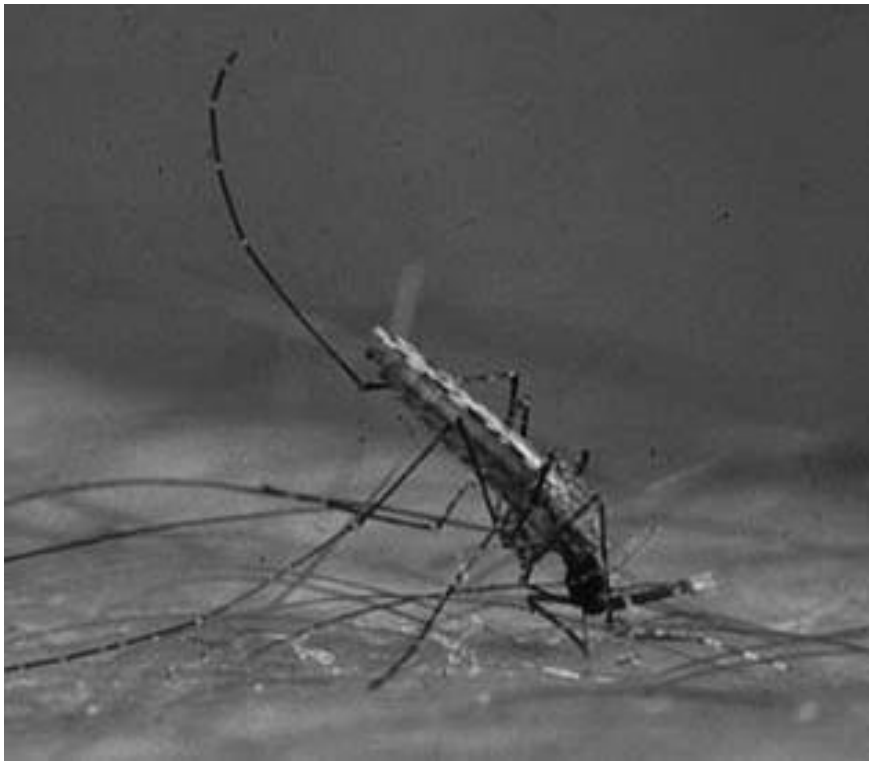
WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

Evaluation of spatial repellents against the malaria mosquito *Anopheles gambiae* s.s.



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Summary

Malaria is still a big problem in the world. Malaria control efforts so far have focused on insecticides, especially Insecticide Treated Nets and Indoor Residual Spraying. Sadly, insecticide resistance is increasing among malaria vectors. The predominant vector of malaria, *Anopheles gambiae* s.s., navigates mainly by odours. Therefore, odour cues could be used to keep mosquitoes away from hosts and thereby reduce transmission. Research into attractive odours is well under way, but research into repellents is still in its infancy. If a spatial repellent could be found that could be applied in houses, it would protect all inhabitants. Sadly, standard dual-choice olfactometers cannot evaluate spatial repellents. Typical African houses are known to have eaves for ventilation. *An. gambiae* s.s mosquitoes are known to enter houses preferentially through these eaves. New bio-assays for evaluating spatial repellents were developed that are based on this house entry behaviour. The bio-assays were tested with worn socks and a synthetic blend, both attracted a large number of mosquitoes. DEET (40%) was tested as a repellent control and was found to be repellent in this setup. Three compounds, benzeneethanol, 6-methyl-5-hepten-2-one and 2-methyl-2-benzoate were tested in 3 concentrations, 1%, 0.1% and 0.01%, in the new setup. Also two commercially available products were tested: citronella and a newly available impregnated bracelet known as Para'Kito©.

None of the three tested compounds were significantly repellent at any of the tested concentrations. Of the two commercially available products, the Para'Kito© bracelet was found to be repellent.

The newly developed bio-assays were shown to be an effective tool in testing for spatial repellents against *An. gambiae* s.s. mosquitoes. Although none of the tested compounds were shown to be spatially repellent in this study, they might be repellent at different concentrations. Benzeneethanol and 6-methyl-5-hepten-2-one were repellent in previous studies, therefore these might show spatial repellence in this setup at other concentrations. The Para'Kito bracelet is too expensive and its range is too short to be applied on a large scale for vector control. More compounds should be tested in this setup at a broader concentration range in order to find an effective spatial repellent.

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1 Introduction

1.1 General Introduction

Every year millions of people worldwide are affected by malaria. Most of these cases occur in the tropical regions, 90% of which occur in Africa (Tanser et al. 2003). In Africa, 300-500 million cases are reported each year, of which 1.5-2.7 million patients do not survive (Greenwood and Mutabingwa 2002). Of these victims, approximately 90% consists of children under 5 years old.

There are several species of malaria mosquitoes that can act as a vector for *Plasmodium* spp, the protozoa which is responsible for malaria. One of the most important vectors of malaria in Africa is *Anopheles gambiae sensu stricto*. It is more efficient as a malaria vector because it is highly antropophilic, as opposed to many other mosquito species which often prefer to bite on animals. In sub-Saharan Africa it is responsible for most of the malaria transmission in humans (Takken and Knols 1999).

Many different strategies have been employed in the effort to combat malaria. Presently the most utilized mechanisms are Indoor Residual Spraying and Insecticide Treated Nets. Unfortunately, both these strategies are suffering from increased insecticide resistance among the mosquito population (Hemingway and Ranson 2000; Awolola et al. 2002; Tia et al. 2006). As a result focus is shifting to the host-seeking behaviour of the vectors. If somehow mosquitoes can be prevented from locating their hosts, they would be unable to transmit the malaria parasites. It has been shown that *An. gambiae* s.s locates its host by odour cues; it responds strongly to human skin emanations (Takken and Knols 1999). Research has mainly focused on finding the compounds of human skin emanations that are attractive to the mosquitoes. The aim is to create a blend that is equally or more attractive to mosquitoes than human hosts (Okumu et al. 2010). Such a synthetic odour blend could then be used to lure mosquitoes away from humans. Many attractive compounds have already been identified, such as CO_2, ammonia, lactic acid and several carboxylic acids (Qiu 2005; Smallegange et al. 2005; Smallegange et al. 2009; Okumu et al. 2010). With the research on attractive compounds well under way, it is now time to look at the opposite side of mosquito olfaction. When there are compounds that attract a mosquito to a location, there are also compounds with a repellent effect. (Logan et al. 2008) investigated the effects of human odours on *Aedes aegypti* and found several compounds that seemed to repel the mosquitoes or mask the attractive odours. Of course, DEET is a well known topical repellent and is very effective. However, the need for repeated application in combination with high costs makes it impossible to apply it on a large scale. In contrast, if a cheap, long lasting spatial repellent could be found that could be applied in houses in malaria-affected areas, it might significantly reduce malaria transmission (Kawada et al. 2005). Such a spatial repellent could also be used in combination with attractants in a push-pull system which could even further increase the reduction of malaria (Cook et al. 2007). Traditional dual-choice olfactometers are not capable of testing for spatial repellence (Dogan and Rossignol 1999). In this research recently developed bio-assay boxes were tested for their effectiveness in evaluating potential spatial repellents.

1.2 The mosquito

Human malaria is transmitted by mosquitoes of the genus *Anopheles*, 30 to 40 of the 430 *Anopheles* species are considered malaria vectors (CDC 2006). Most notorious among these are the members of the *Anopheles gambiae sensu lato* complex, as its members are responsible for most of the transmission among humans. *Anopheles gambiae sensu stricto* is a member of the complex and is the principal malaria vector in Sub-Saharan Africa.

1.2.1 Mosquito life-cycle

Females of the *Anopheles gambiae* s.l. complex, the most important vectors of malaria in tropical Africa, lay their eggs in shallow puddles, which can be foot prints, tire tracks filled with rainwater, or agricultural fields. Its preference for these kinds of breeding sites causes its high occurrence in and around human houses, in particular in agricultural areas. The mosquito lays 50-200 eggs per oviposition. The eggs take 2-3 days to hatch, although it takes the eggs longer to hatch as the temperature decreases.
When the eggs hatch, the mosquito larvae appear. Most mosquito species prefer clean, unpolluted water. The larvae feed on algae, bacteria, and other micro-organisms in the surface micro layer. They spent all their time at the water surface, and leave it only when disturbed. The larvae go through four stages, or instars, before they develop into pupae. The pupae, like the larvae, stay close to the water surface to be able to breathe. After 4 or more days, depending on temperature, the pupa bursts open and the adult mosquito emerges.
The mosquitoes usually mate within a few days after emerging from the pupa. Usually the males fly in large swarms and the female mosquitoes fly into the swarm to mate. Mating usually occurs near the larval habitat, and this one mating provides the female with enough sperm to lay al her eggs, for the rest of her life. The male mosquitoes feed solely on nectar. Although females also rely on nectar as an energy source, they are dependent on blood meals to provide the proteins necessary for developing their eggs. Mosquito species responsible for malaria transmission feed predominantly on humans, although they can feed on animals as well. The female rests about 2-3 days, digesting the blood meal and developing her eggs, before she can lay her eggs. After laying her eggs she will search for a new host for her next blood meal. This is repeated multiple times until the mosquito dies. In the field a female mosquito usually survives up to a maximum of 6 weeks (Snow 1990).

1.2.2 Mosquito olfaction

When looking for mating partners, oviposition sites or hosts, mosquitoes utilize multiple cues, such as temperature and humidity. However, in *An. gambiae* s.s. mosquitoes, olfaction is the most important factor in mosquito navigation. Research has shown that mosquitoes are attracted by host odours (Costantini et al. 1993). *Anopheles gambiae* s.s. is a highly anthropophilic mosquito and therefore will react most strongly to human odours (Torr et al. 2008; Lefevre et al. 2009). Among the compounds of human sweat that have been identified to be attractive to *An. gambiae* s.s. mosquitoes are ammonia, L-lactic acid and carboxylic acids (Braks et al. 2001; Smallegange et al. 2005). The olfactory sensors of the mosquito are located in the sensilla, which are located on the antennae and maxillary palps. There are four types of sensilla, two of these, sensilla coeloconica and sensilla ampullacae, have a thermoreception function. The other two, sensilla trichodea and sensilla basiconica are olfactory. Sensilla basiconica, also called the “grooved peg” sensilla, is also capable of registering CO_2 (Clements 1999). All sensilla contain at least one receptor neuron and are surrounded by a glia, epidermis and cuticle. Odour molecules can enter the sensillum lymph

through pores in the wall of the sensillum. Proteins within the sensillum lymph bind the odour molecules and transport them to receptor molecules (Clements 1999; Qiu 2005).

1.2.3 Anopheline house entry behaviour

Female *An. gambiae* s.s. mosquitoes bite humans when they are most vulnerable: when they are asleep within their houses (Gillies and DeMeillon 1968). To do so, they must enter the house where their intended host is sleeping. There are three typical ways of entering a typical African house: the doors, the windows or the eaves. Eaves are a gap between the walls and the roof, to increase ventilation in the house. (Njie et al. 2009) investigated the relative importance of these different entryways for mosquitoes looking for hosts. They found that closing up the eaves reduced the number of *Anopheles gambiae* sensu lato mosquitoes by 66%. Mosquitoes of the *Anopheles gambiae* s. l. complex have been shown to exhibit "climbing behaviour", which means they will follow an odour cue upwards when encountering a vertical obstacle, thus permitting them to fly through the eaves (Snow 1987). Eaves are the most important entryway for *Anopheles gambiae* s.s. mosquitoes(Lindsay et al. 2002; Njie et al. 2009).

1.3 The disease

1.3.1 The malaria parasite

Human malaria is caused by four species of *Plasmodium*; *P. falciparum, P. vivax, P. ovale and P. malariae,* these are protozoan parasites that are very common in the tropics, especially Africa. Of these four species, *P. falciparum* is without a doubt the most dangerous, since it is the only one that is able to cause cerebral malaria and therefore often results in death. The other malaria species are hardly ever fatal on their own. The malaria parasite is dependent on both mosquito and man for the completion of its life-cycle. The mosquitoes are responsible for the transmission of the parasite and provide the environment for its sexual reproduction, whereas in humans it completes its asexual reproduction.

1.3.2 A short history

Malaria, or a disease with symptoms resembling malaria, is an ancient disease. It has been known for over 4000 years. The symptoms were first described in ancient China in 2700 BC. It became widely recognized in 400 BC in ancient Greece, when Hippocrates reported the symptoms of the disease responsible for a decline in city populations at that time. Roman writers attributed malarial diseases to foul-smelling swamps, hence the name 'mal'-'aria', which is Latin for bad air. In the Sanskrit manuscript *Susruta*, symptoms of malaria were said to be caused by the bites of certain insects. The malaria parasite was not discovered until 1880, when the French army surgeon Charles Louis Alphonse Laveran noticed the parasites in the blood of a patient suffering from malaria. For his discovery Laveran was awarded the Nobel Prize in 1907.

Six years after Laveran had discovered the parasites, the Italian neurophysiologist Camillo Golgi, concluded that there were at least two different types of malaria; one type caused fevers every other day (tertian periodicity) and the other caused fevers every third day (quartan periodicity), also, the two types differed in the number of merozoites they produced upon maturity. He also discovered that the fevers coincided with the rupture of red blood cells and the release of merozoites into the bloodstream.

In 1890 malaria parasites got their current name *Plasmodium*, when two Italian scientists, Grassi and Filetti, named two of the malaria parasites infectious to humans *Plasmodium vivax* and *Plasmodium ovale.* In 1897 the third, and most malignant parasite was named

Plasmodium falciparum by the American William H. Welch. In 1922 John W. W. Stephens described the fourth and last known human parasite, *P. ovale*.
The first one to prove that mosquitoes could transmit malaria between hosts was Ronald Ross in 1897; in his experiments he used mosquitoes to transmit avian malaria between birds. In 1902 he received the Nobel Prize for his research. After Ross's discovery, a team of Italian scientists, led by Giovanni B. Grassi, were quick to provide evidence that human malaria was also transmitted by mosquitoes in 1899. Mosquitoes, fed on volunteers infected with malaria in Rome, were flown from there to London, were they were fed on two volunteers, who both developed benign malaria.
Now that the transmission cycle was clear, the war against malaria had begun, and the discovery of chloroquinine and DDT led to the belief that malaria could be eradicated. Although there were successes, malaria was eradicated in North-America and Europe, in the areas that suffered most from the disease, South-America but mainly Sub-Saharan Africa, little progress was made. Nowadays, efforts for eradication in these areas have been abandoned for the more realistic primary goal of malaria control (Takken et al. 2004).

1.3.3 Epidemiology and Transmission

The occurrence of malaria depends mainly on two factors: the presence of *Anopheles* mosquitoes and their ability to survive and multiply, and a high enough temperature for the parasites to complete their reproductive cycle within the mosquito. The parasite needs both its human and mosquito hosts to complete its life-cycle, but although the part of the reproductive cycle that occurs in humans (the "exo-erythrocytic" and "erythrocytic" cycle) is independent of temperature, the part that takes place inside the mosquito ("sporogonic" life-cycle) is dependent on high temperatures for two reasons: firstly, the parasite has a temperature threshold (15°C for *Plasmodium viv*ax, 20°C for *P. falciparum*), below which it cannot complete its sporogonic cycle in the mosquito mid-gut (Takken et al. 2004). In that case, the parasite will be unable to migrate to the mosquito's salivary glands, and therefore the mosquito will be unable to transmit malaria to its human host. Secondly, mosquito longevity is determined by, among other things, ambient temperature. Considering it takes the parasite 9-21 days to complete its sporogonic cycle within the mosquito, the mosquito will have to survive that long to be able to transmit the parasite to its next host. If temperatures are too low, mosquitoes will not live long enough to be able to transmit malaria. Also, the time necessary for mosquitoes to develop from egg to adult decreases dramatically with higher temperatures, reaching peak development rates at temperatures between 22 and 28 °C (Bayoh and Lindsay 2003). Temperatures in tropical and sub-tropical areas are perfect for mosquito and malaria parasite survival and development; this explains in part why malaria is predominant in these regions. It also raises concern that the current trend of global warming might lead to an expansion of malaria-affected areas worldwide.
Besides temperature, there is another important climatic factor for malaria transmission. In places where there is little or no water drainage, rainfall will create puddles which are perfect breeding sites for *Anopheles* mosquitoes. Although variation in preference for breeding sites exists between species, by far most *Anopheles* species seem to prefer small rain puddles to deposit their eggs (Clements 1999). This explains the seasonality of malaria in some areas. Usually, there are two rainy seasons in sub-Saharan Africa, the short rains in November and the long rains in April and May, but with global warming, the rains have become unpredictable and less severe, causing severe droughts in some areas. During the dry season there is hardly any rainfall and therefore it is difficult for mosquitoes to find breeding sites, however, as soon as the rains start, the mosquitoes will be able to lay their eggs virtually anywhere, and soon the mosquito population (and therefore malaria transmission) increases dramatically. In agricultural areas irrigation may provide year-long

breeding sites for mosquitoes, these areas will show little or no seasonality compared to non-irrigated areas (Sissoko et al. 2004). In some areas, heavy rain may eventually cause the temperature to drop below the threshold value in an area, thereby lowering or even stopping malaria transmission in this location. Malaria transmission usually also declines with altitude, as it gets cooler at higher altitudes, and mosquito densities therefore tend to be low in mountainous areas.

1.4 Bio-assay development

Dual-choice, Y-tube and other types of olfactometers that are used for research into attractive compounds cannot be used for spatial repellent research. The mechanism behind these setups is that the mosquito can move towards the odour sources, and choose to move towards the most attractive source. If a spatial repellent is placed within such a setup mosquitoes would stay inside or close to the release cage. Although this is a nice indication for spatial repellence, the mosquitoes might also be affected by other influences, for example differences between days. A repellent would override the control in the other chamber, therefore it cannot be used to correct for other factors that might influence the mosquito's flight behaviour. Because of the absence of a proper control,it is impossible to distinguish whether the mosquitoes remained at the release end of the tunnel because of the tested compounds or another influence. Therefore, a new bio-assay was developed to efficiently evaluate the spatial repellent effects of selected compounds. The design of the new bio-assay is based upon the house entry behaviour of Anopheline mosquitoes as described above.

1.5 Compounds

1.5.1 Controls

Ammonia is a naturally occurring compound in human sweat (Braks et al. 2001). It has been found to be attractive to *An. gambiae* s.s. on its own, but has been found to especially increase attractiveness of a blend, in combination with other attractive odours (Braks et al. 2001; Smallegange et al. 2005). Human sweat has uniquely high concentrations of L-lactic acid (Dekker et al. 2002). Therefore, it might play a role in the host seeking behaviour of antropophilic mosquitoes. L-lactic acid on its own is attractive to *Aedes aegypti* mosquitoes (Geier et al. 1999), but not to *An. gambiae* s.s. (Braks et al. 2001; Smallegange et al. 2005). However, it has been shown to increase attractiveness when used in a blend of odours (Smallegange et al. 2005; Smallegange et al. 2009). Volatile carboxylic acids have been shown to be attractive to *An. gambiae* s.s. mosquitoes (Knols et al. 1997). The level of attraction is dependent on the chain length of the carboxylic acid and the concentration. Tetradecanoic acid has been shown to increase attractiveness relative to a binary mixture of ammonia and L-lactic acid (Smallegange et al. 2009).
DEET was used as the control repellent for this study. There is some discussion on the spatial repellent effects of DEET (Dogan and Rossignol 1999; Kline et al. 2003; Syed and Leal 2008). However, until a proven spatial repellent is found, DEET is the only standard that can be used for these studies.

1.5.2 Selection of test compounds

Benzeneethanol

Benzeneethanol was selected because of effects seen in previous olfactometer studies and semi-field results. Benzeneethanol decreased the attractiveness of Basic Blend at the two lowest concentrations of the 3 concentrations that were tested in dual-choice olfactometer

experiments (N. Verhulst, in preparation). In 4-choice semi-field experiments in Mbita (Kenya), all 3 concentrations that were tested in traps baited with benzeneethanol + Basic Blend + CO_2 caught significantly fewer mosquitoes than traps with the Basic Blend + CO_2 (R. Smallegange, personal communication).

6-Methyl-5-hepten-2-one
6-Methyl-5-hepten-2-one was selected because of results in the previous study by Qiu et al. (2005) where it was found to be repellent at a flow rate of 50 ml/min (in combination with ammonia and lactic acid). Also, it has been shown to be repellent for *Aedes aegypti* in a study by Logan et al. (2008). In a 4-choice semi-field experiments in Mbita (Kenya) mosquito traps baited with carbon dioxide + the Basic Blend (ammonia, L-lactic acid and tetradecanoic acid) + 6-Methyl-5-hepten-2-one , caught significantly fewer *An. gambiae s.s.* females than traps baited with CO_2 + the Basic Blend in 2 out of 3 concentrations tested (R. Mukabana, unpublished results).

Methyl-2-methylbenzoate
Methyl-2-methylbenzoate was shown by Yale University to have a receptor response profile similar to that of DEET. They found that DEET elicited a stronger response from a receptor named AgOR15 than from any other receptor. In fact, no other adult receptor gave even a modest response to DEET. Methyl-2-methyl-benzoate elicited responses in a very similar manner as DEET. These results suggested the possibility that methyl-2-methyl-benzoate could have a repellent effect like that of DEET. Behavioral evidence was found to support this possibility in *Drosophila*: Methyl-2-methyl-benzoate did in fact act as a repellent, in a dose-dependent manner, and its effect lasted longer than DEET.

1.5.3 Commercially available products

Citronella
Citronella has long been used as a mosquito repellent (Müller et al. 2009). Usually it is applied in the form of candles or diffusers. Citronella essential oil is derived from different species of *Cymbopogon* (citronella grass).
Much research has been done on the repellent effects of citronella, some found repellent effects and others found little to no effect (Lindsay et al. 1996; Fradin and Day 2002).

Para'Kito© bracelet
Para'Kito© (France) is a newly commercially available bracelet with a pellet consisting of essential oils, that claims to repel most species of mosquitoes (www.parakito.com). The company does not divulge exactly which essential oils are used, only essential lavender oil is mentioned. During the experiment, the pellet that is usually inserted into the bracelet, was now hung from the wire rack along with the Basic Blend sachets.

1.6 Grand Challenges of Global Health Project

The research described in this thesis is part of the Grand Challenges of Global Health project 32 28. It is part of a international collaboration between institutions in the U.S, The Netherlands, Tanzania, Kenya and The Gambia. These institutions combine their knowledge of molecular, behavioral and physiological research to identify compounds that interfere with mosquito host-seeking behaviour. Cloned genes that encode odour receptors in *Anopheles gambiae* s.s. mosquitoes are used to assess the responses of these receptors to chemical stimuli associated with the insect's behaviour. Compounds of interest are subsequently tested in laboratory and field conditions for their effect on mosquito behaviour.

Collaborators:

- Yale University, Connecticut, United States
- Wageningen University and Research Centre, the Netherlands
- Ifakara Health Research and Development Centre, Tanzania
- Medical Research Laboratories, The Gambia
- University of Nairobi, Kenya
- ICIPE, Kenya

1.7 Research questions

- Is the newly developed bio-assay an effective tool for evaluating spatial repellents against the malaria mosquito *An. gambiae* s.s.?
- Is any of the tested compounds a significant spatial repellent against the malaria mosquito *An. gambiae* s.s.

1.8 Relevance

Malaria is still taking a huge toll on human lives every year. With insecticide resistance among malaria mosquitoes on the rise, it is important to look to alternatives to lower the number of people getting bitten by infected mosquitoes. Because malaria is mostly a disease located in impoverished areas, it is important that we look for a cheap and effective method to protect as many people as possible. If an effective, long lasting spatial repellent can be found, it could protect large numbers of people at low costs. Combined with attractants found in previous research, a push-pull mechanism could be set up to further improve the chances of preventing malaria mosquitoes from finding their hosts and infecting them. This newly developed bio-assay could provide us with a fast and effective way to test compounds for spatial repellent effects against malaria mosquitoes.

2 Material and Methods

2.1 Mosquito rearing and selection

The *An. gambiae* s.s. mosquitoes reared in the Laboratory of Entomology originate from Suakoko, Liberia (courtesy Prof. M. Coluzzi) and have been cultured there since 1988. They are kept at 27 ± 1°C, 80 ± 5% RH and a photo-scotophase of 12L:12D, which is similar to conditions in Sub-Saharan countries in Africa during the rainy season (Takken et al. 2004). Mosquitoes are given the opportunity to feed on membranes with human blood twice a week, and have continuous access to a 6% glucose solution on filter paper.
Larvae were reared on tap water in plastic trays and fed with Tetramin® baby fish food (Melle, Germany). After the development of fourth instar larvae into pupae, pupae are collected and placed in plastic cups with tap water. The cups with pupae are then placed in gauze cages (30x30x30 cm) for emergence of the adult mosquitoes. Three to five days after emergence the mosquitoes will mate. After the females have taken a blood meal a plastic cup with water and filter paper is provided in the cages for oviposition. The eggs can then easily be collected to be placed in the larval trays for emergence.
Because all experiments have to take place at the time of the peak activity of the mosquitoes, the light-dark regime in the climate room is shifted from a light period from 06:00 to 18:00 to a light period from 00:30 to 12:30. This way the mosquitoes' peak activity is in the early morning, and all experiments were conducted during that time (between 09:00 and 12:30).
Seven to eight days old female mosquitoes were selected randomly from adult cages 14-18 hours before experiments. These mosquitoes have mated, but have not yet been blood fed. Using a mouth aspirator 200 mosquitoes were placed in 10 cylindrical release cages (20 mosquitoes per cage, ø 7cm., height 10 cm) and provided with damp cotton wool placed on the top netting. The cages were left in the mosquito rearing conditions until the beginning of the experiments, when they were transported to the bio-assays in a polystyrene box, preventing them from perceiving any (day)light.

2.2 Compounds

2.2.1 Delivery

All compounds (except worn socks and Para'Kito© pellet) were placed within the air stream in the bio-assays in Low Density Poly Ethylene (LDPE) sachets. LDPE sachets allow for a continuous and constant release of compounds (Torr et al. 2008; Verhulst et al. 2009). Release rate of the compounds and therefore the concentration of that compound within the bio-assay is dependent on the thickness and the size of the sachets. Thickness of the LDPE sachets varied per compound, and will be described per compound below. The sachets were hung on a small wire rack (see Figure 1), which was placed in front of the gauze at the opening where the air flow entered the bio-assays (location labeled "odour source" in figure 2)For every compound, 100 µl of the solution was pipetted into a LDPE sachet of approximately 2x3 cm.

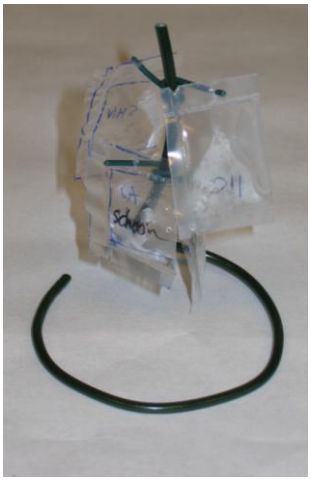


Figure 1: Wire rack for placing LDPE sachets in air stream

2.2.2 Controls

Before the bio-assay could be used to test for spatial repellents, a series of experiments should be done to test how the mosquitoes behave within the setup without any spatial repellents. First a series of experiments was done with worn socks. This was done to verify whether the mosquitoes would enter the inner compartment when a attractive odour source is present. Worn socks usually have a very high response rate for *An. gambiae* s.s. mosquitoes, however, there can be differences between the attractiveness of worn socks. Also the blend of odour that emanates from the sock is complex and unknown. It is preferable to use an odour source withhout variation, to minimize the number of influences that can affect the mosquitoes apart from the tested compounds. Therefore, we tested a synthetic odour blend, to assess whether this blend could function as a more constant attractant control than worn socks in our research. Subseqently, DEET was tested to investigate whether fewer mosquitoes would enter the inner compartment when a repellent is present.

Worn socks

Black nylon socks were worn by a volunteer (male) for 24 hours. Socks were stored in clean glass bottles at -20°C when they were not used in experiments. Worn socks have previously been shown to be very attractive to *An. gambiae* s.s. mosquitoes (Pates et al. 2001; Qiu 2005; Smallegange et al. 2010). The socks were placed within the air stream, upon the same wire rack that was used for the LDPE sachets.

Attractant control

As a control for attractants a blend was used of ammonia, L-lactic acid and tetradecanoic acid. This blend will hereafter be referred to as "Basic Blend". This Basic Blend is used as an attractant control in all olfactometer experiments at the Laboratory of Entomology, and has previously been shown to be attractive for *An. gambiae* s.s. mosquitoes (Smallegange et al. 2009). 0.03 mm LDPE sachets were used for ammonia and tetradecanoic acid. 0.05 mm sachets were used for the L-lactic acid. All LDPE sachets were approximately 2x3 cm and filled with 100 µl of ammonia (25%) solution and L-lactic acid (88-92%) solution and 0.05 mg of tetradecanoic acid.

Repellent control

For this study we used a commercially available 40% solution of DEET in ethanol (CarePlus® DEET anti-insect spray).
0.03 mm LDPE sachets were used for the DEET solution. A sachet filled with 60% ethanol (Merck, 96%) was added to the control. All LDPE sachets were approximately 2x3 cm and filled with 100 µl of solution.

Clean air

Some of the flight behaviour of the mosquitoes might be caused simply by the current of warm moist air, and not by the odours within the air plume. Tests were run with LDPE sachets filled with distilled water. LDPE sachets had the same thickness as the sachets used for the Basic Blend, two 0.03 mm sachets and one 0.05 mm sachet. All LDPE sachets were approximately 2x3 cm and filled with 100 µl of water.

2.2.3 Test compounds

All tested compounds can be found in table 1. The purity and brand of used compounds can be found in Appendix B.

Table 1: Tested compounds, the concentrations in which they were tested and the number of times the experiments were repeated.

Tested compounds	Tested concentrations			# of repetitions
	1%	0.10%	0.01%	
Benzeneethanol	x	x	x	6
6-methyl-5-hepten-2-one	x	x	x	6
2-methyl-2-benzoate	x	x	x	6
Citronella		x		5
Para'Kito	concentration unknown			5

Repetitions
Experiments with benzeneethanol, 6-methyl-5-hepten-2-one and 2-methyl-2-benzoate were repeated 6 times for each concentration tested. Due to time constraints citronella and the Para'Kito bracelet could only be tested 5 times.

Concentrations
All tested compounds were tested in 3 concentrations; 1%, 0.1% and 0.01%, diluted in paraffin oil. These were tested in the same concentrations at which the test compounds were originally tested in the dual-choice olfactometer (G. Bukovinzkine kiss, personal communication). Citronellal was tested at a concentration of 0.1%. All test compounds were tested in 0.2 mm LDPE sachets. All LDPE sachets were approximately 2x3 cm and filled with 100 µl of compound.

2.3 Description of the bio-assays

Two identical bio-assay boxes were developed to resemble a brief stretch of the eave of an African house. The outside dimensions of the boxes measured 30x30x50 cm. The front side consists of translucent acrylate, the other sides are made from white trespa. Figure 2 gives a schematic overview of the bio-assays. On the left side there is an opening where the mosquito release cage can be placed. When the mosquitoes are released into the bio-assay box, they first enter the larger compartment, representing the "outside" of the house. They can then continue to fly to the "inside" of the house by flying through the eave opening. The eave opening is adjustable with a system of small metallic balls and a clasp. For this series of experiments, however, the eave opening was kept constant at approximately 1 cm. At the end of the experiment, the eaves can be closed, and the mosquitoes in each compartment can be counted. In the rest of this report, the left compartment will be referred to as the "outer" compartment, and the right compartment as the "inner" compartment.

From the right side pressurized air is blown into the bio-assay. Before the air streams enters the bio-assay it first passed through an active charcoal filter, then it is split in two (Tubing: Saint Gorman Performance Plastic, Versitec silicone 7x10mm). The two air streams first pass through a flow meter (Brooks Sho-Rate™ 1355) to regulate air flow (set at ± 4.5) and are then moistened by letting the air pass through a water bottle filled with warmed, distilled water (Figure 3)(Warmed waterbath, Julabo U3, ±40ºC). Tubing between elements was kept equal between both bio-assays, to prevent differences. Thus, when the air enters the bio-assay box it is clean, with a temperature of 27 ± 2° C and a relative humidity of 95 ± 2%.
As *An. gambiae* especially bites during the last four hours of the night, all experiments were conducted in darkness, with one dimmed spotlight (15W, covered with filter paper to diffuse the light), opposite the bio-assays to simulate moonlight.

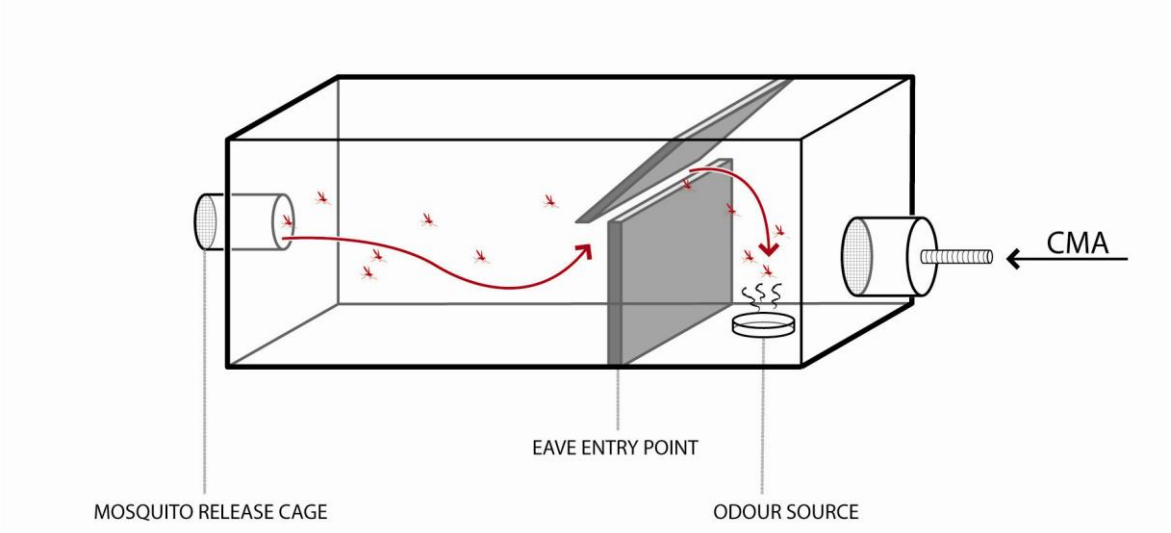


Figure 2: Schematic representation of one of the repellent bio-assay boxes. Clean moist air (CMA) is blown in on the right, mosquitoes are released on the left. The representation of the odour source is not how odours were offered in the experiments. Check the chapter Compounds>Delivery for an explanation how compounds were offered into the air stream (figure 1).

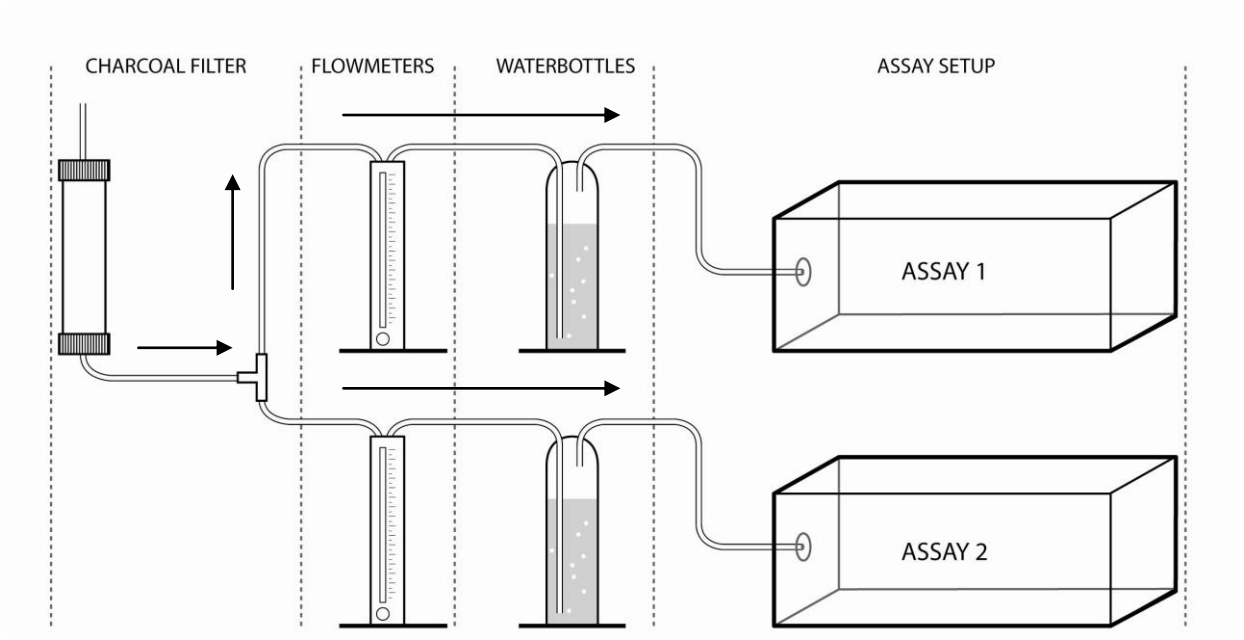


Figure 3: Path of the air flow before it enters the bio-assays. Arrows indicate direction of air stream. Contrary to this schematic representation, length of tubing between all elements was kept equal for both bio-assays.

2.4 Experimental procedure

All experiments took place between 9:00 and 12:30 am. The bio-assays were placed within a tent through which no light could get in (see figure 4). Within the setup space a heater (Hellen thermostat) and a humidifier (ultrasonic humidifier YC-F670)were placed to create similar environmental conditions as within the bio-assay boxes. Temperature was kept at about 27°C and relative humidity was approximately 65%. Timers, a thermostat and a hydrostat were used to ensure that conditions were optimal at the start of the experiments. A half hour before the experiments were started, the air flow was opened up, to ensure a secure and regular air flow before each series of experiments. An anemometer was used to determine air speed. Air speed should be around 20-22 cm/s at the point where the air enters the bio-assays (Smallegange et al. 2005; Verhulst et al. 2009). The same air speed should be obtained in both bio-assays. At the start of each experiment, two release cages were taken from the transport box and placed in the release openings at the left of the bio-assay boxes. One of the bio-assays contained the Basic Blend and the solvent of the tested compound, the other the Basic Blend and the compound to be tested. Next, the release cages were opened and the mosquitoes were released into the boxes. The experimenter then left the setup area and the mosquitoes were left in the bio-assays for 15 minutes. After this period the release cages and the eaves are closed. The mosquitoes in each compartment and the release cages were then counted, they were removed from the bio-assays via mouth aspirator or vacuum cleaner. On each experimental day, this procedure was repeated 5 times. Each concentration was tested once every day. Two controls: Basic Blend against Basic Blend and DEET with Basic Blend against Basic Blend and ethanol were also tested each day. For each compound, 6 days of experiments were conducted. Tests were randomized for different days, times, assays and positions. The scheme for the experiments can be found in appendix A.

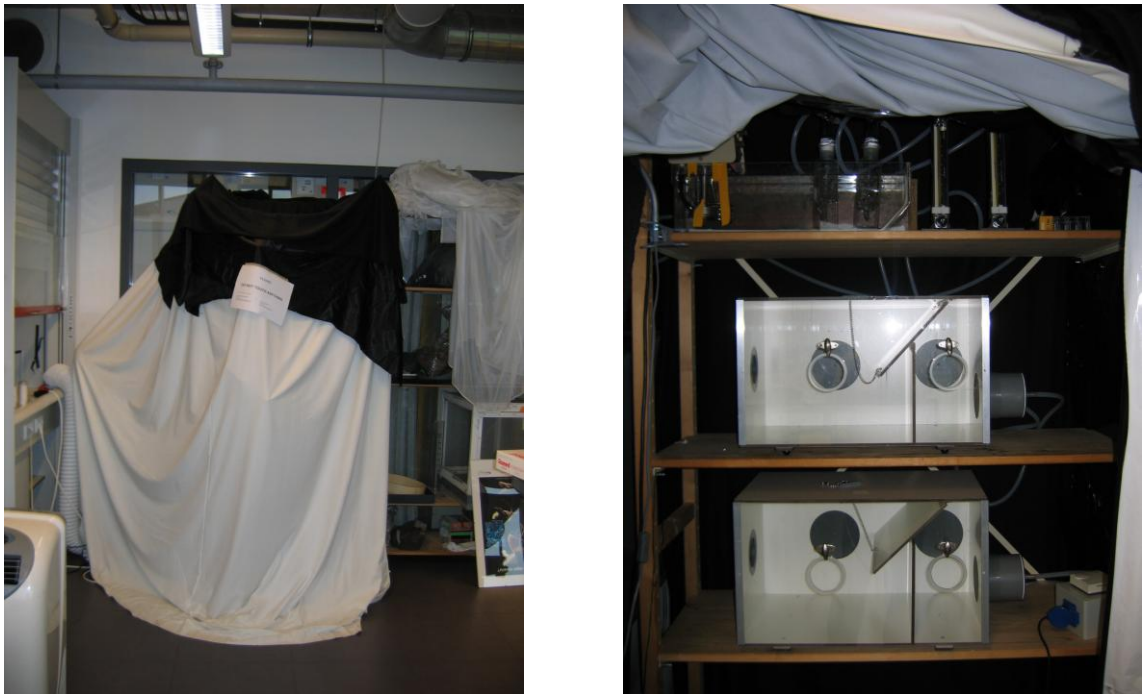


Figure 4: Experimental setup
Left: The “tent” in which the setup was placed. Curtains were darkening, no light came through. Right: The setup as it was used in experiments. The top shelf contained the charcoal filter, waterbath, waterbottles and flowmeters. The two bio-assays were placed on the two shelves below. Heater and humidifier were placed on the floor inside the tent.

2.5 Prevention of contamination

The experimenter wore latex gloves (Romed, powderfree) at all times during the experiments. Gloves were replaced regularly to prevent contamination with odours, human or otherwise. During the assays, the experimenter left the setup area, so as not to influence the mosquitoes in any way. All materials used were cleaned regularly . The LDPE sachets used as control against the test compounds were stored separately in a freezer at -20°C , so they could not be contaminated by the test compounds. For each new series of 6-day experiments new sachets for Basic Blend, DEET, ethanol and paraffin oil were made.
The bio-assays and wire racks were cleaned at the end of every day of experiments. Materials were washed first with distilled water, then with 60% ethanol, and then washed with distilled water again.

2.6 Measurements of environmental conditions

At the beginning of each day of experiments temperature, wind speed and relative humidity were measured using a thermo-anemometer (Lambrecht, 642) and a thermo-hygrometer (WM, DHM200). When the equipment was available, measurements were also taken in between each experiment.

2.7 Statistical analysis

Data were analyzed using SPSS 15.0. A generalized linear model (binomial distribution, link in logit) was used to analyze the effect of the test compounds. Responses were considered to be statistically significant at $p<0.05$. Response was measured as the number of mosquitoes in the inner compartment. The response measured in the bio-assay box containing the test compound was then compared to the bio-assay box containing the control. The null hypothesis was that mosquito responses for both bio-assay boxes were 1:1.

3 Results

3.1 Control experiments

Worn socks

Before experiments were conducted with worn socks, to test whether mosquitoes would fly into the inner compartment of the bio-assays when an attractive odour source is present. On average worn socks attracted 63% of the mosquitoes that were released within the 15 min period N=26).

Symmetry of the bio-assays

During experiments, Basic Blend was tested against Basic Blend to test the symmetry of the bio-assays. A Generalized Linear Model (Binomial distribution; linked in Logit) was used to test if there was a difference between the bio-assays boxes (# 1 or #2) and their position (top or bottom). No significant difference was found between the bio-assay boxes themselves (p=0.64). There was a significant difference in number of mosquitoes in the inner compartment for the positions of the bio-assays. A bio-assay positioned at the bottom attracted significantly more mosquitoes than the top position (p<0.001, N=18), see table 2. See figures 5 A and B. Positions were rotated throughout the experiments, therefore this should not influence our results. Also, one day, clean air was tested against clean air in the bio-assays, no significant difference was found for position (p=0.96, N=6) or bio-assay (p=0.96, N=6).

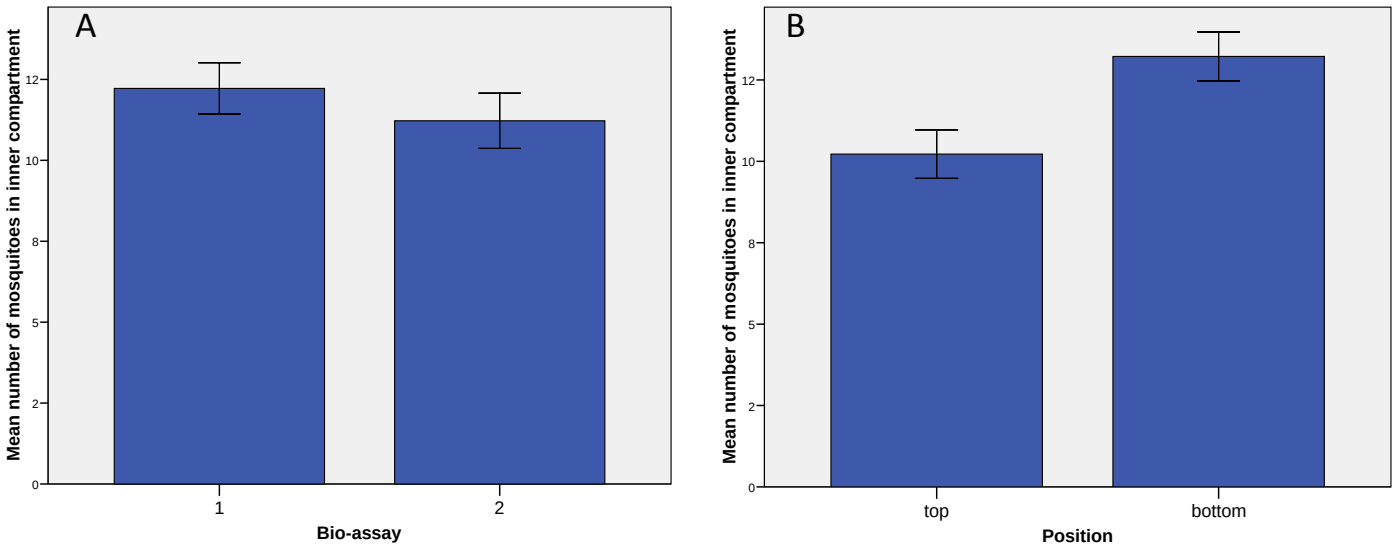


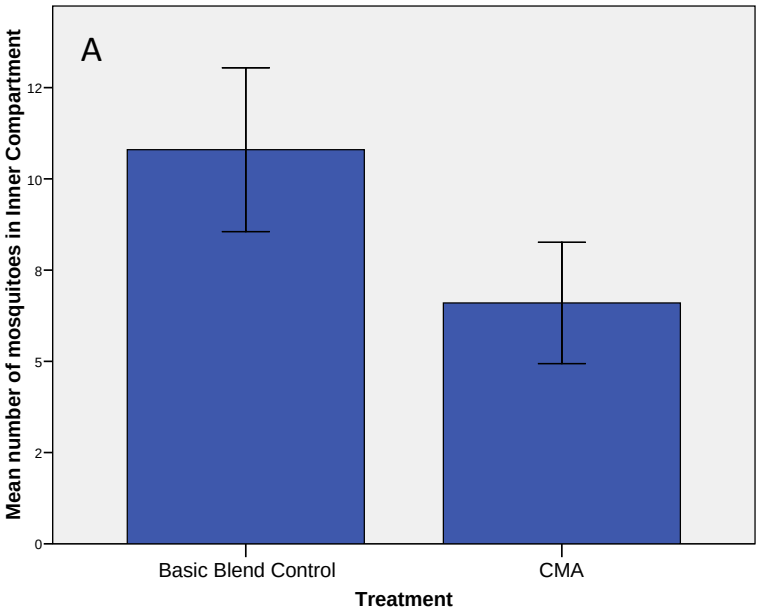
Figure 5: Series of control experiments to test the symmetry of the bio-assays:
A: No significant difference was found in the number of mosquitoes in the inner compartment between box #1 and box #2.
B: Significantly more mosquitoes were found in the inner compartment of assays at the bottom position.
Error bars represent standard error of mean.

Table 2: Contingency table: number of mosquitoes in the different compartments for position of the boxes (N=18)

Compartment	Top	Bottom	Total
Inner compartment	184	238	422
Outer compartment	107	57	164
Release cage	69	65	134
Total	360	360	720

Attractant and repellent control

Basic Blend was tested against clean air (3 LDPE sachets filled with water 2x 0.03mm and 1x 0.05mm) to test the attractiveness of the blend. The blend was significantly more attractive than clean air (p=0.002). 40% DEET in ethanol with Basic Blend was tested against Basic Blend with ethanol to assess the spatial repellence of DEET. DEET was significantly repellent, but not very strong (p=0.038, N=18). See figures 6 A and B. No significant differences were found between Basic Blend, Basic Blend with ethanol and Basic Blend with paraffin oil (p=0.20)



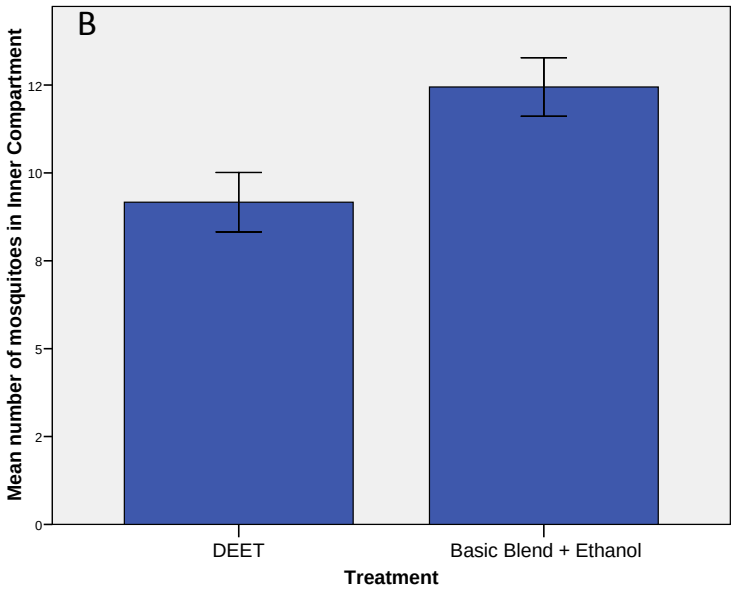


Figure 6: Series of control experiments to test the attractiveness of Basic Blend and the repellence of DEET

A: Basic Blend against Clean Moistened Air (CMA)
Basic Blend attracted significantly more mosquitoes than Clean Moistened Air.

B: Basic Blend with DEET against Basic Blend with Ethanol
Basic Blend with ethanol attracted significantly more mosquitoes than Basic Blend with DEET.

Error bars represent standard error of mean.

3.2 Tested compounds

Benzeneethanol

None of the tested concentrations of benzeneethanol in combination with Basic Blend yielded lower numbers of mosquitoes than the Basic Blend with paraffin oil (1% p=0.59; 0.1% p=0.63; 0.01% p=0.21). See figure 7. No significant difference was found between the bio-assays (p=0.96). The bottom position yielded significantly more mosquitoes in the inner compartment than the top position (p<0.001).

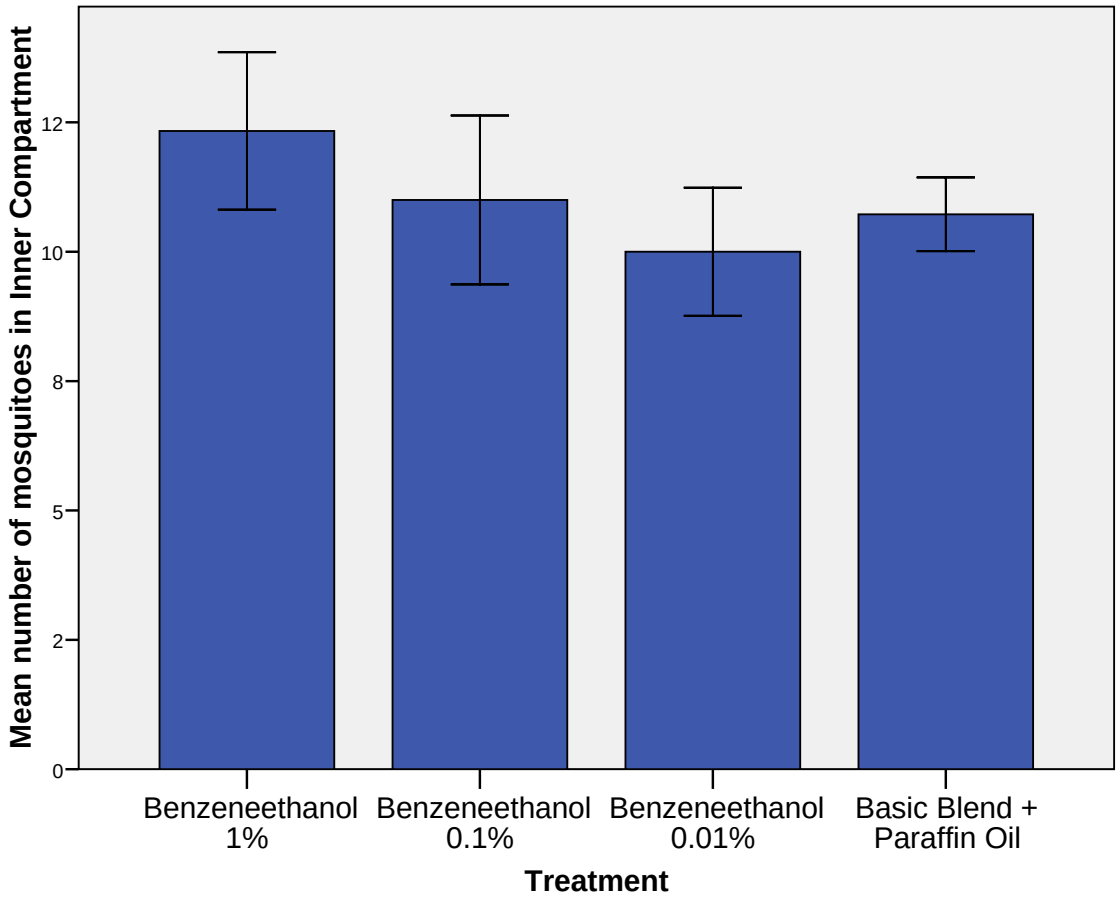


Figure 7: Mosquito response to different concentrations of Benzeneethanol with Basic Blend against Basic Blend with Paraffin Oil. None of the concentrations yielded a significant difference in mosquito response. Error bars represent standard error of mean.

2-Methyl-2-benzoate

None of the tested concentrations of 2-Methyl-2-benzoate in combination with Basic Blend yielded lower numbers of mosquitoes than the Basic Blend with paraffin oil (1% p=0.86; 0.1% p=0.66; 0.01% p=0.67). See figure 8. No significant difference was found between the bio-assays (p=0.06). The bottom position yielded significantly more mosquitoes in the inner compartment than the top position (p=0.002).

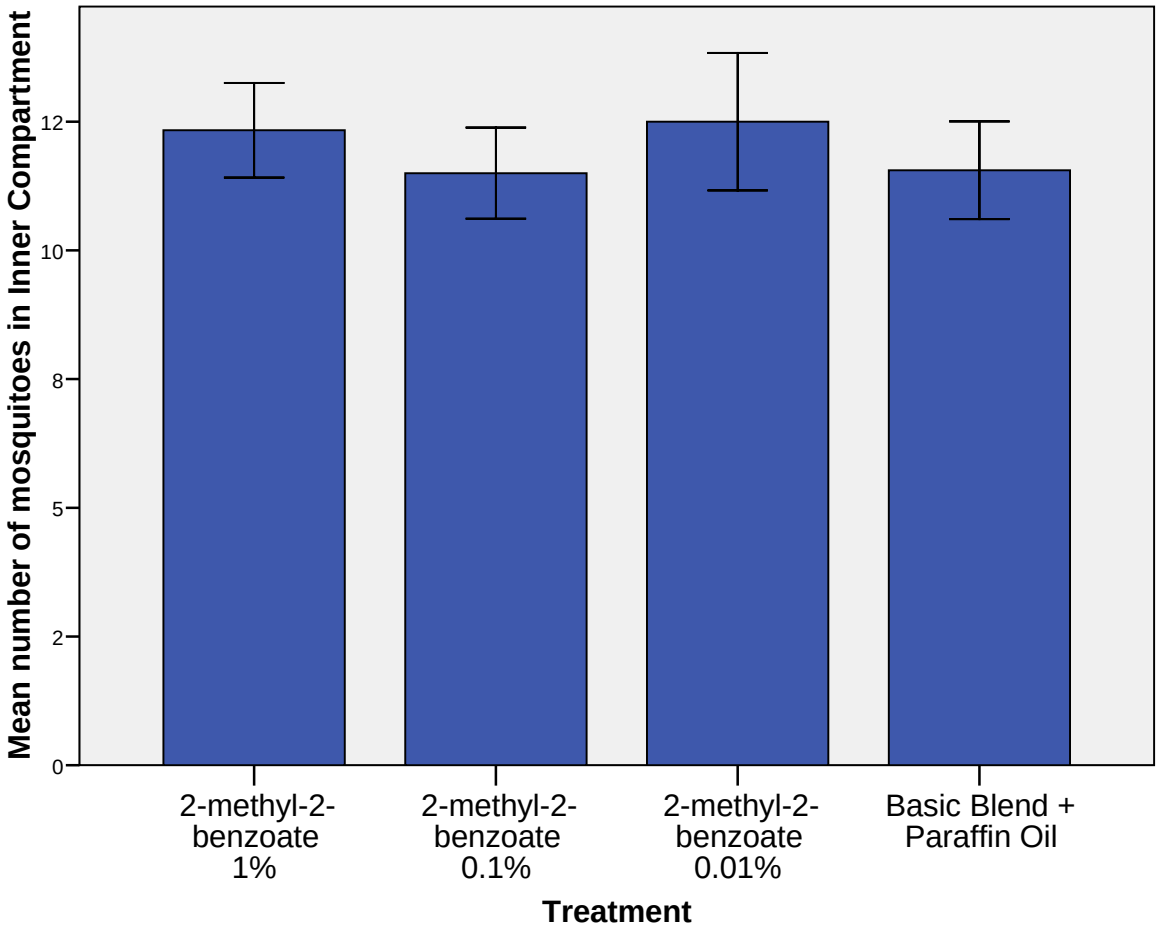


Figure 8: Mosquito response to different concentrations of 2-methyl-2-benzoate with Basic Blend against Basic Blend with Paraffin Oil. None of the concentrations yielded a significant difference in mosquito response. Error bars represent standard error of mean.

6-Methyl-hepten-2-one

None of the tested concentrations of 2-Methyl-2-benzoate in combination with Basic Blend yielded lower numbers of mosquitoes than the Basic Blend with paraffin oil (1% p=0.98; 0.1% p=0.30; 0.01% p=0.30). See figure 9. No significant difference was found between the bio-assays (p=0.92). The bottom position yielded significantly more mosquitoes in the inner compartment than the top position (p<0.001).

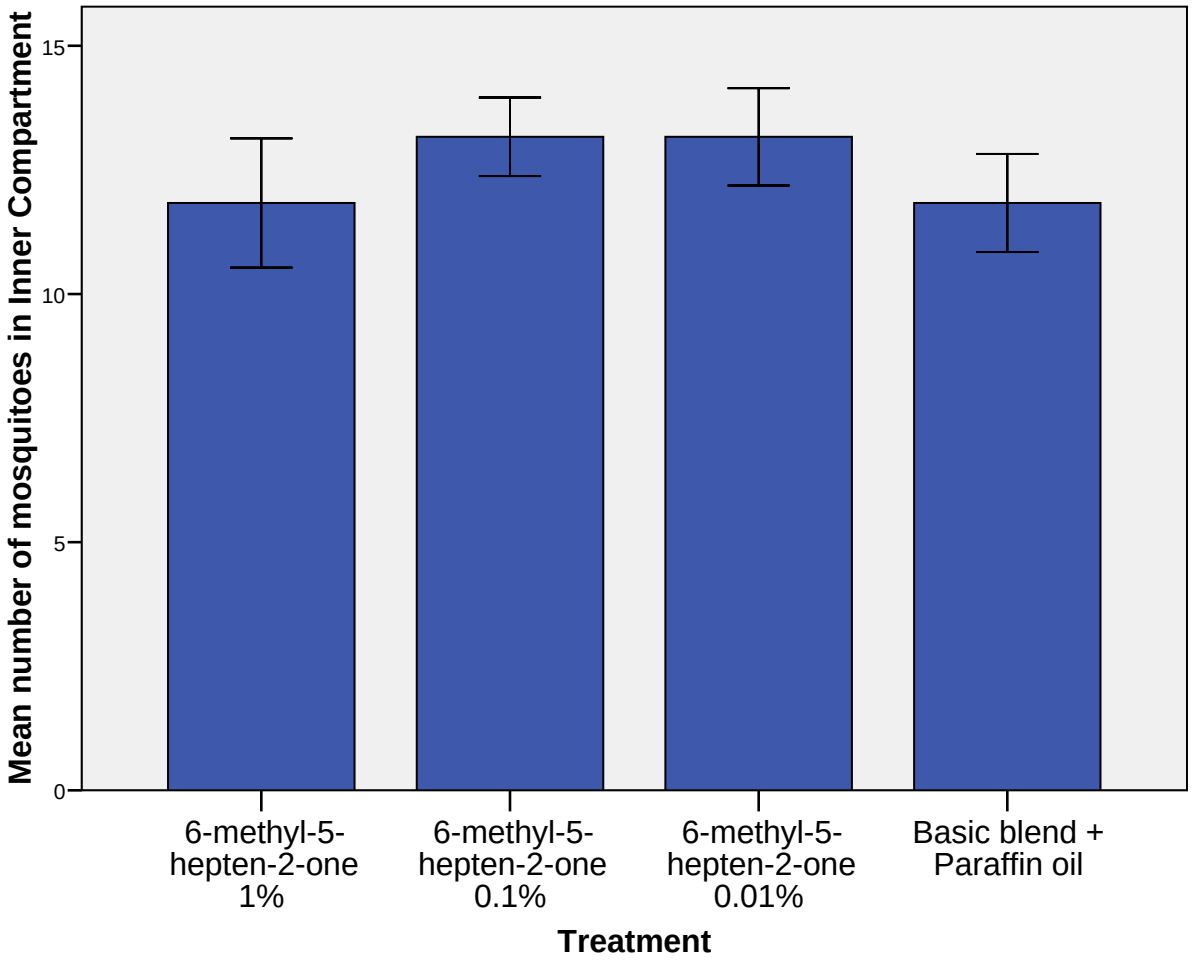


Figure 9: Mosquito response to different concentrations of 6-methyl-5-hepten-2-one with Basic Blend against Basic Blend with Paraffin Oil. None of the concentrations yielded a significant difference in mosquito response. Error bars represent standard error of mean.

3.3 Commercially available compounds

Citronellal

Although Citronellal with Basic Blend did attract fewer mosquitoes than Basic Blend with Paraffin Oil, this difference was not significant at the concentration we tested (0.1%, p=0.39). See figure 10A. No significant difference was found between the bio-assays (p=0.73) or their position (p=0.73).

ParaKito© bracelet

The ParaKito© bracelet in combination with Basic Blend attracted significantly fewer mosquitoes than Basic Blend alone (p<0.001). See figure 10 B. No significant difference was found between the bio-assays (p=0.73) or their position (p=0.73).

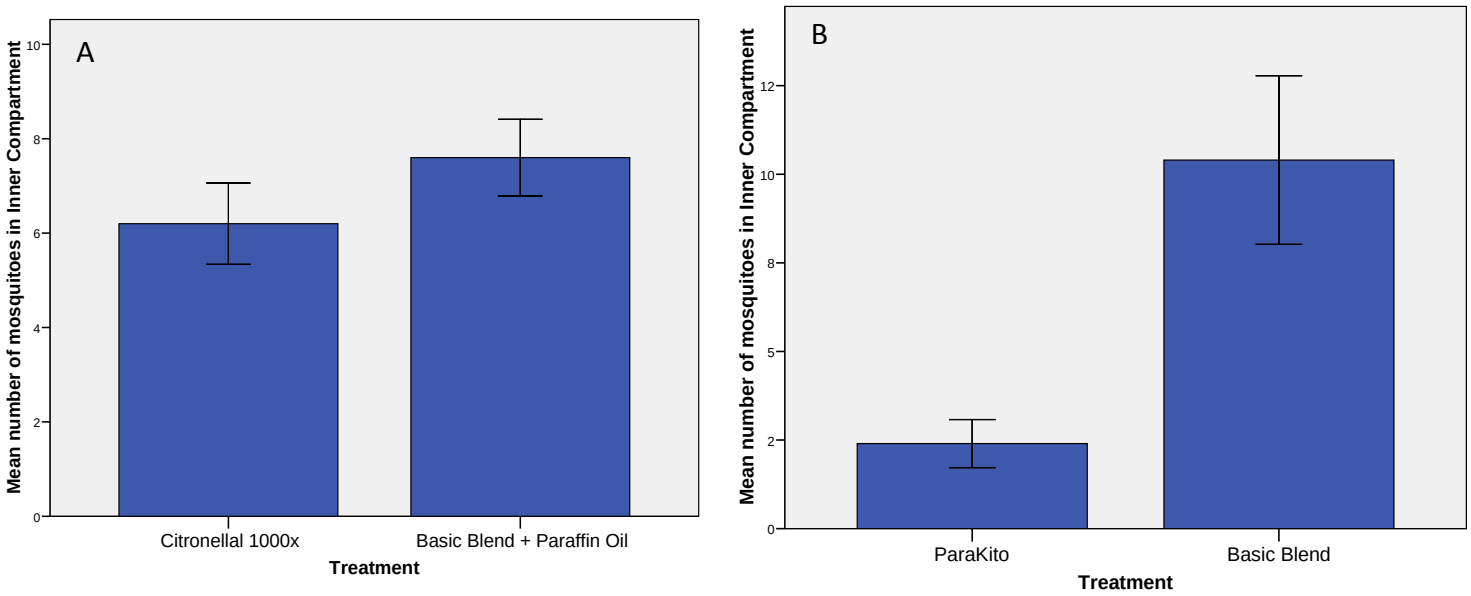


Figure 10: Mosquito response to commercially available compounds.
A: Mosquito response to Citronellal with Basic Blend against Basic Blend with Paraffin Oil. No significant difference in response was found.
B: Mosquito response to the ParaKito bracelet with Basic Blend against Basic Blend only. The Para'Kito bracelet with Basic Blend attracted significantly fewer mosquitoes than Basic Blend only.
Error bars represent standard error of mean.

3.4 Overview mosquito response

When we look at mosquito response as the number of mosquitoes trapped in the inner compartment as a fraction of the total number of mosquitoes that were released, we can easily compare all treatments at once. Mosquito response to Basic Blend (without any solvents) was high, on average 58.61%. Both 2-methyl-2-benzoate and 6-methyl-5-hepten-2-one yielded higher overall average responses than Basic Blend (60.56% and 63.61%, respectively), although this was not significant. The ParaKito bracelet yielded the lowest overall response, 12%. See figure 11 for an overview of mosquito response for all tested compounds.

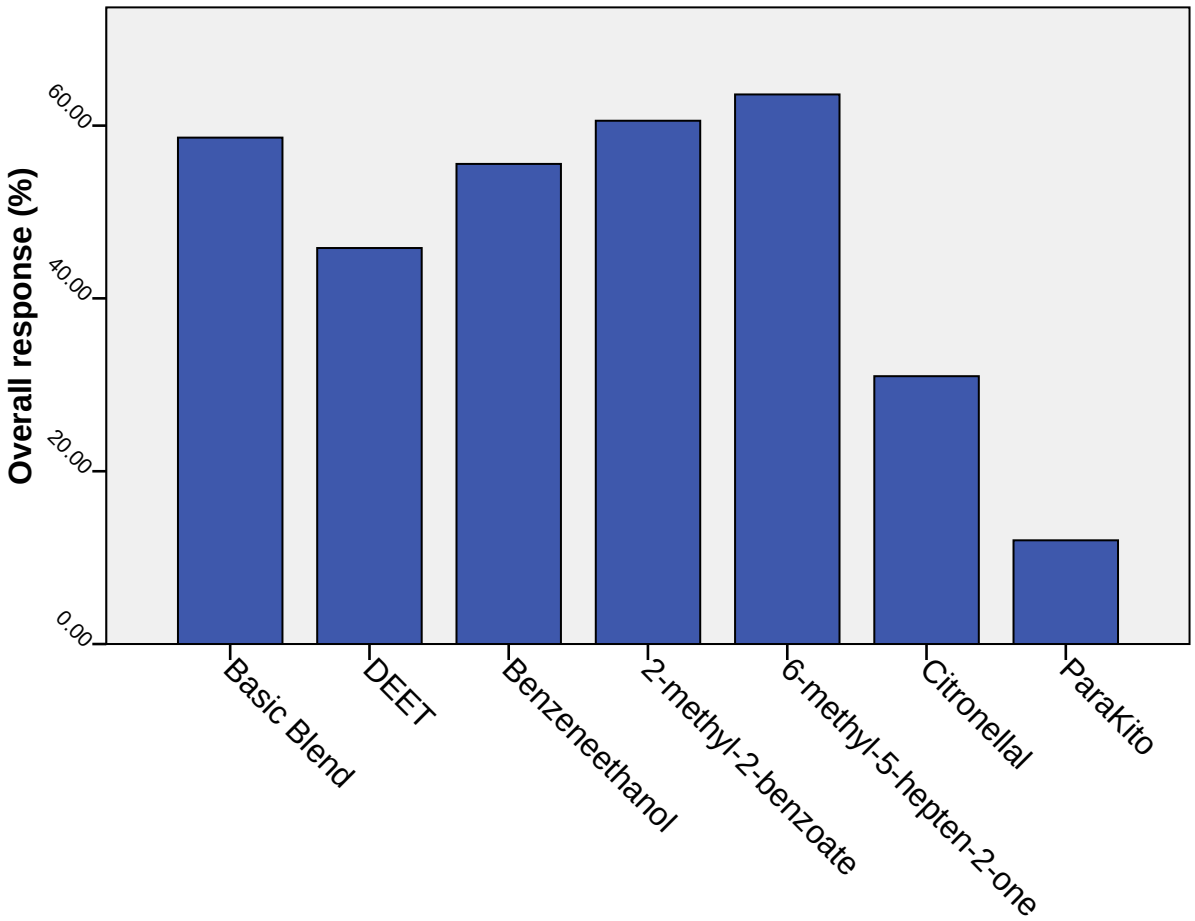


Figure 11: Overall response in percentages for all tested compounds. For compounds of which multiple concentrations were tested, the mean response of all concentrations was used.

3.5 Environmental influences

Temperature, relative humidity and wind speed were kept as constant and as equal between the two bio-assay boxes as possible. A Generalized Linear Model was used to investigate if any of these environmental conditions had any effect on mosquito response throughout all experiments. The mosquito response was significantly lower when wind speed was 19 cm/s ($p=0.023$, $n=29$ of 158), and when temperature was at $27\circ C$ ($p=0.008$, $n=16$ of 158) compared to other wind speeds and temperatures. Relative humidity was found to have no influence on mosquito response ($p=0.49$). Light intensity from the small lamp to simulate moonlight was not possible to measure since the only available photometer was not sensitive enough to measure such low lighting conditions.

4 Conclusions and discussion

4.1 Control experiments

Worn socks

The first series of tests was executed with worn socks to assess whether mosquitoes would fly into the inner compartment when an attractive odour source was present. Worn socks have previously been shown to be very attractive, and are the most attractive odour source found so far (Pates et al. 2001; Qiu 2005). Worn socks were found to be highly attractive in this research, and could potentially be used as an attractant control. However, even when the same volunteer is used to wear the socks, there can still be differences between the socks, due to diet or exercise or even what foot it was worn on. Also, nylon socks contain many non-human components, that might influence the results (Qiu et al. 2004). Also the exact composition of odours and their concentration are unknown. These differences are hard to quantify or control, therefore, in this research, worn socks were replaced with a synthetic odour blend as the attractive odour source in all experiments.

Symmetry of the bio-assays

During experiments tests were conducted to test the symmetry of the bio-assay boxes. No difference was found between bio-assays, but a significant difference was found for position of the bio-assays (N=18). No significant difference was found for the smaller experiments, probably due to the smaller amount of repetitions (N=6). Since positions were rotated throughout all experiments this should not have influenced the results. Because all other environmental conditions could be measured and were kept constant, the difference between the positions must have been caused by the lighting conditions. A small light was provided in front of the bio-assays to simulate moonlight and to help the mosquitoes navigate in the bio-assays. No sufficiently sensitive photometer was present to measure how much light each bio-assay received. Presumably, because of the difference in light intensity, mosquitoes are able to navigate more effectively in the bottom bio-assay.

Attractiveness of Basic Blend

Experiments had to be conducted with an attractive control in order to evaluate the effect of the different compounds. The Basic Blend used in this study has been shown to be attractive in other setups in previous studies (Smallegange et al. 2009). For the experiments in this setup to be successful, this Basic Blend had to be attractive in the new bio-assays as well. The Basic Blend was found to be highly attractive to *An. gambiae* s.s mosquitoes in the new bio-assays. In fact, Basic Blend was almost as attractive as worn socks in this setup (BB: 58.6%, WS:63%). In dual-choice olfactometer experiments, the mosquito response to Basic Blend is much lower, usually below 30% (G. Bukovinzkine Kiss, personal communication (Smallegange et al. 2009)). The higher entry response in this setup is probably due to the smaller scale of the bio-assays, the concentration of the compounds is higher, so the mosquitoes can more easily locate the attractive odour source.

Repellence of DEET

Because no true spatial repellents are known for *An. gambiae* s.s. , DEET was used as a repellent control. Even though results on the spatial repellence of DEET vary (Dogan and Rossignol 1999; Kline et al. 2003; Syed and Leal 2008), it is still the golden standard for

repellents until a truly effective spatial repellent is found. DEET was found to be spatial repellent in this study, but not very strongly so.

4.2 Tested compounds

Benzeneethanol

Although benzeneethanol showed promising results in previous olfactometer and semi-field experiments, it was not able to repel mosquitoes from an attractive odour source in any of the tested concentrations in this study. Possibly the difference in results can be attributed to a difference in concentrations perceived by the mosquitoes due to the scale of the setups that were used. The results in semi-field experiments are very promising and are a good indication that this compound may be used as a spatial repellent in future setups. Benzeneethanol could show spatial repellence in this setup at a different concentration.

6-methyl-5-hepten-2-one

6-Methyl-5-hepten-2-one was selected because of results in the previous study by Qiu et al. (2005) where it was found to be repellent at a flow rate of 50 ml/min (in combination with ammonia and lactic acid). However, in that study, a different method was used: 3 ml of pure (99%) 6-methyl-5-hepten-2-one was placed in a glass bottle through which air was led before entering the olfactometer that was used. Concentrations were varied by adjusting the air flow. These concentrations are far higher than the ones used in the current study. Logan et al. (2008) showed 6-Methyl-5-hepten-2-one to be repellent for *Aedes aegypti* mosquitoes. It might be that *Aedes aegypti* responds differently to 6-methyl-5-hepten-2-one, but the results cannot be compared. Logan et al. analyzed human hand emanations, therefore the exact concentrations of 6-methyl-5-hepten-2-one in his study are unknown. Also, it was part of a blend of odours, but in the current study it was examined as a single odour only. As with benzeneethanol the difference with the results found in the 4-choice semi-field experiments, might be due to concentration differences. It is very probable that 6-methyl-5-hepten-2-one will show spatial repellent effects at higher concentrations.

2-methyl-2-benzoate

2-Methyl-2-benzoate showed a similar receptor response profile to DEET, but in this research it did not show any repellence at any concentration tested. DEET did show spatial repellence, but at a much higher concentration (40%, 0.03 mm LDPE) than 2-methyl-2-benzoate (0.01-1%, 0.2mm LDPE). It is possible that at the same concentration, 2-methyl-2-benzoate, will show a similar effect on mosquito response. However, if 2-methyl-2-benzoate is similar to DEET, it will probably not be an effective spatial repellent. DEET will only repel mosquitoes at close range and at a high concentration. 2-methyl-2-benzoate might, however, be applied as a topical repellent. It might be an improvement on DEET, since its effect seems to last longer.

4.3 Commercially available compounds

Citronella

The one tested concentration of citronella in this study did not significantly repel mosquitoes from the attractive odour source. In the recent study by Müller et al. (2009), citronella did show spatial repellent effects for *Aedes* and *Culex* mosquitoes. It is possible that *An. gambiae* s.s. responds differently to citronella. However, the candles and diffusers that were used in the study by Müller et al. also utilized much higher concentrations than were tested in this study. The candles contained 88 g of 5% citronella, and the diffusers 20 g of 100%

citronella. The candles repelled 14% of mosquitoes and the diffusers 68%. In Lindsay et al. (1996) citronella was also found to be repellent at concentrations of 3 and 5%. These results indicate that citronella should be tested at higher concentrations in order for it to show spatial repellent effects. No influence was shown for position of the bio-assays for the test series with citronella, probably this is due to the lower number of repetitions in this experiment.

Para'Kito© bracelet

The Para'Kito© bracelet showed a clear spatial repellent effect in this study. However, it is probably not applicable a scale that would reduce malaria transmission in malaria-endemic area. The bracelets are quite expensive (± 5 euro per pellet) and according to the manufacturer they only work on a short range. Wearers are advised to wear one bracelet on the wrist and another around the opposite ankle. This indicates a active range of approximately one meter, which is a far too short range for application in houses. The producer of the bracelets does not divulge what components are used, although essential oils and lavender are mentioned. The bracelet smelled quite strongly of citronella as well (personal observation). The overall smell of the bracelet was quite overwhelming which would make application on a larger scale unfeasible. However, it would still be interesting to investigate what components within the bracelet repel the mosquitoes. No influence was shown for position of the bio-assays for the test series with Para'Kito©, probably this is due to the lower number of repetitions in this experiment.

4.4 Evaluation of the bio-assays

The primary aim of this research was to evaluate whether these newly developed bio-assays could function as an effective tool to evaluate spatial repellents against *An. gambiae* s.s. mosquitoes. Basic Blend attracted higher numbers of mosquitoes to the inner compartment than the dual-choice olfactometer used in previous research. DEET and the Para'Kito© bracelet were found to be spatial repellent. These results show that the bio-assays are a quick and effective tool to evaluate spatial repellents for this mosquito species. Since the bio-assays were specifically designed with the flight behaviour of *An. gambiae* mosquitoes in mind, they might not work to evaluate spatial repellents for other mosquito species. On the other hand, because of the small scale of the bio-assays, the climbing capability of *An. gambiae* is not necessarily needed (Snow 1987). It might be interesting to test it on other mosquito species. The circumstances in which these experiments were conducted was not ideal, environmental conditions were hard to keep constant, and the space was quite cramped. Possibly the effectiveness of the bio-assays could be further increased by moving them to a climate cell with continuous environmental conditions.

4.5 Used methods

Chosen concentrations

The concentrations that were used in this study, were quite conservative. Because of the short time that was available for this study, there was only time to study a limited amount of concentrations. The 3 concentrations that were used for this study, were investigated in previous studies and therefore seemed a good starting point. For future research, a broader concentration range should be examined. It could be that the tested compounds studied in this report will show repellence at different concentrations.

Mosquitoes

All behavioural experiments are done with live mosquitoes. Using living animals in research influences results in a way that cannot be controlled. The conditions among which the *An. gambiae* s.s. mosquitoes are raised at the Laboratory of Entomology are as constant as possible, but still there will always be differences among the mosquitoes. There are many factors that could influence the mosquitoes' behaviour. For example, even though the mosquitoes are kept in climate cells the weather outside seems to influence their behaviour. Especially when it was raining outside, the mosquitoes tended to exhibit less flight behaviour. The mechanisms behind this phenomenon are not yet known. Because experiments in the two bio-assays were always run at the same time and treatments were randomized, this should not have interfered with our results.

4.6 Research questions

- Is the newly developed bio-assay an effective tool for evaluating spatial repellents against the malaria mosquito *An. gambiae* s.s.?

The newly developed bio-assay performed well in this study. It attracted high percentages of mosquitoes to the Basic Blend and worn socks. DEET and the Para'Kito© bracelet were found to be spatial repellent. The results of this study confirm that this new bio-assay can be used to evaluate spatial repellents for *An. gambiae* s.s. mosquitoes.

- Is any of the tested compounds a significant spatial repellent against the malaria mosquito *An. gambiae* s.s.?

None of the compounds selected because of previous results was found to be an effective spatial repellent for *An. gambiae* s.s. mosquitoes. However, benzeneethanol and 6-methyl-5-hepten-2-one might be repellent at other concentrations. One of the commercially available products, the Para'Kito© bracelet did significantly repel *An. gambiae* s.s. mosquitoes, the other product, citronella, did not. Citronella was only tested at one, quite low, concentration, it might be spatial repellent at a higher concentration.

4.7 Suggestions for further research

- Bio-assays should be moved to a more spacious location with continuous environmental conditions
- The moonlight simulation should be adjusted so that both positions for the bio-assays attract equal amounts of mosquitoes
- A broader range of concentrations should be tested
- More compounds that have shown indications of spatial repellence should be tested in this setup
- The effect of adding CO_2 to the air flow should be investigated
- In this research, eaves were kept at ± 1 cm, tests should be done with different eave sizes to find the optimum eave size
- Tested compounds were placed within the air stream, along with the attractive odour source in this research. To more closely resemble the situation in an African house, tests should be done with the tested compound near the eaves.
- The attractive odour source could be placed in the tube where the air stream passes through.
- A smoke machine could be attached to the bio-assays to see how the airstream travels through the setup

5 Acknowledgements

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6 References

Awolola, T. S., B. D. Brooke, R. H. Hunt and M. Coetze (2002). **Resistance of the malaria vector *Anopheles gambiae* s.s. to pyrethroid insecticides, in south-western Nigeria**. Annals of Tropical Medicine and Parasitology **96**: 849-52.

Bayoh, M. N. and S. W. Lindsay (2003). **Effect of temperature on the developmentof the aquatic stages of *Anopheles gambiae* sensu stricto (diptera: Culicidae)**. Bulletin of Entomological Research **93**: 375–381.

Braks, M. A. H., J. Meijerink and W. Takken (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**: 142-148.

CDC, c. f. d. c. (2006). **Malaria**. www.cdc.gov.

Clements, A. N. (1999). The biology of mosquitoes. Wallingford, U.K., CABI Publishers.

Cook, S. M., Z. R. Khan and J. A. Pickett (2007). **The use of push-pull strategies in integrated pest management**. Annual Review of Entomology **52**: 375-400.

Costantini, C., G. Gibson, J. Brady, L. Merzagora and M. Coluzzi (1993). **A new odour-baited trap to collect host-seeking mosquitoes**. Parassitologia **35**: 5-9.

Dekker, T., B. Steib, R. T. Carde and M. Geier (2002). **L-lactic acid: A human-signifying host cue for the anthropophilic mosquito *Anopheles gambiae***. Medical and Veterinary Entomology **16**: 91-98.

Dogan, E. B. and P. A. Rossignol (1999). **An olfactometer for discriminating between attraction, inhibition, and repellency in mosquitoes (diptera: Culicidae)**. Journal of Medical Entomology **36**: 788-93.

Fradin, M. S. and J. F. Day (2002). **Comparative efficacy of insect repellents against mosquito bites**. New England Journal of Medicine **347**: 13-8.

Geier, M., O. J. Bosch and J. Boeckh (1999). **Ammonia as an attractive component of host odour for the yellow fever mosquito, *Aedes aegypti***. Chemical Senses **24**: 647-653.

Gillies, M. T. and B. DeMeillon (1968). **The Anophelinae of africa south of the sahara**. The South African Institute for Medical Research. Johannesburg, South Africa.

Greenwood, B. and T. Mutabingwa (2002). **Malaria in 2002**. Nature **415**: 670-672.

Hemingway, J. and H. Ranson (2000). **Insecticide resistance in insect vectors of human disease**. Annual Review of Entomology **45**: 371-91.

Kawada, H., N. T. Yen, N. T. Hoa, T. M. Sang, V. A. N. D. N and M. Takagi (2005). **Field evaluation of spatial repellency of metofluthrin impregnated plastic strips against mosquitoes in hai phong city, vietnam**. American Journal of Tropical Medicine and Hygiene **73**: 350-3.

Kline, D. L., U. R. Bernier, K. H. Posey and D. R. Barnard (2003). **Olfactometric evaluation of spatial repellents for *Aedes aegypti***. Journal of Medical Entomology **40**: 463-7.

Knols, B. G. J., W. Takken, A. Cork and R. DeJong (1997). **Odour-mediated, host-seeking behaviour of *Anopheles* mosquitoes: A new approach**. Annals of Tropical Medicine and Parasitology **91**: S117-S118.

Lefevre, T., L. C. Gouagna, K. R. Dabire, E. Elguero, D. Fontenille, C. Costantini and F. Thomas (2009). **Evolutionary lability of odour-mediated host preference by the malaria vector *Anopheles gambiae***. Tropical Medicine and International Health **14**: 228-36.

Lindsay, L. R., G. A. Surgeoner, J. D. Heal and G. J. Gallivan (1996). **Evaluation of the efficacy of 3% citronella candles and 5% citronella incense for protection against field**

populations of *Aedes* mosquitoes. Journal of the American Mosquito Control Association **12**: 293-4.

Lindsay, S. W., P. M. Emerson and J. D. Charlwood (2002). **Reducing malaria by mosquito-proofing houses**. Trends in Parasitology **18**: 510-514.

Logan, J. G., M. A. Birkett, S. J. Clark, S. Powers, N. J. Seal, L. J. Wadhams, A. J. Mordue Luntz and J. A. Pickett (2008). **Identification of human-derived volatile chemicals that interfere with attraction of *Aedes aegypti* mosquitoes**. Journal of Chemical Ecology **34**: 308-22.

Müller, G. C., A. Junnila, J. Butler, V. D. Kravchenko, E. E. Revay, R. W. Weiss and Y. Schlein (2009). **Efficacy of the botanical repellents geraniol, linalool, and citronella against mosquitoes**. Journal of Vector Ecology **34**: 2-8.

Njie, M., E. Dilger, S. W. Lindsay and M. J. Kirby (2009). **Importance of eaves to house entry by Anopheline, but not Culicine, mosquitoes**. Journal of Medical Entomology **46**: 505-10.

Okumu, F. O., G. F. Killeen, S. Ogoma, L. Biswaro, R. C. Smallegange, E. Mbeyela, E. Titus, C. Munk, H. Ngonyani, W. Takken, H. Mshinda, W. R. Mukabana and S. J. Moore (2010). **Development and field evaluation of a synthetic mosquito lure that is more attractive than humans**. PLoS One **5**: -.

Pates, H. V., W. Takken, K. Stuke and C. F. Curtis (2001). **Differential behaviour of *Anopheles gambiae* sensu stricto (diptera: Culicidae) to human and cow odours in the laboratory**. Bulletin of Entomological Research **91**: 289-96.

Qiu, Y., R. Smallegange, H. Smid, J. v. Loon, A. Galimard, M. Posthumus, T. v. Beek and W. Takken (2004). **Gc-eag analysis of human odours that attract the malaria mosquito *Anopheles gambiae* sensu stricto**. Groningen.

Qiu, Y. T. (2005). **Sensory and behavioural responses of the malaria mosquito *Anopheles gambiae* to human odours**. Entomology. Wageningen, Wageningen University.

Sissoko, M. S., A. Dicko, O. J. T. Briët, M. Sissoko, I. Sagara, H. D. Keita, M. Sogoba, C. Rogier, Y. T. Touré and O. K. Doumbo (2004). **Malaria incidence in relation to rice cultivation in the irrigated sahel of mali**. Acta Tropica **89**: 161–170.

Smallegange, R. C., B. G. Knols and W. Takken (2010). **Effectiveness of synthetic versus natural human volatiles as attractants for *Anopheles gambiae* (diptera: Culicidae) sensu stricto**. Journal of Medical Entomology **47**: 338-44.

Smallegange, R. C., Y. T. Qiu, G. Bukovinszkine-Kiss, J. J. Van Loon and W. Takken (2009). **The effect of aliphatic carboxylic acids on olfaction-based host-seeking of the malaria mosquito *Anopheles gambiae* sensu stricto**. Journal of Chemical Ecology **35**: 933-43.

Smallegange, R. C., Y. T. Qiu, J. J. A. v. Loon and W. Takken (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**: 145-152.

Snow, K. R. (1990). Mosquitoes, School of Science Polytechnic of East Londen.

Snow, W. F. (1987). **Studies of house-entering habits of mosquitoes in the gambia, west africa: Experiments with prefabricated huts with varied wall apertures**. Medical en Veterinary Entomology **1**: 9-21.

Syed, Z. and W. S. Leal (2008). **Mosquitoes smell and avoid the insect repellent deet**. Proceedings of the National Academy of Sciences of the United States of America **105**: 13598-603.

Takken, W., W. M. Docters van Leeuwen and H. Verhoek (2004). **Malaria**. Wageningen, Wageningen University and Research Centre.

Takken, W. and B. G. Knols (1999). **Odor-mediated behavior of afrotropical malaria mosquitoes**. Annu Rev Entomol **44**: 131-57.

Tanser, F. C., B. Sharp and D. le Sueur (2003). **Potential effect of climate change on malaria transmission in africa**. Lancet **362**: 1792-1798.

Tia, E., M. Akogbeto, A. Koffi, M. Toure, A. M. Adja, K. Moussa, T. Yao, P. Carnevale and E. Chandre (2006). **Pyrethroid and ddt resistance of *Anopheles gambiae* s.s. (diptera: Culicidae) in five agricultural ecosystems from Cote-d'ivoire**. Bulletin de la Société de Pathologie Exotique **99**: 278-82.

Torr, S. J., A. Della Torre, M. Calzetta, C. Costantini and G. A. Vale (2008). **Towards a fuller understanding of mosquito behaviour: Use of electrocuting grids to compare the odour-orientated responses of *Anopheles arabiensis* and *An. quadriannulatus* in the field**. Medical en Veterinary Entomology **22**: 93-108.

Verhulst, N. O., H. Beijleveld, B. G. Knols, W. Takken, G. Schraa, H. J. Bouwmeester and R. C. Smallegange (2009). **Cultured skin microbiota attracts malaria mosquitoes**. Malaria Journal **8**: 302.

APPENDIX

Appendix A: Experiment scheme

Time	Assay	day 1		day 2		day 3		day 4		day 5		day 6	
		top	bottom	top	bottom	top	bottom	top	bottom	top	bottom	top	bottom
1	1		BB	BB		BB			100x	DEET		BB	1000x
	2	BB			1000X		10000X	BB			BB	BB	
2	1		100x	BB		BB			BB	1000X			BB
	2	BB			DEET		BB	10000X			BB	100X	
3	1	1000x		BB		DEET			BB	BB			BB
	2		BB		10000X		BB	BB			100X	BB	
4	1	10000x		BB		100X			BB	10000X	B		BB
	2		BB	BB			BB	1000X			BB	DEET	
5	1	DEET			BB	BB			BB	BB			10000x
	2		BB	100X			1000X	DEET			BB	BB	
	deet	40% deet											
	BB	basic blend+solvent											

Appendix B: Compounds

Table 1: Compounds used in experiments

Compounds	Purity	Company
Ammonia	25%	Merck
L-lactic acid	88-92%	Riedel- de Haën
Tetradecanoic acid	>99%	Sigma
Paraffin oil		
Benzeneethanol	≥99.5%	Fluka
6-methyl-5-hepten-2-one	99%	Sigma Aldrich
2-methyl-2-benzoate	99%	Sigma Aldrich
Citronella	≥95%	
Ethanol	96%	Merck
Nylon Socks		Hema
Para'Kito bracelet		www.parakito.com

Appendix C: SPSS outputs

Table 2: Worn socks output

Descriptives

			Statistic	Std. Error
Percentage of mosquitoes in Inner Compartment	Mean		.6320	.04212
	95% Confidence Interval for Mean	Lower Bound	.5451	
		Upper Bound	.7189	
	5% Trimmed Mean		.6417	
	Median		.6500	
	Variance		.044	
	Std. Deviation		.21059	
	Minimum		.10	
	Maximum		.95	
	Range		.85	
	Interquartile Range		.30	
	Skewness		-.628	.464
	Kurtosis		-.019	.902

Table 3: Basic Blend output (symmetry test)

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.623	.1464	.336	.910	18.130	1	.000	1.865	1.400	2.485
[pos=1]	-.608	.1571	-.915	-.300	14.955	1	.000	.545	.400	.741
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	.074	.1569	-.234	.381	.222	1	.638	1.077	.792	1.464
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 4: Basic Blend against clean air output:

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.649	.0335	.584	.715	375.448	1	.000	1.914	1.792	2.044
[Treatment=1]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.146	.0371	-.219	-.073	15.451	1	.000	.864	.804	.929
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	.020	.0371	-.053	.093	.290	1	.590	1.020	.949	1.097
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 5: DEET output, Treatment 2=DEET+BB treatment 7=ETH+BB

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.420	.1372	.151	.689	9.379	1	.002	1.522	1.163	1.992
[Treatment=2]	-.404	.1952	-.787	-.022	4.291	1	.038	.667	.455	.978
[Treatment=7]	0[a]	.	.	.	.	.	.	1	.	.
[Pos=1]	-.417	.2085	-.826	-.008	4.001	1	.045	.659	.438	.992
[Pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[Assay=1]	.315	.1759	-.029	.660	3.214	1	.073	1.371	.971	1.935
[Assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, Pos, Assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 6: Benzeneethanol output: Treatments: 3=1%, 4=0.1% 5=0.01%.

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.547	.1987	.157	.936	7.570	1	.006	1.728	1.170	2.550
[Treatment=1]	.112	.2345	-.348	.572	.228	1	.633	1.119	.706	1.771
[Treatment=2]	.014	.2758	-.527	.554	.002	1	.961	1.014	.590	1.741
[Treatment=3]	.148	.2701	-.382	.677	.299	1	.585	1.159	.683	1.968
[Treatment=4]	-.130	.2669	-.653	.393	.239	1	.625	.878	.520	1.481
[Treatment=5]	-.333	.2661	-.855	.188	1.570	1	.210	.716	.425	1.207
[Treatment=6]	-.187	.2201	-.618	.244	.722	1	.396	.829	.539	1.277
[Treatment=7]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.433	.1231	-.675	-.192	12.394	1	.000	.648	.509	.825
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	.007	.1175	-.223	.237	.003	1	.954	1.007	.800	1.268
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 7 : 6-methyl-5-hepten-2-one output: Treatments: 3=1%, 4=0.1% 5=0.01%

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.664	.2094	.253	1.074	10.041	1	.002	1.942	1.288	2.927
[Treatment=1]	-.148	.2325	-.603	.308	.403	1	.525	.863	.547	1.361
[Treatment=2]	-.563	.2716	-1.095	-.031	4.294	1	.038	.570	.335	.970
[Treatment=3]	-.008	.2684	-.535	.518	.001	1	.975	.992	.586	1.678
[Treatment=4]	.282	.2730	-.254	.817	1.064	1	.302	1.325	.776	2.263
[Treatment=5]	.282	.2730	-.254	.817	1.064	1	.302	1.325	.776	2.263
[Treatment=6]	-.009	.2203	-.440	.423	.002	1	.969	.991	.644	1.527
[Treatment=7]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.564	.1209	-.801	-.327	21.790	1	.000	.569	.449	.721
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	.011	.1212	-.227	.248	.008	1	.929	1.011	.797	1.282
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 8: 2-methyl-2-benzoate results: Treatments: 3=1%, 4=0.1% 5=0.01%

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.498	.1993	.107	.889	6.245	1	.012	1.646	1.113	2.432
[Treatment=1]	-.045	.2354	-.507	.416	.037	1	.847	.956	.602	1.516
[Treatment=2]	-.738	.2788	-1.284	-.191	6.999	1	.008	.478	.277	.826
[Treatment=3]	.061	.2716	-.471	.593	.050	1	.823	1.063	.624	1.809
[Treatment=4]	-.117	.2687	-.644	.409	.190	1	.663	.889	.525	1.506
[Treatment=5]	.093	.2715	-.439	.625	.117	1	.732	1.098	.645	1.869
[Treatment=6]	-.105	.2221	-.540	.330	.224	1	.636	.900	.583	1.391
[Treatment=7]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.381	.1242	-.624	-.137	9.399	1	.002	.683	.536	.872
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	.229	.1189	-.004	.462	3.711	1	.054	1.257	.996	1.587
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 9: Citronella output

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	-.388	.2374	-.853	.078	2.668	1	.102	.679	.426	1.081
[Treatment=1]	-.260	.3049	-.857	.338	.725	1	.394	.771	.424	1.402
[Treatment=2]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.260	.3049	-.857	.338	.725	1	.394	.771	.424	1.402
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
[assay=1]	0[a]	.	.	.	.	.	.	1	.	.
[assay=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos, assay

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 10: Para'Kito output

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.127	.2426	-.349	.602	.274	1	.601	1.135	.706	1.827
[Treatment=1]	-2.050	.3722	-2.780	-1.321	30.352	1	.000	.129	.062	.267
[Treatment=2]	0[a]	.	.	.	.	.	.	1	.	.
[pos=1]	-.117	.3417	-.787	.553	.117	1	.732	.890	.455	1.738
[pos=2]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment, pos

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 11: different Basic Blends output, Treatments: 1=BB, 2=BB+PF oil, 3=BB+ETH

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	.499	.1087	.286	.712	21.070	1	.000	1.647	1.331	2.038
[Treatment2=1]	-.151	.1324	-.411	.109	1.301	1	.254	.860	.663	1.115
[Treatment2=2]	-.223	.1249	-.468	.022	3.195	1	.074	.800	.626	1.022
[Treatment2=3]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), Treatment2

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Table 12: environmental conditions output

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval		Hypothesis Test			Exp(B)	95% Wald Confidence Interval for Exp(B)	
			Lower	Upper	Wald Chi-Square	df	Sig.		Lower	Upper
(Intercept)	1.110	.5408	.050	2.170	4.212	1	.040	3.034	1.051	8.757
[WindspdIn=19]	-.643	.2828	-1.197	-.089	5.172	1	.023	.526	.302	.915
[WindspdIn=20]	-.340	.2754	-.880	.199	1.528	1	.216	.711	.415	1.221
[WindspdIn=21]	.052	.2851	-.506	.611	.034	1	.854	1.054	.603	1.842
[WindspdIn=22]	-.205	.2776	-.749	.339	.546	1	.460	.815	.473	1.403
[WindspdIn=23]	0[a]	.	.	.	.	.	.	1	.	.
[TempIn=26]	-.623	.4542	-1.514	.267	1.882	1	.170	.536	.220	1.306
[TempIn=27]	-.908	.3433	-1.581	-.235	7.000	1	.008	.403	.206	.790
[TempIn=28]	-.259	.3269	-.899	.382	.626	1	.429	.772	.407	1.465
[TempIn=29]	-.245	.3288	-.889	.400	.553	1	.457	.783	.411	1.492
[TempIn=30]	0[a]	.	.	.	.	.	.	1	.	.
[RelHum=94.00]	-.556	.4665	-1.470	.359	1.420	1	.233	.574	.230	1.431
[RelHum=95.00]	-.314	.3348	-.970	.342	.880	1	.348	.730	.379	1.408
[RelHum=96.00]	0[a]	.	.	.	.	.	.	1	.	.
(Scale)	1[b]									

Events: Inner Compartment
Trials: # of mosquitoes
Model: (Intercept), WindspdIn, TempIn, RelHum

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

Appendix D: Raw data

Date	BA	Position	Compound	[Conc.]	# mos	Temp surr.	RH surr.	WS	RH	Temp	Cage	Outer	Inner	Eave entry%	Comments
12/4/2010	2	top	sok jeroen		15	32	45	18	95	30	0	12	3	0.2	muggen vliegen
	1	bottom	sok jeroen		15			19	95	30	5	9	1	0.06667	wel naar sok, maar
	2	top	sok jeroen		15	32	45	19-20	95	30	0	11	4	0.26667	dan door eave
	1	bottom	sok jeroen		15			19	95	30	2	11	2	0.13333	weer terug
13/4/2010	2	top	sok jeroen		15	32	50	21	93	29	0	5	10	0.66667	Thermostaat verkeerd
	1	bottom	sok jeroen		15			19	95	29	3	8	4	0.26667	afgesteld, temp-in
	2	top	sok jeroen		15	32	50	22	93	31	0	3	12	0.8	lijkt afhankelijk van
	1	bottom	sok jeroen		15			20	95	31	3	9	3	0.2	omgevingstemp.
14/4/2010	2	top	sok jeroen		20	30	54	20	94	29	0	1	19	0.95	
	1	bottom	sok jeroen		20			19	95	29	1	3	16	0.8	
	2	top	sok jeroen		20	30	53	22	95	29	0	2	18	0.9	
	1	bottom	sok jeroen		20			19	95	29	0	3	17	0.85	
	2	top	sok jeroen		20	30	57	20	95	29	0	3	17	0.85	
	1	bottom	sok jeroen		20			19	95	29	0	11	9	0.45	assay geraakt arm
15/4/2010	2	top	BB		20	30	58	22	95	29	0	9	11	0.55	
	1	bottom	BB		20			22	95	29	2	14	4	0.2	
	2	top	BB		20	29	58	22	95	29	1	9	10	0.5	
	1	bottom	BB		20			21	95	29	2	12	6	0.3	
	2	top	BB		20	28	59	23	95	28	0	15	5	0.25	0.05 mm LDPE NH3
	1	bottom	BB		20			21	95	28	1	7	12	0.6	
	2	top	BB		20	30	60	22	95	29	7	10	3	0.15	
	1	bottom	BB		20			22	95	29	2	12	6	0.3	

16/4/2010	1	top	BB		20	28	62	22	95	29	3	3	14	0.7	Top en bottom bio-
	2	bottom	BB		20			21	95	29	4	7	8	0.4	assays
	1	top	BB		20	29		22	95	29	4	4	12	0.6	omgewisseld
	2	bottom	BB		20			22	95	29	1	7	12	0.6	
	1	top	BB		20	30		23	95	29	3	7	10	0.5	
	2	bottom	BB		20			22	95	29	2	8	10	0.5	
20/4/2010	1	top	BB		20	30	62	23	95	30	3	4	13	0.65	
	2	bottom	BB		20			23	95	30	6	5	9	0.45	
	1	top	BB		20	30		21	95	29	5	4	11	0.55	
	2	bottom	BB		20			20	95	29	7	5	8	0.4	
	1	top	BB		20	30		23	95	29	4	4	12	0.6	
	2	bottom	BB		20			21	95	29	7	4	9	0.45	
	1	top	BB		20	30		23	95	29	2	7	11	0.55	15w lamp kapot
	2	bottom	BB		20			22	95	29	0	6	14	0.7	gegaan
21/4/2010	1	top	cma		20	28	60	23	95	28	3	16	1	0.05	LDPE-zakjes gevuld
	2	top	cma		20			20	95	28	4	14	2	0.1	met 100microl.
	1	bottom	cma		20	30		22	95	29	3	17	0	0	Gedistilleerd
	2	bottom	cma		20			22	95	29	1	19	0	0	water.
	1	top	cma		20	29		22	95	29	2	17	1	0.05	
	2	bottom	cma		20			22	95	29	3	15	2	0.1	
22/4/2010	1	top	sok jeroen		20	29	60	45?	95	29	0	10	10	0.5	controle
	2	bottom	sok jeroen		20			30?	95	29	3	4	13	0.65	
	1	top	deet	40%	20	29	58	?	95	29	2	4	14	0.7	problemen met
	2	bottom	sok jeroen		20			?	95	29	1	6	11	0.55	windsnelheid
23/4/2010	1	top	sok jeroen		20	30	58	19	95	29	1	3	16	0.8	controle
	2	bottom	sok jeroen		20			20	95	29	7	6	7	0.35	
	1	top	deet	40%	20	30	59	20	95	29	6	1	13	0.65	Deet en ethanol op
	2	bottom	sok jeroen	+eth	20			18	95	29	8	2	10	0.5	filtreerpapier, met
	1	top	deet	40%	20	29	59	20	95	29	5	5	10	0.5	plakband bij eave
	2	bottom	sok jeroen	+eth	20			21	95	29	8	5	7	0.35	
	1	top	deet	40%	20	29	57	21	95	29	6	6	8	0.4	
	2	bottom	sok jeroen	+eth	20			21	95	29	7	11	2	0.1	
28/4/2010	1	top	sok jeroen		20	30	60	21	95	29	6	4	10	0.5	controle
	2	bottom	sok jeroen		20			20	95	29	1	2	17	0.85	
	1	top	sok jeroen	+eth	20	29	61	19	95	29	3	2	15	0.75	Deet en ethanol op
	2	bottom	deet	40%	20			20	95	29	5	1	14	0.7	filtreerpapier, met
	1	top	sok jeroen	+eth	20	31	60	19	95	29	2	2	16	0.8	plakband bij eave
	2	bottom	deet	40%	20			19	95	29	0	3	17	0.85	
	1	top	sok jeroen	+eth	20	30	58	20	95	29	2	3	15	0.75	
	2	bottom	deet	40%	20			19	95	29	9	3	8	0.4	
	1	top	sok jeroen	+eth	20	28	57	19	95	29	3	4	13	0.65	
	2	bottom	deet	40%	20			19	95	29	4	2	14	0.7	
29/4/2010	1	top	BB		20	30	50	19	95	30	4	5	11	0.55	
	2	bottom	BB		20			20	95	30	4	4	12	0.6	
	2	top	BB		20	29	55	20	95	29	5	4	11	0.55	
	1	bottom	BE	100x	20			21	95	29	1	1	18	0.9	
	1	top	BE	1000x	20	29	60	20	95	29	9	1	10	0.5	
	2	bottom	BB		20			22	95	29	9	1	10	0.5	
	2	top	BB		20	28	60	19	95	28	5	0	15	0.75	
	1	bottom	BE	10000x	20			20	95	28	4	2	14	0.7	
	1	top	DEET	40%	20	28	60	19	95	28	5	2	13	0.65	
	2	bottom	BB		20			20	95	28	4	2	14	0.7	
3/5/2010	1	top	BB		20	29	58	22	95	29	3	10	7	0.35	
	2	bottom	BE	1000X	20			23	95	29	2	4	14	0.7	
	1	top	BB		20	28	60	20	95	28	2	5	13	0.65	
	2	bottom	DEET	40%	20			20	95	28	2	4	14	0.7	
	2	top	BE	10000X	20	28	57	19	95	28	8	5	7	0.35	
	1	bottom	BB		20			22	95	28	7	4	9	0.45	
	2	top	BB		20	28	58	20	95	28	4	2	14	0.7	
	1	bottom	BB		20			21	95	28	3	4	13	0.65	
	2	top	BE	100X	20	29	60	20	95	29	6	4	10	0.5	
	1	bottom	BB		20			20	95	29	7	3	10	0.5	
4/5/2010	1	top	BB		20	28	50	20	95	27	2	8	10	0.5	
	2	bottom	BE	10000X	20			22	95	27	4	5	11	0.55	
	1	top	BB		20	28	58	22	95	27	3	5	12	0.6	
	2	bottom	BB		20			22	95	27	2	2	16	0.8	
	1	top	DEET	40%	20	29	58	21	95	28	5	3	12	0.6	
	2	bottom	BB		20			22	95	28	3	6	11	0.55	
	1	top	BE	100X	20	29	60	22	95	28	7	2	11	0.55	
	2	bottom	BB		20			22	95	28	4	1	15	0.75	
	1	top	BB		20	29	58	19	95	29	10	2	8	0.4	
	2	bottom	BE	1000X	20			20	95	29	4	3	13	0.65	

	2	bottom	BB		20			22	95	28	3	6	11	0.55	
	1	top	BE	100X	20	29	60	22	95	28	7	2	11	0.55	
	2	bottom	BB		20			22	95	28	4	1	15	0.75	
	1	top	BB		20	29	58	19	95	29	10	2	8	0.4	
	2	bottom	BE	1000X	20			20	95	29	4	3	13	0.65	
5/5/2010	2	top	BB		20	29	57	22	95	29	3	4	13	0.65	
	1	bottom	BE	100X	20			22	95	29	1	3	16	0.8	
	2	top	BE	10000X	20			21	95	29	2	6	12	0.6	
	1	bottom	BB		20			22	95	29	4	2	14	0.7	
	2	top	BB		20			19	95	29	2	5	13	0.65	
	1	bottom	BB		20			21	95	29	2	6	12	0.6	
	2	top	BE	1000X	20			20	95	29	1	4	15	0.75	
	1	bottom	BB		20			21	95	29	4	2	14	0.7	
	2	top	DEET	40%	20			20	95	29	2	5	13	0.65	
	1	bottom	BB		20			22	95	29	5	5	10	0.5	
7/5/2010	1	top	DEET	40%	20	29	58	19	95	29	5	5	10	0.5	Weer buiten erg
	2	bottom	BB		20			20	95	29	7	4	9	0.45	regenachtig,
	1	top	BE	1000X	20			20	95	29	4	12	4	0.2	slechte
	2	bottom	BB		20			21	95	29	4	10	6	0.3	muggenrespons
	1	top	BB		20			20	95	29	6	2	12	0.6	
	2	bottom	BE	100X	20			22	95	29	4	6	10	0.5	
	1	top	BE	10000X	20			19	95	29	9	5	6	0.3	
	2	bottom	BB		20			20	95	29	7	4	9	0.45	
	1	top	BB		20			19	95	29	11	4	5	0.25	
	2	bottom	BB		20			20	95	29	9	0	11	0.55	
11/5/2010	2	top	BB		20	28	55	22	95	27	4	11	5	0.25	Rare geur in lab,
	1	bottom	BE	1000X	20			22	95	27	2	8	10	0.5	droogoven
	2	top	BE	100X	20			22	95	28	3	8	9	0.45	
	1	bottom	BB		20			20	95	28	2	5	13	0.65	
	2	top	BB		20	30		22	95	29	1	8	11	0.55	
	1	bottom	BB		20			22	95	29	4	0	16	0.8	
	2	top	DEET	40%	20			22	95	29	6	10	4	0.2	
	1	bottom	BB		20			21	95	29	2	1	17	0.85	
	2	top	BB		20			21	95	29	7	1	12	0.6	
	1	bottom	BE	10000X	20			22	95	29	4	6	10	0.5	
12/5/2010	2	top	BB		20	28	50	?	95	26	2	10	8	0.4	Windsnelheidsmet
	1	bottom	BB		20			?	95	26	3	9	8	0.4	er bij Remco
	2	top	BB		20			20	95	26	5	11	4	0.2	
	1	bottom	MHO	100X	20			22	95	26	2	3	15	0.75	
	1	top	MHO	1000X	20	29	55	19	95	28	3	4	13	0.65	
	2	bottom	BB		20			20	95	28	5	3	12	0.6	
	1	top	MHO	10000X	20	30		20	95	29	7	4	9	0.45	
	2	bottom	BB		20			22	95	29	4	0	16	0.8	
	1	top	DEET	40%	20			22	95	29	10	2	8	0.4	
	2	bottom	BB		20			22	95	29	7	3	10	0.5	
14/5/2010	1	top	BB		20	28	60	21	95	28	1	15	4	0.2	C14 afgevallen
	2	bottom	MHO	1000X	20			19	95	28	2	4	14	0.7	
	1	top	BB		20			22	95	28	1	6	13	0.65	
	2	bottom	DEET	40%	20			20	95	28	8	4	8	0.4	
	1	top	BB		20			21	95	28	5	4	11	0.55	
	2	bottom	MHO	10000X	20			20	95	28	4	3	13	0.65	
	2	top	BB		20			21	95	28	5	4	11	0.55	
	1	bottom	BB		20			21	95	28	5	0	15	0.75	
	2	top	MHO	100X	20	29		19	95	29	2	6	12	0.6	
	1	bottom	BB		20			23	95	29	2	3	15	0.75	
18/5/2010	1	top	BB		20	28	50	19	95	28	0	11	9	0.45	
	2	bottom	MHO	1000X	20			22	95	28	1	6	13	0.65	
	1	top	BB		20			19	95	28	1	11	8	0.4	
	2	bottom	BB		20			22	95	28	0	14	6	0.3	
	1	top	DEET	40%	20			19	95	28	3	7	10	0.5	
	2	bottom	BB		20			22	95	28	6	2	12	0.6	
	1	top	MHO	100X	20			22	95	28	2	11	7	0.35	
	2	bottom	BB		20			22	95	28	2	3	15	0.75	
	1	top	BB		20	29		19	95	29	2	10	8	0.4	

	2	bottom	MHO	10000X	20			22	95	29	2	5	13	0.65	
19/5/2010	2	top	BB		20	28	55	21	95	28	0	7	13	0.65	
	1	bottom	MHO	100X	20			22	95	28	5	6	9	0.45	
	2	top	MHO	10000X	20			19	95	28	1	6	13	0.65	
	1	bottom	BB		20			22	95	28	4	2	14	0.7	
	2	top	BB		20			20	95	28	3	7	10	0.5	
	1	bottom	BB		20			22	95	28	1	4	15	0.75	
	2	top	MHO	1000X	20			19	95	28	2	5	13	0.65	
	1	bottom	BB		20			21	95	28	1	3	16	0.8	
	2	top	DEET	40%	20			20	95	29	4	6	10	0.5	
	1	bottom	BB		20			22	95	29	2	1	17	0.85	
20/5/2010	1	top	DEET	40%	20	28	50	19	95	28	2	10	8	0.4	
	2	bottom	BB		20			22	95	28	2	4	14	0.7	
	1	top	MHO	1000X	20	30		20	95	29	1	3	16	0.8	
	2	bottom	BB		20			21	95	29	1	1	18	0.9	
	1	top	BB		19			21	95	29	0	4	15	0.78947	mug ontsnapt
	2	bottom	MHO	100X	20			20	95	29	3	3	14	0.7	
	1	top	MHO	10000X	20			21	95	29	4	1	15	0.75	
	2	bottom	BB		20			21	95	29	1	3	16	0.8	
	1	top	BB		20	28		20	95	28	1	5	14	0.7	
	2	bottom	BB		20			20	95	28	2	2	16	0.8	
21/5/2010	2	top	BB		20	28	55	20	95	28	3	9	8	0.4	Windsnelheid
	1	bottom	MHO	10000X	20			21	95	28	1	3	16	0.8	assay 1
	2	top	MHO	100X	20			20	95	28	3	3	14	0.7	onregelmatig,
	1	bottom	BB		20			22	95	28	4	6	10	0.5	slecht in te stellen,
	2	top	BB		20			20	95	28	8	3	9	0.45	laatste run beter.
	1	bottom	BB		20			22	95	28	4	2	14	0.7	
	2	top	DEET	40%	20			20	95	28	9	5	6	0.3	
	1	bottom	BB		20			22	95	28	9	1	10	0.5	
	2	top	BB		20			22	95	28	8	3	9	0.45	
	1	bottom	MHO	1000X	20			22	95	28	9	1	10	0.5	
26/5/2010	2	top	BB		20	28	45	20	95	28	2	12	6	0.3	
	1	bottom	BB		20			20	95	28	4	5	11	0.55	
	2	top	BB		20			19	95	28	3	12	5	0.25	
	1	bottom	MMB	100X	20			20	95	28	5	4	11	0.55	
	1	top	MMB	1000X	20			20	95	28	0	7	13	0.65	
	2	bottom	BB		20			20	95	28	10	4	6	0.3	
	1	top	MMB	10000X	20			20	95	29	5	7	8	0.4	
	2	bottom	BB		20			20	95	29	7	2	11	0.55	
	1	top	DEET	40%	20			20	95	29	7	7	6	0.3	
	2	bottom	BB		20			20	95	29	5	1	14	0.7	
27/5/2010	1	top	BB		20	28	50	19	95	28	0	15	5	0.25	
	2	bottom	MMB	1000X	20			19	95	28	4	7	9	0.45	
	1	top	BB		20			19	95	28	2	4	14	0.7	
	2	bottom	DEET	40%	20			19	95	28	4	6	10	0.5	
	1	top	BB		20						0	4	16	0.8	Windsnelheids- en
	2	bottom	MMB	10000X	20						2	1	17	0.85	luchtvochtigheids-
	2	top	BB		20						4	3	13	0.65	meter bij Renate
	1	bottom	BB		20						3	1	16	0.8	
	2	top	MMB	100X	20						1	9	10	0.5	
	1	bottom	BB		20						4	0	16	0.8	
28/5/2010	1	top	BB		20	28		19		28	3	10	7	0.35	
	2	bottom	MMB	10000X	20			19		28	5	3	12	0.6	
	1	top	BB		20						2	6	12	0.6	Windsnelheids-
	2	bottom	BB		20						3	0	17	0.85	meter bij Renate,
	1	top	DEET	40%	20						2	2	16	0.8	luchtvochtigheids-
	2	bottom	BB		20						0	0	20	1	meter werkt niet
	1	top	MMB	100X	20						3	2	15	0.75	
	2	bottom	BB		20						3	2	15	0.75	
	1	top	BB		20						1	0	19	0.95	
	2	bottom	MMB	1000X	20						5	2	13	0.65	

1/6/2010	2	top	BB		20	28	65	20	95	28	4	4	12	0.6	
	1	bottom	MMB	100X	20	28		20	95	28	4	6	10	0.5	
	2	top	MMB	10000X	20	28		21	95	28	1	6	13	0.65	
	1	bottom	BB		20	28		22	95	28	1	4	15	0.75	
	2	top	BB		20	28		20	95	28	1	4	15	0.75	
	1	bottom	BB		20	28		20	95	28	4	1	15	0.75	
	2	top	MMB	1000X	20	27		20	95	29	4	5	11	0.55	
	1	bottom	BB		20	27		20	95	29	5	1	14	0.7	
	2	top	DEET	40%	20	28		20	95	29	5	11	4	0.2	
	1	bottom	BB		20	28		20	95	29	4	3	12	0.6	
2/6/2010	1	top	DEET	40%	20	28	50	21	95	28	1	5	4	0.2	
	2	bottom	BB		20	28		22	95	28	5	10	5	0.25	
	1	top	MMB	1000X	20						3	3	14	0.7	Windsnelheids-
	2	bottom	BB		20						3	5	12	0.6	meter en thermo-
	1	top	BB		20						5	4	11	0.55	hygrometer bij
	2	bottom	MMB	100X	20						4	2	14	0.7	Gabriella
	1	top	MMB	10000X	20						6	4	10	0.5	
	2	bottom	BB		20						6	2	12	0.6	
	1	top	BB		20						10	3	7	0.35	
	2	bottom	BB		20						9	2	9	0.45	
3/6/2010	2	top	BB		20	28	60	20	95	28	4	8	8	0.4	
	1	bottom	MMB	1000X	20	28		23	95	28	3	8	9	0.45	
	2	top	MMB	100X	20						1	5	14	0.7	Windsnelheids-
	1	bottom	BB		20						3	5	12	0.6	meter en thermo-
	2	top	BB		20						5	10	5	0.25	hygrometer bij
	1	bottom	BB		20						3	1	16	0.8	Gabriella
	2	top	DEET	40%	20						7	6	7	0.35	
	1	bottom	BB		20						8	1	11	0.55	
	2	top	BB		20						5	3	12	0.6	
	1	bottom	MMB	10000X	20						3	2	15	0.75	
8/6/2010	2	top	BB		20	28	50	21	95	28	7	10	3	0.15	LDPE-zakjes gevuld
	1	bottom	H20		20	28		21	95	28	5	13	2	0.1	met 100microl.
	2	top	H20		20	28		21	95	28	5	9	6	0.3	Gedistilleerd
	1	bottom	BB		20	28		21	95	28	6	5	9	0.45	water. 2X0.03mm
	2	top	BB		20	29		19	95	29	6	1	13	0.65	1x0.05mm
	1	bottom	H20		20	29		19	95	29	5	7	8	0.4	1x0.2mm voor H20.
	2	top	H20		20	28		20	95	28	8	7	5	0.25	1x0.2mm bij BB
	1	bottom	BB		20	28		20	95	28	4	3	13	0.65	voor Controle.
	2	top	BB		20	29		19	95	29	2	2	16	0.8	
	1	bottom	H20		20	29		21	95	29	4	4	12	0.6	
9/6/2010	2	top	PK		20	27	60	19	95	27	3	14	3	0.15	
	1	bottom	BB		20			21	95	27	2	15	3	0.15	
	2	top	BB		20						3	10	7	0.35	
	1	bottom	PK		20						8	8	4	0.2	
	2	top	PK		20						6	11	7	0.35	
	1	bottom	BB		20						1	3	16	0.8	
	2	top	BB		20						5	3	12	0.6	
	1	bottom	PK		20						9	11	0	0	
	2	top	PK		20						8	10	2	0.1	
	1	bottom	BB		20						5	1	14	0.7	
10/6/2010	2	top	CTAL		20	28	65	20	95	28	3	12	5	0.25	
	1	bottom	BB		20			20	95	28	2	13	5	0.25	
	2	top	BB		20						5	8	7	0.35	
	1	bottom	CTAL		20						6	5	9	0.45	
	2	top	CTAL		20						4	9	7	0.35	
	1	bottom	BB		20						6	6	8	0.4	
	2	top	BB		20						4	8	8	0.4	
	1	bottom	CTAL		20						3	11	6	0.3	
	2	top	CTAL		20						6	10	4	0.2	
	1	bottom	BB		20						8	2	10	0.5	