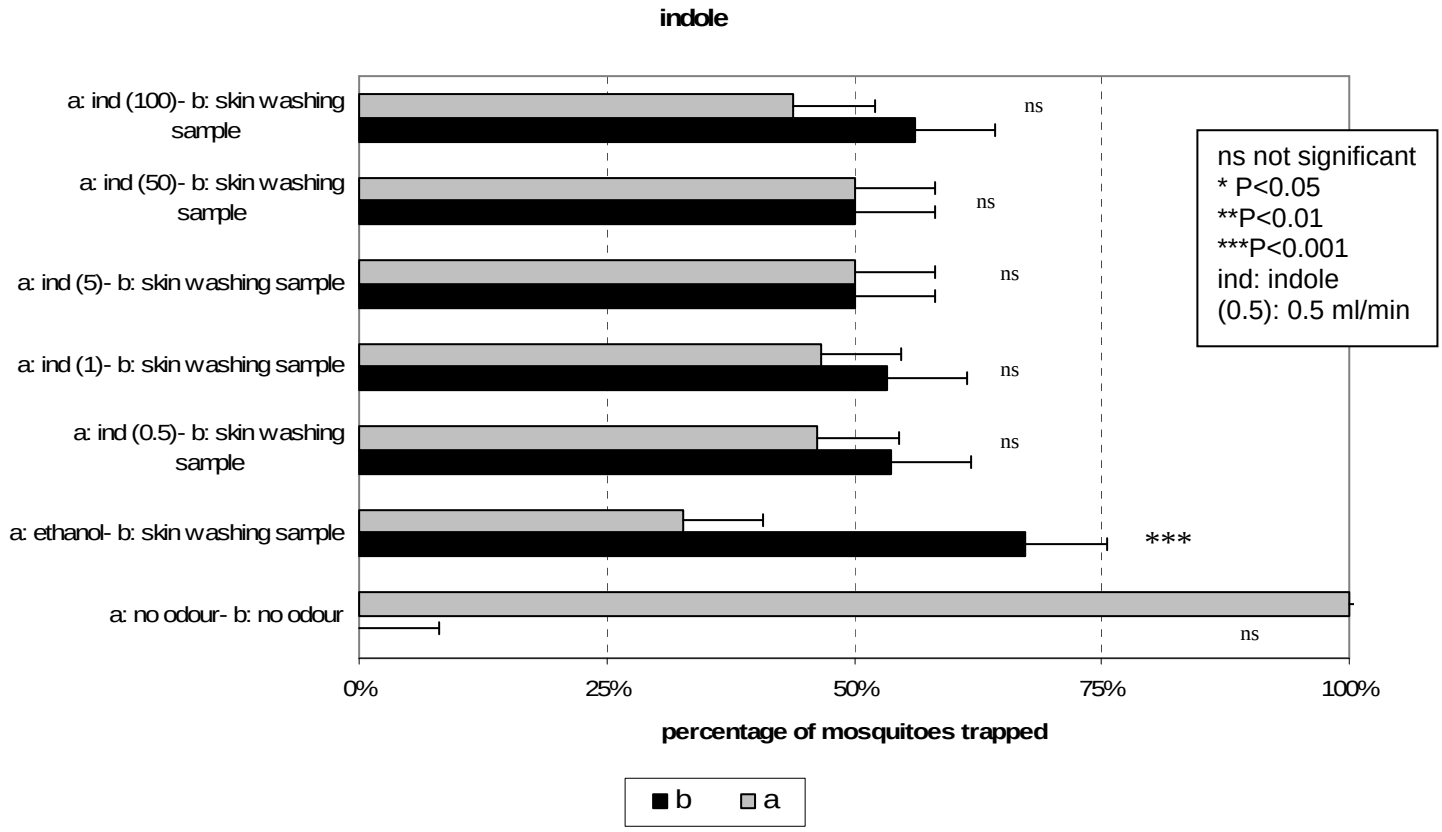

Figure 10 Responses of mosquitoes to different concentrations of indole and skin washing sample tested against skin washing sample alone. The total percentage of mosquitoes that flew into either port is shown. The error bars show the standard error of mean for each treatment. Ethanol is tested against skin sample as control experiment and is significantly different (P<0.001). None of the treatments with indole gave significan (P<0.05) results.

3.1.6 Mixture of two test compounds (6-methyl-5-hepten-2-one and nonanal)

The experiments with clean air at both sides do not show any differences between both ports. This indicates that the dual port olfactometer is symmetrically. The mixture which contains 6-methyl-5-hepten-2-one and nonanal is tested in two concentrations. Differences between the number of trapped mosquitoes in the odour trap with mixture combined with skin sample and just skin sample were not significant for both concentrations. The control experiment for measuring response showed a clear difference between skin sample and the control (ethanol). This was significant (P>0.01). (See Figure 11)

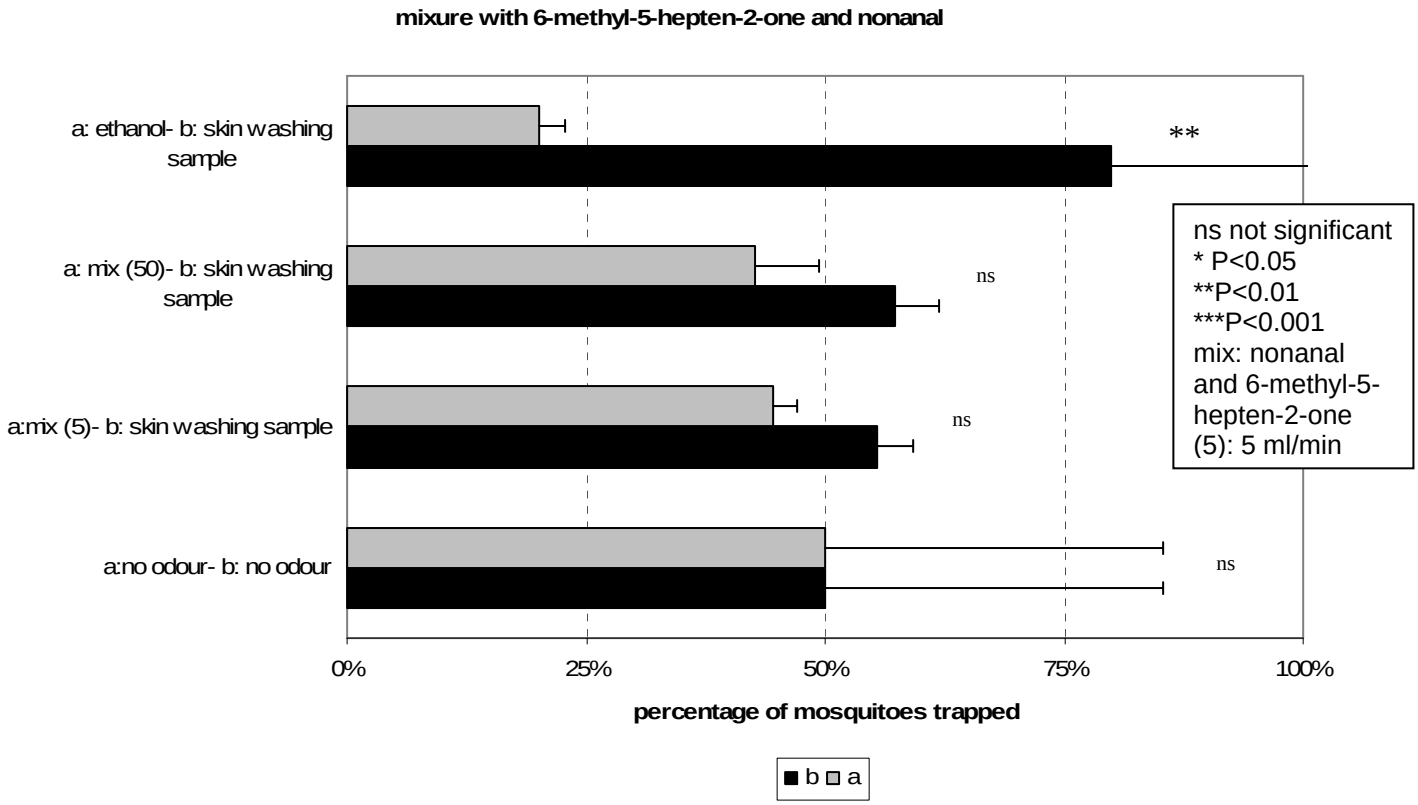

Figure 11 Responses of mosquitoes to different concentrations the blend with 6-metyl-5-hepten-2-one and nonanal combined skin sample tested against a skin sample alone. The total percentage of mosquitoes that flew into either port is shown. The error bars show the standard error of mean for each treatment. Ethanol is tested against skin sample as control experiment and the skin sample attract significant (P<.0.01) more mosquitoes.

3.2 Total responses for each test series

The total response is the number of trapped mosquitoes as fraction of the total number of mosquitoes that flew into the flight chamber. The lowest reaction of female mosquitoes was on the treatment without odour: In all test series less than 25% of the released mosquitoes were trapped (See Figure 12-18).

The summed total response of all treatments with the skin sample as human odour equivalent was 37.5% and the summed total response of all treatments with a worn sock as human odour equivalent was 28.5%.

For each treatment the total responses of each treatment were statistical tested and compared. Significant differences between responses for each treatment were calculated for each test series.

3.2.1 Control experiments

No significant (P>0.05) differences between total responses of each treatment were seen in the control experiment test series. See Figure 12.

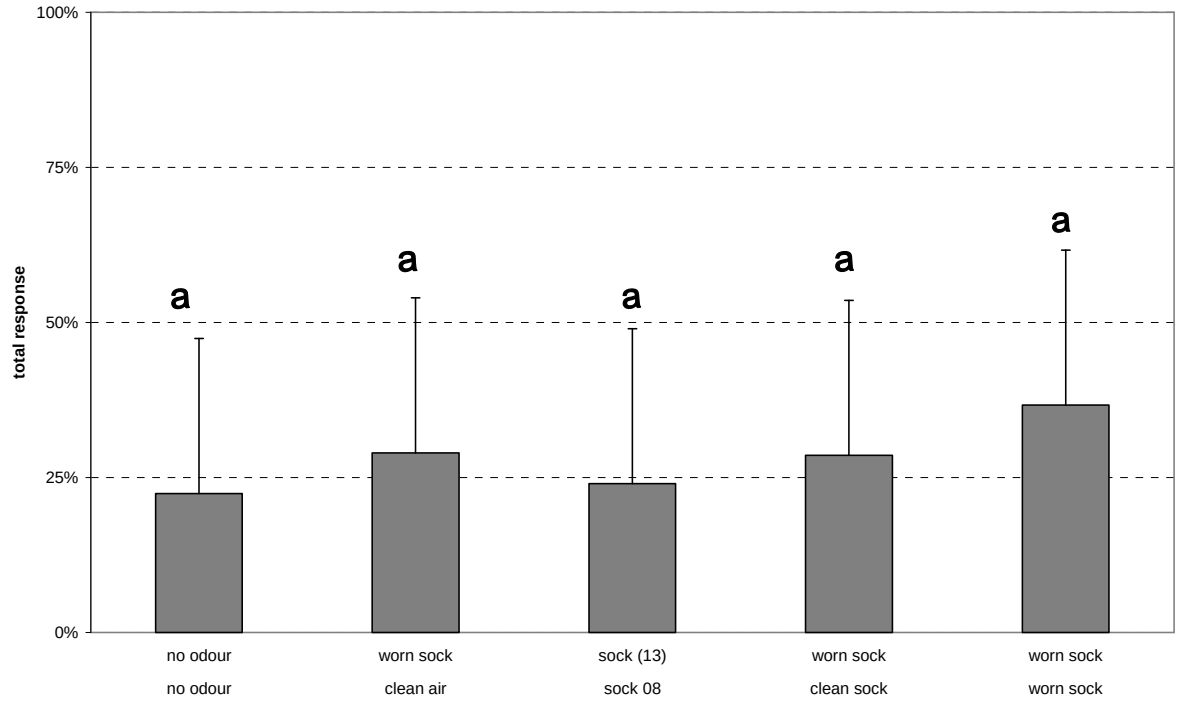

Figure 12 Total responses of mosquitoes on different treatments.

3.2.2 Geranyl acetone

The statistical test (GLM) showed significant effect of the treatments (P<0.05). The post-hoc test showed that the total response from the experiment without any offered odour was significant lower (P=0.022) than the total response of the test with geranyl acetone in the concentration of 5 ml/min. Other treatments did nit show differences (P> 0.05). See Figure 13.

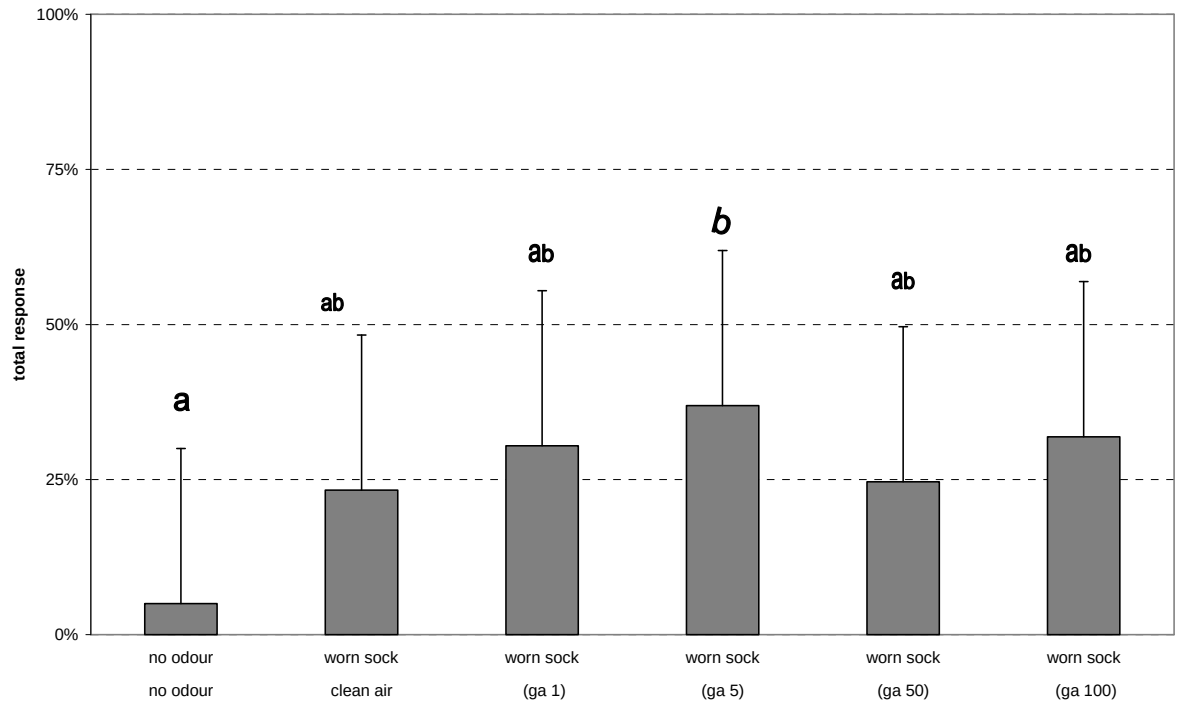

Figure 13 Total responses of mosquitoes to different treatments with geranyl acetone combined with worn socks in different concentrations. Differences in total response were seen between no odour-no odour and the test series with ga (5) (P 0.022). The mark *a* means significant different from the other bars (*b*). Ga (1): geranyl acetone at 1 ml/min.

3.2.3 6 methyl-5-hepten-2-one with worn socks

The statistical test (GLM) did not show significant differences between the total responses of each geranyl acetone treatment (P>0.05). But because it was very close to the significant level and there were strong evidences that a post-hoc test would show differences this test was done afterwards. The post-hoc test showed that the total response from the experiment without any offered odour differed significant (P=0.03) from the total response of the test with a worn sock tested against clean air. No other significant differences (P<0.05) were seen. See Figure 14.

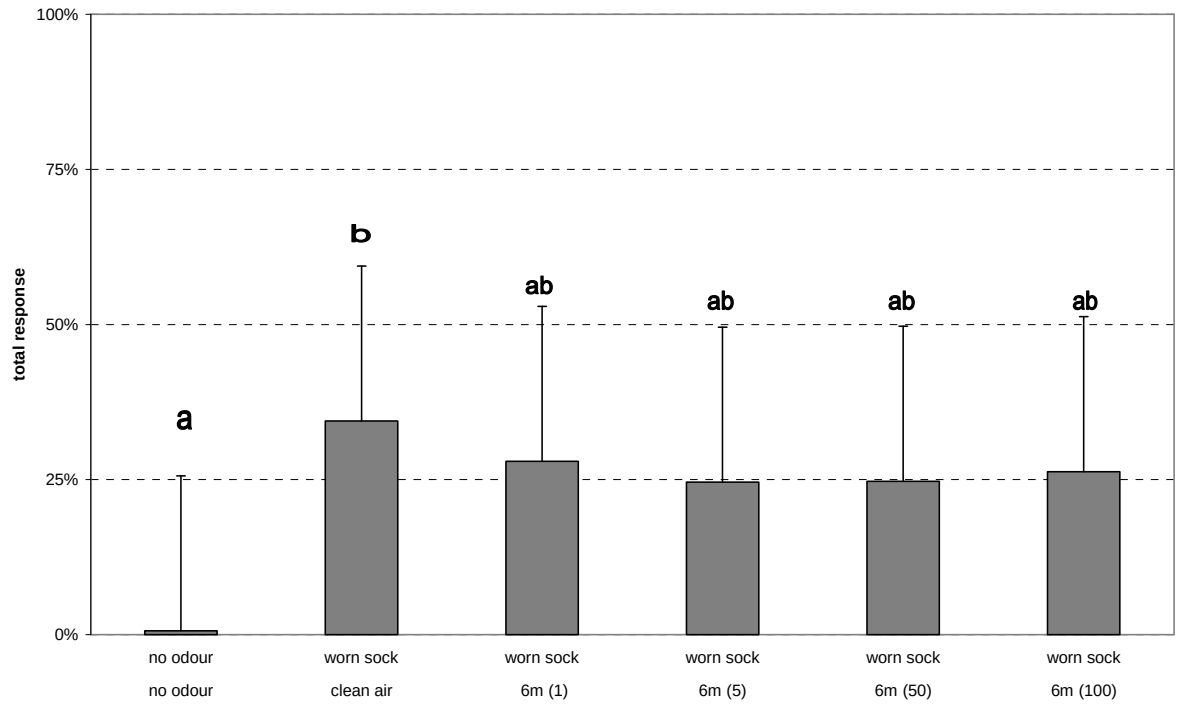

Figure 14 Total response of *An. gambiae* mosquitoes on treatments with different concentrations of 6-methyl-5-hepten-2-one. Significant differences were seen between the no-odour treatment and the worn sock-clean air experiment (P 0.03). 6m: 6-methyl-5-hepten-2-one, each number represents a concentration in ml/min.The bar marked with *a* is significant different from one of the other series (*b*).

3.2.4 6-methyl-5-hepten-2-one with skin washing samples

Significant differences between total responses of the odourless treatment and the treatment with ethanol against skin washing sample could be seen. Also differences in total response of 6-methyl-5-hepten-2-one in the concentrations of 50 an 100 ml/min differ significant from the odourless treatment ($P<0.001;0.023;0.043$).

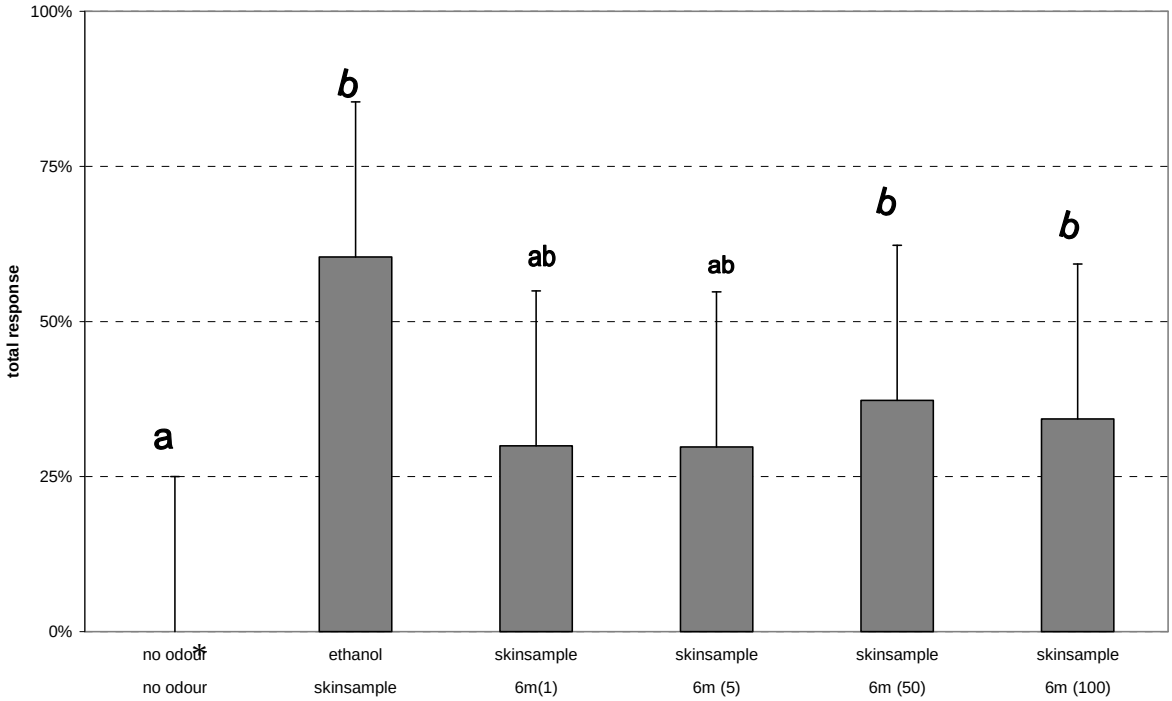

Figure 15 Total response of mosquitoes that flew into one of the traps in each treatment. Significant differences could be seen between no odour- no odour and ethanol/skin sample and 6-methyl-5-hepten-2-one in to concentrations (50 and 100 ml/min) (P<0.001; 0.023; 0.043). The bar marked with *a* is significant different from the bars marked with *b*.

3.2.5 Nonanal

The statistical test (GLM) did show significant differences between the total responses of each of the tests with nonanal. A post-hoc test showed that the total response from the experiment without any offered odour was significant lower than the total response of the test with nonanal in the concentrations of 5, 50 and 100 ml/min (P: 0.03;0.025;0.025).

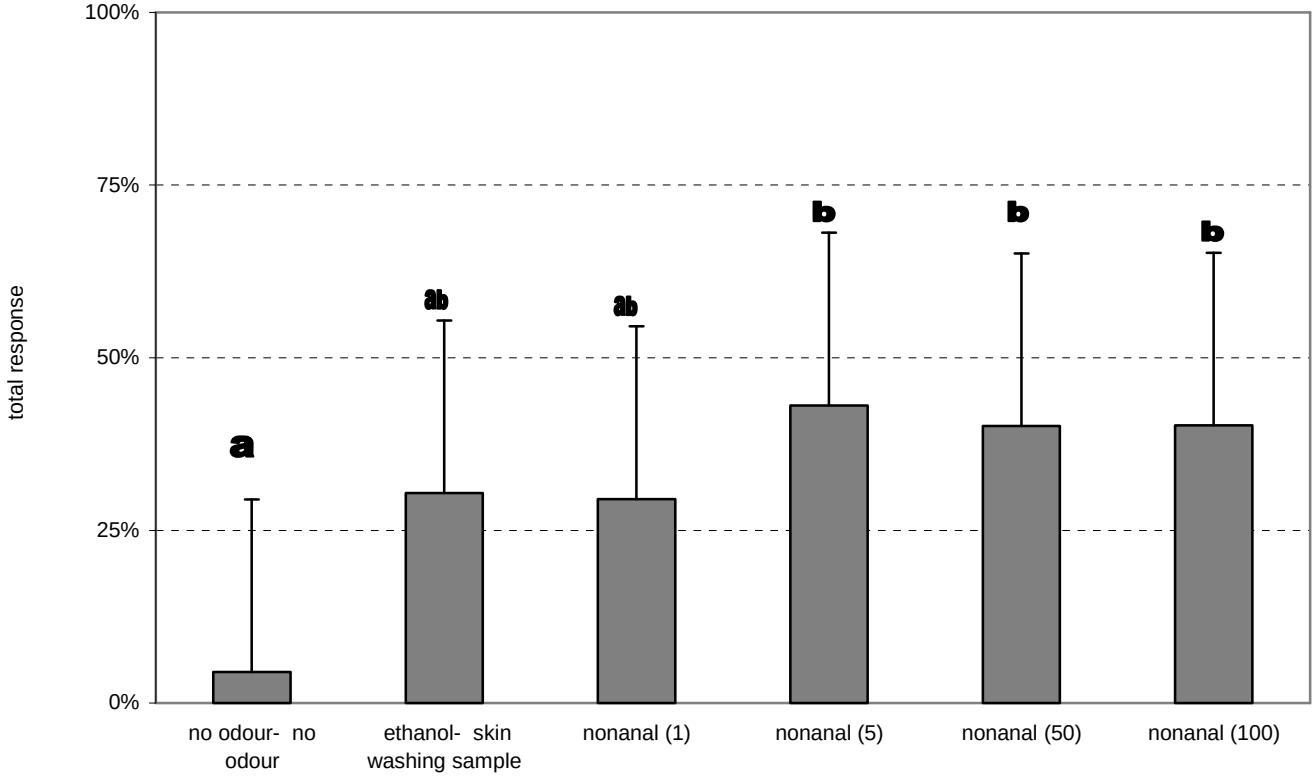

Figure 16 Total responses *An. gambiae* mosquitoes on treatments with different concentrations of nonanal. Each number represents a concentration in ml/min. Significant differences were seen between the test without offered odours and nonanal in concentrations 5, 50 and 100 ml/min (P: 0.03;0.025;0.025). *a* is significant different from one of the other series (*b*).

3.2.6 Indole

No significant differences between the number of mosquitoes that flew into the ports were seen between the different treatments with indole (P>0.05).

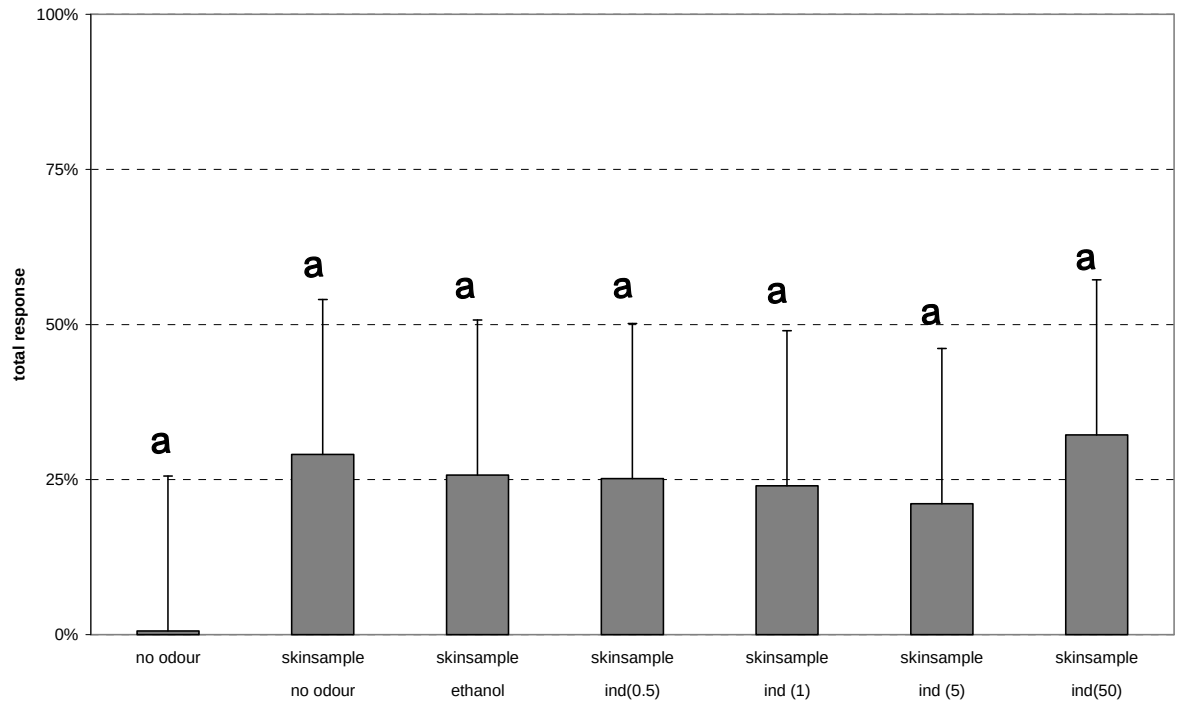

Figure 17 The total number of mosquitoes that flew into a port for each treatment as function of total number of mosquitoes that flew into the flight chamber. No significant differences (P> 0.05) in total responses between the treatments were seen.

3.2.7 Mixture

The total responses of all the different treatments in the test series with the mixture of nonanal and 6-methyl-5-hepten-2-one did not differ significantly from each other except for the series with no added odour compared with the series with skin sample and mix at the flow rate of 5 ml/min. Significant less mosquitoes (P 0.032) responded tot the no odour treatment compared with the treatment with the mixture (5 ml/min).

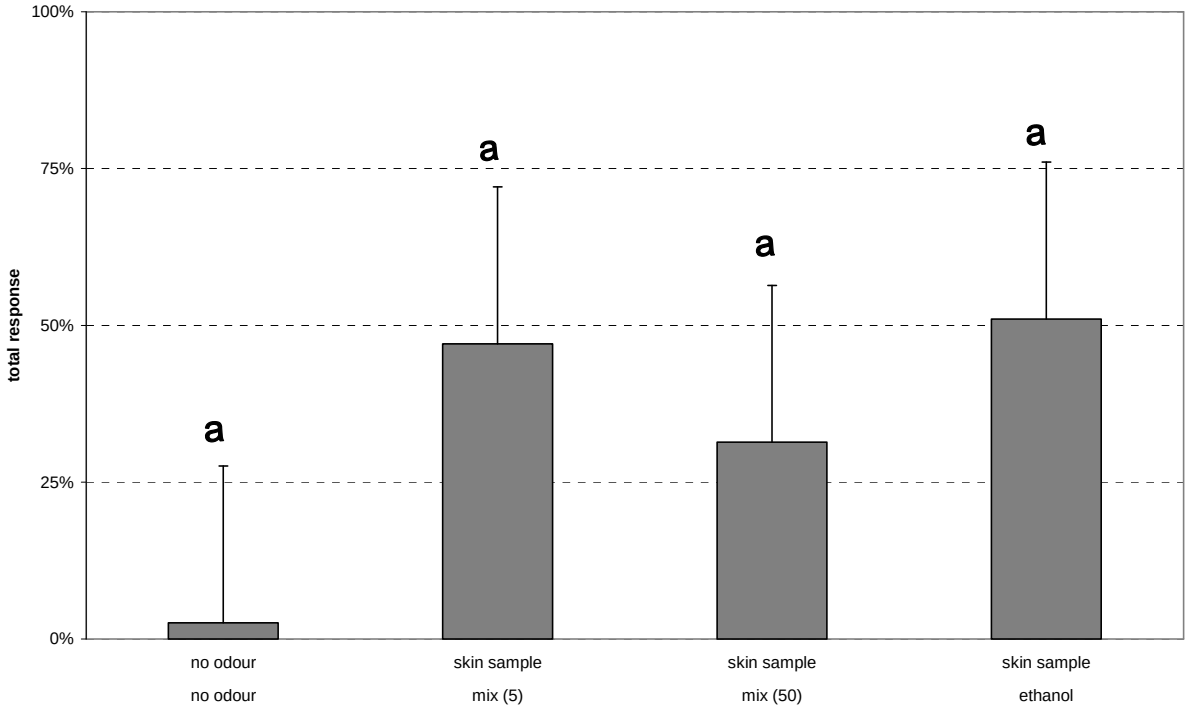

Figure 18 Total responses of female *An. gambiae* mosquitoes on treatments with different concentrations of the mixture. Each number e.g. (5) represents a concentration in ml/min. Significant differences in total response can be seen between no odour-no odour and skin sample with mix at the flow rate of 5 ml/min (P 0.032). * means significant different from one of the other series.

4 Conclusions and discussion

Why are people attractive to malaria mosquitoes? Why are some people more attractive to mosquitoes then other people? Many previous studies are based on these questions. In these studies the aim was to find specific components of human emanations that act as key compounds in mosquito host seeking for example Bernier and others 2000 and Smallegange and others 2003 .This present study had the same basic question; which odours play a role in mosquito host seeking behaviour?
The goal in this research was to test several known and putative repellent compounds in different concentrations in a bioassay. The main question was:

Which concentrations of known and putative allomones decrease or overrule the attractiveness of human odour for female *An. gambiae*?

This question is split in two research questions;

1. **What is the effect of concentration on repellency of the selected compounds?**
2. **What is the effect of these compounds in a blend?**

To test these research questions an experiment was designed in which different test compounds were tested for a behavioural response of *An*. *gambiae* females. Four test compounds were selected and tested with worn socks or skin sample as human odour equivalent. Conclusions and discussion points from each of these test series are worked out for each of the different compounds.

4.1 Control experiments

- **Symmetry tunnel**

Before the experiments with the test compounds could start first the dual port olfactometer had to be tested. The olfactometer should generate clear data and the aim of the control experiments was to test the symmetry of the olfactometer. No significant differences were found between both ports. The dual port olfactometer was symmetric and could be used in the experimental setup.

- **Attractiveness of worn socks**

The experiments have to be accomplishing with human odour in combination with the one of the selected test compounds. As human odour equivalent worn socks were chosen in the first three test series. These worn socks were higly attractive in previous studies (Qiu 2005; Pates and others 2004). Before the experiments with the test compounds could start first the worn socks had to be tested if they were really attractive to female *An. gambia* mosquitoes and could be used in this setup. Worn socks were found to be highly attractive and could be used for the further experiments.

- **Attractiveness of skin washing sample**

Four test series were done with another human odour equivalent, a skin washing sample. Previous research showed that human skin washing with ethanol were reliable mosquito attractants (Pates 2002). Before the experiments with the test compound could start first different concentrations of skin washing sample were tested to determine which concentration could be used. In these tests diluted (0.01%) human skin sample was most attractive to the female mosquitoes.

- **Differences between days**

In the control experiment series also socks worn at two different days were tested against each other to see whether they were significant different in attractiveness. This was to determine significant day effects. The socks that were used in this test were used in the later test series. The worn socks were slightly different between both days, this could be explained by differences in diet, differences in materials that contacted the worn socks or due to different behaviour of the person that donates the socks on different days.
Results from this test series show slightly differences between attractiveness, but no significant differences. This is important because otherwise no comparing of results could be done.

4.1.1 Conclusions

- The dual port olfactometer was symmetrical
- Worn sock were highly attractive and could be used as human odour equivalent
- No significant differences between socks worn at different days were found

4.1.2 Discussion

Human odour equivalents

In the experiments are two different human odour equivalents used. First the experiments were started with worn socks. These were found out to be highly attractive studies (Qiu 2005; Pates and others 2004). A special construction should prevent contaminating with test odours. The use of worn socks in the experimental setup has some disadvantages. Although the worn socks were presented in a special construction, there are evidences that socks were contaminated with test odour in one test series.

The worn socks were used intensive. It could be that the attractiveness of the socks would reduce with time. Therefore in each test series a new pair of worn socks is used. But they were not totally equal. For practical reasons (future research that would require socks from the same volunteer to be comparable) worn socks are not a preferred method. The continuity of the research would require a more standard human odour sample.

Because of these disadvantages there is chosen to try another human odour sample. Human skin washing samples which were presented on glass slides were used only once, this prevents reduction of attractiveness after a few test days and also contamination with the added test compound. These washing samples could also be used for future research and are standard. But this method is only useful if it appears to be highly attractive too and will give a high behavioural response. Results from this study show a good total response for each test series with skin washing samples as human odour equivalent and this human odour sample is therefore appropriate for behavioural experiments.

4.2 Geranyl acetone

For geranyl acetone the expectation was that this compound would show repellency. Geranyl acetone is present in both fresh collected and incubated sweat (Meijerink and others 2000) in a relative high amount. This could suggest that it can induce a behavioural response in *An. gambiae.* In Qiu (2005) for this compound repellency is reported at the concentration 5 ml/min. Geranyl acetone is tested in a dual port olfactometer in four different concentrations; 1, 5, 50 and 100 ml/min. These different amounts are tested in combination with a worn sock which was the human odour equivalent and highly attractive. None of the four concentrations tested in combination with a worn sock showed significant difference with the control which contained only a worn sock. Geranyl acetone is therefore not able to decrease or overrule the attractiveness of human odour.

4.2.1 Total response

For al of the treatments the total response was higher compared with the control with only clean air and worn sock. This indicates that the treatment didn't influence the total response.

4.2.2 Conclusions

- No repellency was found in experiments with geranyl acetone. No significant attraction was found neither.

4.2.3 Discussion

It was expected that in the experiment with the worn sock against clean air the worn sock would attract significant more mosquitoes. Although there were more female mosquitoes trapped with the sock-treatment the differences between both treatments were not significant. A reason for these unexpected data could be contamination of the mosquito trap with human odour.

Less attractive people are likely to have some repellent compounds in their odour spectrum that overrule the attractiveness of other present (attractive) compounds and are therefore less frequently bitten by mosquitoes. The concentration is very important, possibly only the right dose could cause this reaction. Also the abundance of different possibly repellent components could be important. Maybe the repellent reaction can only be seen when a combination of odours is present.

Geranyl acetone is tested previously with different insects. In this studies geranyl acetone is tested in combination with other test compounds, in combination with ammonia and lactic acid (Smallegange and others 2005) and in combination of at least 7 other compounds (Jumean 2004a; Jumean 2004b). This could indicate that geranyl acetone can only act in combination with other compounds or has a stronger reaction acting in a blend. Geranyl acetone is also reported to be part of a larval aggregation pheromone of the codling moth, *Cydia pomonella*, in the study of Jumean and others 2004a and Jumean and others 2004b. This mixture of different volatile compounds (which contains for example geranyl acetone, 6-methyl-5-hepten-2-one and nonanal) attract a parasitoid species *Mastrus ridibundus.* In this mixture geranyl acetone is one of the essential components to elicited attraction.

For each of the experiments with test compounds the expectation was that the chosen test compound would influence the attractiveness of the human odour equivalent. This compound did not show this effect. Geranyl acetone did show a repellent effect in the study of (Smallegange and others 2005). At the flow rate of 5 ml/min significant less mosquitoes flew in the port with the test compound. A bit contra dictionary with the results of (Smallegange

and others 2005), the results in this report did not show any effect of the concentration of 5 ml/min and neither significant effects of the other tested concentrations were seen.
An explanation of the difference between both studies could be the experimental procedure. In (Smallegange and others 2005) the test compounds are tested against a standard attractive blend containing ammonia, and lactic acid. This is different from the method in this report, the test compounds are tested against a human odour equivalent. This human odour equivalent contains lots of different components with unknown concentrations. The actual offered concentration of geranyl acetone is due to the combination of the test compound with human odour and is unknown and could be quite different from the concentration that is offered in in the study of (Smallegange and others 2005).

4.3 6-methyl-5-hepten-2-one

In a behavioural study in a dual port olfactometer 6-methyl-5-hepten-2-one has been sown repellency at a flow rate of 50ml/min Qiu (2005). In the study of Bernier and others (2000) this component was slightly more abundant in attractive people, however 6-methyl-5-hepten-2-one has been found to be slightly decreased at a more attractive day. In the study of Curran and others (2005) not all persons that were tested contained this component. The expectation in the experiments with 6-methyl-5-hepten-2-one was that this component could show repellency in a concentration comparable with the found concentration in Qiu (2005). However the behavioural response is, due to (a bit) contradictory literature, partly unknown. In different studies with insects 6-methyl-5-hepten-2-one was reported to induce behavioural effects. In the study of Birkett and others 2004 6-methyl-5-hepten-2-one was tested on cattle flies using gas chromatography-electrophysiology, coupled gas chromatograpy-mass spectrometry, electrophysiology, laboratory behaviour and field studies. For all tested species this compound was physiologically active. In a wind tunnel the upwind flight behaviour was tested, 6-methyl-5-hepten-2-one increased upwind flight but in the field study a tendency towards a repellent effect of 6-methyl-5-hepten-2-one was reported and fly loads were reduced by this component. In identification study of volatile emissions from *Platyphus mutates*, a coleopteran species 6-methyl-5-hepten-2-one is reported to act as attractant for females and this component is part of a sex pheromone (Audino 2005). 6-Methyl-5-hepten-2-one is also reported to be part of a larval aggregation pheromone of the codling moth, *Cydia pomonella* (Jumean 2004a; Jumean 2004b). This mixture of different volatile compounds (which contains for example 6-methyl-5-hepten-2-one, nonanal and geranyl acetone) attract a parasitoid species *Mastrus ridibundus.*

6-Methyl-5-hepten-2-one was tested twice. The first test series was done with worn socks as human odour equivalent and the experiment was repeated with skin washing samples as human odour equivalent.
The reason to repeat the test series with this test compound was to see whether both human odour equivalents, the worn socks and the skin washing samples, would generate the same data.

4.3.1 6-methyl-5-hepten-2-one combined with worn socks

In the test series with 6-methyl-5-hepten-2-one in combination with worn socks the test compound was tested in four different concentrations. Flow rates were tested are 1, 5, 50 and 100 ml/min. Significant differences between the control port which contained only a worn sock compared with the treatment port which contained the test compound in combination with the worn sock were seen at two concentrations, 1 and 5 ml/min. The other two concentrations (50 and 100 ml/min) did not show a significant difference. At the flow rate of 1 ml/min and at the flow rate of 5 ml/min significant less mosquitoes were trapped: 6-methyl-5-hepten-2-one decreased the attractiveness of human odour and showed to be repellent at these concentrations.

4.3.2 Total response

The total responses for this test series showed the highest response to the experiment with clean air against worn socks. For all the tested concentrations 6-methyl-5-hepten-2-one the total response was lower. This would indicate that 6-methyl-5-hepten-2-one influenced the total response and reduced the number of catched mosquitoes. But the responses to the different concentrations were not significant different from the responses to the experiment without added test compound.

4.3.3 6-methyl-5-hepten-2-one combined with skin washing samples

In the test series with 6-methyl-5-hepten-2-one in combination with skin washing samples the test compound was also tested in four different concentrations. The tested flow rates were 1, 5, 50 and 100 ml/min. No significant differences between control and treatment were seen at the concentration of 1 m/min and for the concentration of 100 ml/min. At the flow rate of 5 and 50 ml/min significant differences between control and added test compound were seen: 6-methyl-5-hepten-2-one decreased the attractiveness of human odour and showed to be repellent at these concentrations.

4.3.4 Total response

The treatment with ethanol at one site and skin washing sample at the other site attract a large amount of mosquitoes. The total responses in the experiments with 6-methyl-5-hepten-2-one in different concentrations were lower. This could indicate that adding 6-methyl-5-hepten-2-one reduced the number of trapped mosquitoes. However the total responses in the experiments with skin washing sample did not significantly differ from the total responses of the experiments with 6-methyl-5-hepten-2-one.

4.3.5 Conclusion

- 6-methyl-5-hepten-2-one was repellent at the flow rates of 1 and 5 ml/min in combination with worn socks
- 6-methyl-5-hepten-2-one was repellent at the flow rates of 5 and 50 ml/min in combination with skin samples

4.3.6 Discussion

No difference between skin washing sample and the ethanol treatment. The skin washing sample did trapped a larger number of female mosquitoes but was not significantly attractive. A good explanation might be contaminating. Evidences for contamination are the unexpected numbers of mosquitoes trapped when only ethanol was added and the relative high total response in this treatment. If the port or the trap with the ethanol treatment is contaminated with human odour it is likely that at that site large numbers of mosquitoes were trapped also. This can also explained the relative high total response.

One of the effects you might expect is you test a repellent is that the total response would be lower because the repellent would reduce the attractiveness of the human odour and therefore you expected a reduction in upwind flights and trapped mosquitoes. The total response was in the control treatment with only human odour equivalent (worn socks and skin washing sample) higher than the experiments with the added test component in different concentrations. This might indicate that 6-methyl-5-hepten-2-one reduced the number of trapped mosquitoes in the port with the compound and also could reduce the total number of trapped mosquitoes. It seem to be a trend but this are evidences because the compound 6-methyl-5-hepten-2-one did not cause significantly differences.

4.3.7 Differences in results between series with worn sock and skin washing

The test series with 6-methyl-5-hepten-2-one was done twice. The first series had worn socks as odour equivalent, the second series skin washing sample. Presumed was that the repetition of the series would give the same outcome. Both series did show a reduction of

number of mosquitoes that flew into the port with added compound at two concentrations. Remarkably these concentrations were not the same.
In the first test series 6-methyl-5-hepten-2-one was added in combination with a worn sock. A repellent effect was seen at two flow rates, 1 and 5 ml/min. In the second series 6-methyl-5-hepten-2-one was combined with skin samples. Repellent effects of the added compound were seen in this case at 5 and 50 ml/min.
Why are the results not the same? What factors could cause the difference? The experimental procedure differs in both series. The only change was in the choice of human odour equivalent. So it would be expected that differences in both odour samples causes the difference and shift of the effective repellent concentration.

The samples were from different persons, people have a unique odour spectrum. This means that people differ in

- Which compounds are abundant
- Concentration of the different compounds
- Skin microflora (they play a major role in body odour composition (Braks and others 2000)).

It might be possible that in the skin samples that were used in the experiments contained a compound that partly reduced the repellent effect of the test compound so that a higher dose was required to induce a repellent effect. Or that compounds present in the socks could improve/enhance the repellent effect of 6-methyl-5-hepten-2-one. This could explain the effect at the lower concentration.
Another cause might be that the worn socks contained a higher concentration of 6-methyl-5-hepten-2-one and less had to be added to induce repellence. In the skin sample might be less present and therefore the allomonal effect of the test compound was seen at a higher concentration compared with the series with worn socks.

4.4 Nonanal

No direct concentration results from previous studies with *An. gambiae* in an olfactometer experiment are known. Evidence for a repellent effect result came from a study of Du and Millar (1999). In this study a dose response EAG showed an increase in response at higher dose and a bioassay with *Cx. quinquefasciatus* nonanal showed to be attractive at 0.01 microgram/liter.

The test series with nonanal is done with four different test concentrations, which are 1, 5, 50 and 100 ml/min. Significant differences were demonstrated at the flow rates of 5 and 50 ml/min. At these concentrations the attractiveness of the skin washing sample was suppressed by the addition of nonanal. The other two tested concentrations did not show any significant differences.

Nonanal is reported to induce behavioural effects in different insects. In (Puri 2006) nonanal is tested with an other mosquito species, *Culex quinquefasiatus*. EAG responses and behavioural responses were tested for different human emanations. Nonanal elicted a maximum EAG response at 0.01 g but a decrease in response was observed with an increase in the dose. Nonanal was also tested in a y-tube. The result of the behavioural test in this experimental setup was a higher flight orientation response. Maximum response was 87,3 % at 0.1 gram (Puri 2006). The response decreased when the dose increased but was still higher with the control. In the study of Puri (2006) nonanal acted as kairomone and dose-dependency is suggested. The result of this present study and the study of Puri (2006) study might indicate that nonanal can act as allomone and as kairomone as well.

Nonanal is also reported to be part of a larval aggregation pheromone of the codling moth, *Cydia pomonella*, in the study of Jumean and others 2004a and Jumean and others 2004b. This mixture of different volatile compounds, which contains nonanal for example and also the tested componets of the present study 6-methyl-5-hepten-2-one and geranyl acetone, attract a parasitoid species *Mastrus ridibundus.* Nonanal in combination with octanal and decanal or 3-carene are necessary to induce a behavioural response of the mixture which contains also (E)-2-octenal, (E)-2-nonenal, 6-methyl-5-hepten-2-one and geranyl acetone (Jumean 2004a; Jumean 2004b).

4.4.1 Total response

The total responses for nonanal we lowest for the first test concentration (nonanal 1 ml/min) and for the control treatment with skin washing sample and at the other site ethanol. The total responses for the other concentrations were a bit higher. None of the total responses differed significantly from one of the others. No evidences for a reduction of the numbers of trapped mosquitoes were found in the experiments of this series.

4.4.2 Conclusion

- Nonanal was repellent a two different concentrations, 5 and 50 ml/min

4.4.3 Discussion

If you added a repellent compound into the dual port olfactometer you will expect that this compound would spread into the flight chamber. As a result of this spreading you would expect that the mosquito host seeking behaviour is influenced and that less mosquitoes would fly into the flight chamber as reaction on the repellent odour. In the test series with nonanal no effect on the total response was seen. But significant less mosquitoes were trapped at two concentrations. So can you speak of a repellent in this case? The mode of action of nonanal is not known. Does nonanal only work at very specific concentrations, or only at short range? It seems that this component does not influence the willingness to fly into the flight chamber and into one of the ports but that there was only a difference in choice of in which port they flew.

4.5 Indole

In Qiu (2005) an olfactometer study was accomplished which includes the compound indole. In this study a repellent effect of indole was found at 0.1~0.2 ml/min. Indole was also tested in combination with ammonia and lactic acid in the same study and in this combination indole acted as repellent at 0.5 ml/min. Because of the results from the study of Qiu (2005) the chosen test concentrations in the experimental setup of the experiments with indole included a fifth test concentration, which was 0.5 ml/min.
None of the tested concentrations gave significant differences with the control experiments.

Total response

The total responses gave a remarkable result. First the total response seems to decrease with concentration. The flow rate 0.5 ml/min had a lower total response than only skin washing sample tested against ethanol. The lowest total response was with flow rate 1 ml/min. At higher doses the total responses seems to increase again. At 5 ml/min the response was a bit higher than at 1 ml/min and at 50 and 100 ml/min the total response was higher than the response to the control with skin washing sample and ethanol.

4.5.1 Conclusion

- Indole did not show any behavioural effect.

4.5.2 Discussion

For indole it was also expected to see allomonal effects of this component. But none was seen. This test component was chosen because of the previous reported allomonal effects (Smallegange and others 2005). For the test compound indole this could also maybe be explained by concentration. The actual concentration that was offered was unknown and therefore it is possible that not the right ranges of concentrations were tested and no significant results were seen.

For indole there is also a previous reported indication that this compound would react different in a blend. Smallegange (2005) reported a repellent effect for this compound tested on its own. In this study this effect of indole was found at 0.1~0.2 ml/min. Indole was also tested in combination with ammonia and lactic acid in the same study and in this combination indole acted as repellent at 0.5 ml/min. In the experimental procedure each test compound is combined with a human odour equivalent and this can be seen as a blend too. Not only in the test series with indole this might have influenced the results, this could also have influenced the results of the series with other test compounds.

The previous reported allomonal effect of indole was at 0.1~0.2 ml/min. In combination with ammonia and lactic acid at 0.5 ml/min. This indicates that the repellent concentration of indole is dependent on the combination of compound in which it is tested in. It could be that indole acted different in the combination of all component that were present in the skin washing sample and that the offered actual concentration, which is unknown, was not accurate to act as repellent. The tests in this series were done at a higher dose as previous tests. Maybe an effect of indole can only be seen in very low concentrations.

The results of the test series with indole did not show any repellent effect. And no kairomonal effect either, although there are evidences that indole can act as kairomone as well (opzoeken refs, increase in ouder zweet oid). In the results the highest dose, which was 100 ml/min attracted slightly more mosquitoes were trapped compared with the skin washing sample.

4.6 Mixture

Based on the results from the test series with geranyl acetone, 6-methyl-5-hepten-2-one, nonanal and indole a mixture is composed which contained 6-methyl-5-hepten-2-one and nonanal. This mixture was tested in two concentrations, at 5 ml/min and at 50 ml/min. The expectation was that the blend of two previous tested compounds would show repellency at the same concentrations as they showed repellency when they were tested separately. But both concentrations of the mixture did not show significance.

Total responses

The total responses in the series with the mixture did not differ significantly from each other

4.6.1 Conclusion

- The mixture did not show any behavioural effect.

4.6.2 Discussion

Chosen concentrations

In the series with the mixture this mixture was tested in two concentrations, in those concentrations that the compounds on its own showed repellency. No significant repellent effects were seen when two compounds that act as allomone on its one were combined. An explanation could be that compound could react differently in a blend and show an effect in different concentrations then reported before (Osterkamp and others 1999; Smallegange and others 2005).

In all test series except the control series a concentration range of the putative repellent was tested. This range was chosen because the same range was used in (Qiu 2005). Most of the chosen test compounds were tested before in that study and did show a repellent effect in this concentration range. Using the same range would make the results comparable.

But was the use of a standard concentration range, which was used in the experiments, appropriate for all the tests? Was the range maybe too small, or too broad or not specific enough? Results of the series with geranyl acetone and indole show that no repellent effects were seen in this concentration range. A compound specific range (for example with concentrations chosen around the previous reported repellent result) could maybe give more reported repellent results and a better insight. An explanation of the difference between previous reported results (Qiu 2005) and results from this study could be that the concentration of the added test compound was not suitable to induce repellence. It might be that in another concentration range this effect could be seen. Disadvantage of specifying the concentration range of each test compound is that comparability between studies and between compounds would be more difficult. Solution for this problem could be a broader concentration range, which contains all the concentrations from previous study and also lower or higher concentrations. The test series with geranyl acetone, indole and the mixture might show a repellent effect at another concentration. Components might act different in a blend, this is previously reported by Osterkamp 2005. A dilution of the mixture would maybe show repellency.

4.7 Used methods

In this paragraph the appropriateness of the used experimental methods are discussed. In special some additions to the use of mosquitoes, the use of both different human odour equivalents will be discussed, and the chosen test concentrations will be evaluated.

4.7.1 Mosquitoes

All behavioural experiments were done with mosquitoes. Experiments with living animals lead to factors which are unable to control. This means that the condition of the animals that are used influence the results. Sometimes there was evidence that the condition of the *Anopheles gambiae s.s.* females, used at a specific test day, did influence the clearness of the results. These results were not analysed. Doubts about the clearness of the results were for example caused by many dead mosquitoes in the release cages (more than 5 in a cage). Mosquitoes were probably too weak for a good behavioural response. Many factors can influence the conditions of the test insects. There are evidences that, although they are kept in climate cells in continuous climate conditions, weather might influence mosquito response. For successfully completion of experiments large numbers of insects are obtained.
The results of different experiments and studies should be comparable and this requires a continuous rearing under the same circumstances. The condition of the mosquitoes might influence experimental results and therefore a healthy mosquito colony is one of the most important conditions for successful experiments and good test results.

4.7.2 Human odour equivalents

In the experiments are two different human odour equivalents used. First the experiments were started with worn socks. These were found out to be highly attractive. A special construction should prevent contaminating with test odours. The use of worn socks in the experimental setup has some disadvantages. Although the worn socks were presented in a special construction, there are evidences that socks were contaminated with test odour in one test series.
The worn socks were used intensive. It could be that the attractiveness of the socks would reduce with time. Therefore in each test series a new pair of worn socks is used. But they were not totally equal. And for practical reasons (future research that would require socks from the same volunteer to be comparable) worn socks are not a preferred method. The continuity of the research would require a more standard human odour sample.
Because of all these disadvantages there is chosen to try another human odour sample. Human skin washing samples which were presented on glass slides were used only once, this prevents reduction of attractiveness after a few test days and also contamination with the added test compound. These washing samples could also be used for future research and

are standard. But this method is only useful if it appears to be highly attractive too and will give a high behavioural response. Results from this study show a good total response for each test series with skin samples as human odour equivalent and this human odour sample is therefore appropriate for behavioural experiments

4.8 Hypothesis

Before the experiments were started the hypothesis, based on previous studies, was formulated. This hypothesis was

- Less attractive people are likely to have some repellent compounds in their odour spectrum that overrule the attractiveness of other present (attractive) compounds and are therefore less frequently bitten by mosquitoes. The concentration is very important, possibly only the right dose could cause this reaction. Also the abundance of different possibly repellent components could be important. Maybe the repellent reaction can only be seen when a combination of odours is present.

Results from this study not always completely agree with the expectation beforehand.

Results from both 6-methyl-5-hepten-2-one series and the nonanal series show that it could be possible that some present repellent compounds reduce attractiveness for malaria mosquitoes. Results show that concentration is important, not all tested concentrations in the series with 6-methyl-5-hepten-2-one and nonanal did show repellency.
A combination of repellent odours, which were nonanal and 6-methyl-5hepten-2-one did not show a repellent effect. This could be due to concentration. In previous studies it is shown that a single component that showed an allomonal effect when tested on its own react different in a blend and did not show this repellent effect when it was combinated with other compounds (Osterkamp and others 1999; Smallegange and others 2005). In these studies a lower concentration was obtained to induce the allomonal effect of the blend.
It is likely that some volatile human emanations mask, reduce or overrule the attractiveness of human odours and might be used for development a mosquito control method.

4.9 Recommendations

In this experiment a combination of a human odour equivalent and a putative repellent compound are offered to mosquito females. The composition of the compounds present in the different human odour equivalents is unknown. Therefore also the actual offered concentration of the tested compound, that likely also is present in the human odour samples

that are offered, is unknown. It would be useful to know what combination of odour compound is present in the worn socks and skin samples that were used and in what amount.

The worn socks used in the test series with 6-methyl-5-hepten-2-one were likely contaminated with test compound at the end of the series. The highly attractive socks did show repellence. It would be very interesting to analyse these socks and compare them with other worn socks of the same volunteer. What is the concentration of test compound in these socks? Is this higher than expected, compared with the uncontaminated pair? Differences in concentrations could give evidence for the concentration that caused the repellent effect.

Very less is known about dose-response reactions from mosquitoes. And in special less is known of dose-response reactions of natural (human born) repellents. There are evidences and previous reported results that show that undiluted offered odour compounds (aliphatic fatty acids and skin samples (Knols and others 1997) ;Smallegange, unpublised data) induce repellence, and very diluted show attraction (Knols and others 1997). Extended knowledge about this topic might lead to better insight in mosquito behaviour and can be used as a tool for the design of future behavioural experiments.
There are evidences that some components could cause a repellent reaction at a particulair concentration and might show attraction on another concentration. This is reported previously in a blend with volatile carboxylic acids which showed repellence but caused attraction when it was diluted (Knols and Meijerink, 1997). Indole could be a component that might show both an allomonal and kairomonal behavioural response. A broader range of concentrations should be tested to investigate this hypothesis. Also nonanal could be a candidate to test a broader range of doses to prove these hypotheses, because it is previously reported to be a kairomone for another, *Culex quinquefaciatus*, mosquito species (Puri 2006).

A disadvantage of the experimental setup is that there are no previous data of a known mosquito repellent in this kind of setup. It would be useful to test a known repellent with the same experimental procedure and to link these data to the results as was done in Birkett and others (2004). Are the same behavioural responses seen with the known and putative repellent? A disadvantage of the use of mosquito repellents that are commercially available is that there is a chance that they will contaminate the olfactometer and can not be removed from the flight chamber, possibly the olfactometer can not be used afterwards. It would be useful to search for a repellent that is able to ‘calibrate’ the experimental setup without the disadvantage of contaminating the olfactometer.

Another disadvantage of the use of a dual-port olfactometer in the present experimental setup is that the flight chamber is closed. There is no ability to fly away of the putative repellent source. In this case the data give evidences which components might act as repellent, but it will only give a possible indication of the natural behavioural response of the *An. gambiae* females. The setup is a kind of unnatural. The results of this study should therefore be tested in a more natural environment. For example in a semi-field where local ambient climatic conditions are and experiments can be done in a controlled situation . In a bigger experimental setup the 'natural' behavioural response of the malaria mosquitoes can be tested in different setups, two-choice and maybe also one-choice situations. In different setups different questions might be answered: On what distance does the repellent work? What will be the results if there is an ability to fly away, would it be the same as in this report? Are the same concentrations required as in the laboratory?
In a semi-field situation further investigations can be done. For example an experiment which investigates the duration of the repellent effect and the working distance. These kinds of experiments will help with the translation of the lab and semi-field results to a field situation.

5 Aknowledgements

My thesis would not have succeeded without the help of some people. In this paragraph I would like to say a few words to thank all people who contributed during my research.
First I would like to thank my supervisors Dr. Willem Takken and Dr. Renate Smallegange.
Willem thanks for your introduction, your remarks and your interest.
Renate, thanks for guiding me during my experiments and while I was writing my report. For all the nice discussions about various subjects. Thanks for always answering my questions, for your advices and support.
Yu Tong thank you for having a look at my data, some short talks, your empathy and the providing of the skin washing samples. Jeroen thanks for technical support and willingness to answering questions and solving problems.
Special thank to the insect rearers; Frans van Aggelen Leo Koopman, and André Gidding. Without your blood donations twice a week no experiments could have taken place.
Finally I would thank all members of the vector group. For the discussions, their commands, and their support during the months I have been working in the laboratory.

6 References

Allan SA, Kline DL. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictus* and *Aedes aegypti* (Diptera: Culicidae). Journal of Chemical Ecology 21(11):1847-1860.

Allan SAK, D.L. 1995. Evaluation of organic infusions and synthetic compounds mediating oviposition in *Aedes albopictu*s and *Aedes aegypti* (Diptera: Culicidae). Journal of Chemical Ecology 21(11):1847.

Audino PGV, Raul; Alfaro, Rene; Zerba, E. 2005. Identification of volatile emissions from *Platypus mutatus* (=*sulcatus*) (Coleoptera: Platypodiae) and their behavioural activity. Journal of Economic Entomology. 98 (5):1506-1509.

Barrozo RB, Minoli SA, Lazzari CR. 2004. Circadian rhythm of behavioural responsiveness to carbon dioxide in the blood-sucking bug *Triatoma infestans* (Heteroptera: Reduviidae). Journal of Insect Physiology 50(2-3):249-254.

Bernier UR, Kline DL, Barnard DR, Schreck CE, Yost RA. 2000. Analysis of human skin emanations by gas chromatography/mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for yellow fever mosquito (*Aedes aegypti*). Analytical Chemistry A 72: 747-756.

Bernier UR, Kline DL, Schreck CE, Yost RA, Barnard DR. 2002. Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). Journal of the American Mosquito Control Association 18:186-195.

Bosch OJ, Geier M, Boeckh J. 2000. Contribution of fatty acids to olfactory host finding of female *Aedes aegypti*. Chemical Senses 25:323-330.

Braks M, Cork A, Takken W. 1997. Olfactometer studies on the attraction of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human sweat. Proceedings Experimental and Applied Entomology, N.E.V. 8:99-104.

Braks MAH, Meijerink J, Takken W. 2001. The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. Physiological Entomology 26(2):142-148.

Braks MAH, Scholte EJ, Takken W, Dekker T. 2000. Microbial growth enhances the attractiveness of human sweat for the malaria mosquito, *Anopheles gambiae sensu stricto*. Chemoecology 10:129-134.

Braks MAH, Takken W. 1999. Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae sensu stricto*. Journal of Chemical Ecology 25(3):663-672.

Clements AN. 1992. The Biology of Mosquitoes. London: Chapman & Hall. 509 p.

Clements AN. 1999. The Biology of Mosquitoes. Wallingford, UK: CABI Publishers. 740 p.

Costantini C, Li SG, Della Torre A, Sagnon N, Colluzzi M, Taylor CE. 1996. Density, survival and dispersal of *Anopheles gambiae* complex mosquitoes in a West-African Sudan savanna village. Medical and Veterinary Entomology 10:203-219.

Costantini C, Birkett MA, Gibson G, Ziesmann J, Sagnon NF, Mohammed HA, Coluzzi M, Pickett JA. 2001. Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. Medical and Veterinary Entomology 15, 259-266.

Costantini C, Gibson G, Sagnon N, Torre Ad, Brady J, Coluzzi M. 1996. Mosquito responses to carbon dioxide in a West African Sudan savanna village. Med. Vet. Entomol. 10:220-227.

Curran AM, Rabin SI, Prada PA, Furton KG. 2005. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. Journal Of Chemical Ecology 31(7):1607-1619.

Dekker T, Geier M, Carde RT. 2005. Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. Journal Of Experimental Biology 208(15):2963-2972.

Dekker T, Steib B, Cardé RT, Geier M. 2002. l-Lactic Acid, a Human-Signifying Host Cue for the Anthropophilic Mosquito *Anopheles gambiae sensu stricto*. Manuscript for Medical and Veterinary Entomology: In Press.

Du YJ, Millar JC. 1999. Electroantennogram and oviposition bioassay responses of *Culex quinquefasciatus* and *Culex tarsalis* (Diptera: Culicidae) to Chemicals in odors from Bermuda grass infusions. J. Med. Entomol. 36(2):158-166.

Foster WA, Takken W. 2004. Nectar-related vs. human-related volatiles: behavioural response and choice by female and male *Anopheles gambiae* (Diptera: Culicidae) between emergence and first feeding. Bulletin of Entomological Research 94(2):145-157.

Geier M, Bosch OJ, Boeckh J. 1999. Ammonia as an attractive component of host odour for the Yellow fever mosquito Aedes aegypti. Chemical Senses 24: 647-653

Gibson G, Torr SJ. 1999. Visual and olfactory responses of haematophagous Diptera to host stimuli. Medical and Veterinary Entomology 13: 2-23.

Gillies MT, Coetzee M. 1987. A supplement to the Anophelinae of Africa South of the Sahara. Johannesburg: The south African Institute for Medical Research. 143 p.

Grant AJ, Wigton BE, Aghajanian JG. 1995. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. Journal of Comparative Physiology. A 177:389-396.

Guerenstein PG, Guerin PM. 2001. Olfactory and behavioural responses of the blood-sucking bug *Triatoma infestans* to odours of vertebrate hosts. The Journal of Experimental Biology 204:585-597.

Healy TP, Copland MJW. 2000. Human sweat and 2-oxopentanoic acid elicid a landing response from *Anopheles gambiae.* Medical and Veterinary Entomology 14: 195-200.

Jumean Z, Gries R, Unruh T, Rowland E, Gries G 2004a. Identification of the larval aggregation pheromone of codling moth, *Cydia pomonell.* Journal of Chemical Ecology 31 (4):911-924

Jumean Z., Tom; Gries, Regine; Gries, Gerhard. 2004b. *Mastrus ridibundus* parasitoids eavesdrop on cocoon-spinning codling moth, *Cydia pomonell*a, larvae. Naturwissenschaften.

Knols BGJ, Dejong R, Takken W. 1994. Trapping System for Testing Olfactory Responses of the Malaria Mosquito *Anopheles gambiae* in a Wind-Tunnel. Medical and Veterinary Entomology 8(4):386-388.

Knols BGJ, Jong Rd. 1996. Limburger cheese as an attractant for the mosquito *Anopheles gambiae s.s*. Parasitology Today 12(4):159-161.

Knols BGJ, Meijerink J. 1997. Odors influence mosquito behavior. Science and Medicine:56-63.

Knols BGJ, van Loon JJA, Cork A, Robinson RD, Adam W, Meijerink J, de Jong R, Takken W. 1997. Behavioural and electrophysiological responses of the female malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) to Limburger cheese volatiles. Bulletin of Entomological Research 87:151-159.

Korenromp E, Miller, J., Nahlen, B., Wardlaw, T., & Young, M. 2005. Roll Back Malaria, World Malaria Report. World Health Organization, UNICEF.

Lehane M. 2005. Biology of blood sucking in insects. cambridge: cambridge university press.

Mboera LEG, Knols BGJ, Takken W, dellaTorre A. 1997. The response of *Anopheles gambiae* sl and *A-funestus* (Diptera: Culicidae) to tents baited with human odour or carbon dioxide in Tanzania. Bulletin of Entomological Research 87(2):173-178.

Meijerink J, Braks MAH, Brack AA, Adam W, Dekker T, Posthumus MA, Beek TAv, van Loon JJA. 2000. Identification of Olfactory stimulants for Anopheles gambiae from human sweat samples. Journal of Chemical Ecology 26(6) 1367-1382.

Millar JG, Chaney JD, Mulla MS. 1992. Identification of oviposition attractants for *Culex Quinquefasciatus* from fermented bermuda grass infusions. Journal of American Mosquito Control Association 8:11-17.
Mukabana WR, Takken W, Killeen GF, Knols BGJ. 2004. Allomonal effect of breath contributes to differential attractiveness of humans to the African malaria vector *Anopheles gambiae*. Malaria Journal 3:8.
Osterkamp J, Wahl U, Schmalfuss G, Haas W. 1999. Host-odour recognition in two tick species is coded in a blend of vertebrate volatiles. J. Comp. Physiol A 185:59-67.
Pates H. 2002. Zoophilic and anthropophilic behaviour in the *Anopheles gambiae* complex. London: university of London.
Pates HV, Takken W, Stuke K, Curtis CF. 2001. Differential behaviour of *Anopheles gambiae sensu stricto* (Diptera: Culicidae) to human and cow odours in the laboratory. Bulletin of Entomological Research 91:289-296.
Puri SN, Mendki, M.J., Sukumara, D., Ganesan, K, Shri Prakashi, Eekhar, K. 2006. Electroantennogram and behavioural responses of *Culex quinquefasciatus* (diptera: Culicidea) females to chemicals found in human skin emanations. Journal of Medical Entomology 43 (2).
Qiu YT. 2005. Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours. Wageningen: Wageningen university.
Qiu YT, Smallegange RC, Smid H, Loon JJAv, Galimard AMS, Posthumus MA, van Beek TA, Takken W. GC-EAG analysis of human odours that attract the malaria mosquito *Anopheles gambiae sensu stricto*; 2004; Groningen. p 59-64.
Smallegange RC, Qiu YT, Galimard AMS, Posthumus MA, van Beek TA, van Loon JJA, Takken W. 2003. Why humans are attractive to malaria mosquitoes. Entomologische Berichten 63:50-53.
Smallegange RC, Qiu YT, van Loon JJA, Takken W. 2005. Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). Chemical Senses 30(2):145-152.
Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. 2005. The global distribution of clinical episodes of Plasmodium falciparum malaria. Nature 434(7030):214-217.
Spitzen J, Takken W. 2005. Malaria mosquito rearing - maintaining quality and qauntity of laboratory-reared insects. Proceedings Netherlands Entomologic Society Meeting 16:95-100.
Takken W. 1991. The role of olfaction in host-seeking of mosquitoes: a review. Insect Science & Applications 12:287-295.
Takken W. 1999. Chemical signals affecting mosquito behaviour. Invertebrate Reproduction and Development 36(1-3):67-71.
Takken W, Knols BGJ. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. Annual Review Of Entomology 44:131-157.
Zwiebel LJ, Takken W. 2004. Olfactory regulation of mosquito-host interactions. Insect biochemistry and molecular biology 34(7):645-652.
http://www.objectivistcenter.org/images/graphics/mosquitolifecycle.gif. mosquito life cycle.

7 Appendices

7.1 List of materials

Table 4 dual port olfactometer materials

olfactometer
dual-port olfactometer 1.60 x 0.66 x 0.43 m
pressured air
charcoal filter
water bottle
heater for water bottle
6 dimmed spotlights
8x olfactometer traps (glass)
14 sandblasted glass slides
1x mosquito box
8 release cages (single)
various glass bottles
T-pieces
Gilmont flowmeter, type 1060 (Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands)
Gilmont flowmeter, type 3060 (Fisher Scientific B.V.,'s Hertogenbosch, The Netherlands)

clean silicon tubes (diameter 5 and 7 mm; Rubber BV, The Netherlands)

Table 5 climate control materials

climate control
humidifier Defensor type3001
humidifier Defensor type 3001
humidifier Defensor type 505
hygrostat Defensor
10 l plastic bottle (demi water)
heater
timer

Table 6 climate measuring materials

measuring climate
PC +
datalogger+cables
Temp anemometer 642 Lambrecht
thermohydrometer, model DHM200

Table 7 materials for cleaning and counting

cleaning/counting
1x vacuum cleaner (LG V-CP243RDS 1400W)
"sucking pistol"
filter paper
cotton wool
CO2 bottle+
siphon ethanol
perfume free soap

Table 8 other materials

other
isolationbox (ice)
gloves
pipet
stopwatch

Table 9 list of test odours

Test compounds	purity	brand	amount
Geranyl acetone	≥98%	Fluka	3ml
6methyl-5-hepten-2-one	99%	Aldrich	3ml
Nonanal			3ml
Indole	≥99%	Sigma	0.384g
Nylon socks		Hema	

7.2 Chi-square test results

Table 10 chi-square results of control experiment series. The marked (***) treatments are significant ($p<0.001$) different from the control.

Chi square test results control	
treatment	Chi square
no odour-no odour	0.25907684
worn sock-clean air	0.00021***
sock 08-sok 13	0.68309
clean sock-worn sock	0.00000***
worn sock-worn sock	0.20083

Table 11 chi-square results of geranyl acetone (Ga) test series. Each number e.q.(1) represent a concentration in ml/min

Chi square test results geranyl acetone	
treatment	chi-square
no odour-no odour	0.41421618
worn sock-clean air	0.38648
sock- Ga (1)	0.10145
sock- Ga (5)	1.0
Sock- Ga (50)	0.20758
Sock- Ga (100)	0.05220

Table 12 chi square test results of treatment with different concentrations (1 to 100ml/min) 6-methyl-5-hepten-2-one. The red coloured treatments are significant different from the control.

Chi square test results 6-methyl-5-hepten-2-one	
treatment	Chi square
no odour-no odour	0.31731081
worn sock-clean air	0.00096***
sock- 6m5h2one (1)	0.04771*
sock- 6m5h2one (5)	0.03481*
Sock- 6m5h2one (50)	0.36571
Sock- 6m5h2one (100)	0.66168

Table 13 chi-square results for different concentrations 6-methyl-5-hepten-2-one (1-100 ml/min, given by the number behind the abbreviation 6m which stands for 6-methyl-5-hepten-2-one)

Chi square test results 6-methyl-5-hepten-2-one	
treatment	Chi square
no odour-no odour	-

skinsample-ethanol	0.39908
6m(1)-skinsample	0.89075
6m(5)-skinsample	0.03936*
6m(50)-skinsample	0.01382*
6m(100)-skinsample	0.79625

Table 14 chi-square test results for different concentrations nonanal (1-100 ml/min) the red colored are significant different from the control.

Chi square test results nonanal	
treatment	Chi square
no odour-no odour	0.15729926
skinsample-ethanol	0.00061***
nonanal(1)-skinsample	0.40538
nonanal(5)-skinsample	0.04965*
nonanal(50)-skinsample	0.02414*
nonanal(100)-skinsample	0.05935

Table 15 chi-square test results for different concentrations indole (0.5 -100 ml/min)

Chi square test results indole	
treatment	Chi square
no odour-no odour	0.31731081
ethanol-skin sample	0.01255**
ind(0.5)-skin sample	0.58621
ind (1)-skin sample	0.65472
ind (5)-skin sample	1.00000
ind (50)-skin sample	1.00000
ind (100)-skin sample	0.35384