WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

The chemical ecology of mating in *Anopheles gambiae* s.s.

The influence of male-produced pheromones on insemination success

Master thesis Entomology – ENT-80436

MSc thesis no: 08.24
Name: Annet van Swaay
Reg. nr.: 840405 818 070
Period: 11/01/2007 – 05/15/2008
1st Examiner: Prof. M. Dicke
2nd Examiner: Dr. B.G.J. Knols

Content

Summary

The methods currently directed towards the control of adult mosquitoes are based on insecticides for indoor residual spraying and for insecticide treated bednets. Unfortunately, these methods are losing their efficacy due to increasing levels of insecticide resistance in mosquito populations. Other vector control methods need to be developed to sustain and safeguard future malaria control activities.

The release of genetically modified or radio-sterilised mosquitoes into nature for the control of existing mosquito populations is under development. For these strategies to succeed, a thorough knowledge of the population structure and possible barriers to gene flow is needed to determine on what scale the modified or sterile mosquitoes have to be released to optimize the spread of the transgene(s) or induce sterility in the target population. Moreover, for these methods to succeed, the released specimens require normal mating behaviour, which can only be evaluated if this behaviour is properly understood.

Mating of anopheline species usually takes place in swarms. Males aggregate during sunset and form swarms over markers which are approached by females. However, the mechanisms that determine mate-choice and prevent hybridisation are not well understood. For species in the *Anopheles gambiae* complex several mechanisms have been proposed but none have provided a satisfactory explanation. It has been suggested that sex pheromones are involved in specific mate recognition by mosquitoes. Recently, four different volatiles have been identified that are produced by swarming anopheline males: octanal, nonanal, decanal and 6-methyl-5-hepten-2-one. Electroantennogram (EAG) experiments have shown responses of female *An. arabiensis* to these compounds.

This present study focused on testing the four identified compounds produced by swarming males in a behavioural experiment with *An. gambiae* s.s.. Furthermore, the efficacy of fluorescent dust marking as a tool for indicating male mating attempts was evaluated.

The presence of fluorescent dust on females was found to be unsuitable as an indicator for male mating attempts. The application of fluorescent dust on the males' abdomen can decrease the males' ability to inseminate females, depending on the quantity of dust applied. Therefore, the observed insemination rate in these experiments is not an accurate representation of mating activity. Moreover, contamination of the powder occurs through the cage and by accidental encounters between individuals. For that reason, presence of fluorescent dust on females is not necessarily a result of a male mating attempt and consequently, the use of fluorescent dust for this purpose can lead to false positives.

Application of a mixture of the four male-produced compounds on the abdomen of females resulted into a decrease of the insemination rate. The decrease was dose-dependent. From the four concentrations of the mixture that were tested (10 ng/µl, 100 ng/µl, 1 µg/µl and 10 µg/µl) the difference between the odour group and the solvent group of the two highest concentrations were the highest (mean difference of the fraction of inseminated females: 0.188 ± 0.089 and 0.231 ± 0.109 respectively). These results were significant.

Methyl eugenol, a mating stimulant for *Ceratitis* fruit flies, was used to test whether this effect was simply a result of applying a chemical. This compound had a different effect on the insemination rate when it was applied in a 10 µg/µl concentration to females. Although not significant, methyl eugenol increased the insemination rate compared to the solvent (mean difference: 0.124 ± 0.059).

Furthermore, the effect of the odour mixture when applied to males was tested. No effect of this treatment on the insemination rate was observed. This was expected since the compounds are male-produced and therefore control group and test group were equal.

As the mixture of the four identified compounds that anopheline males produce during swarming decreases the insemination rate when applied to female *An. gambiae s.s.* mosquitoes, it is concluded

that these compounds influence the sexual behaviour of *An. gambiae s.s.*. Whether or not these compounds play a role in sexual behaviour under natural (field) conditions needs to be evaluated next.

1. Introduction

Malaria is one of the major insect-transmitted diseases interfering with development of the world's tropical and sub-tropical regions. Worldwide, some 250 million people became infected with malaria in 2006, with nearly one million deaths, mostly in the sub-Saharan region of Africa (WHO, 2008). Especially in young children and pregnant women malaria causes high fatality rates yet, the protection of children against malaria increases by the use of bednets and by receiving appropriate malaria treatment (WHO, 2008). Malaria causes low birth weight, prevents children from going to school, and has a major influence on people's productivity, contributing to poverty (Alnwick, 2000).

Malaria is caused by a genus of protozoan vertebrate blood parasites, *Plasmodium,* of which *P. falciparum* is the dominant and most lethal species in most regions of Africa (Guerra *et al*., 2006). The parasite has a highly complex life cycle in both vector and human host and is transferred between humans by the bite of a female *Anopheles* mosquito (Hyde, 2007).

The term malaria comes from the Italian "mal'aria", which means "bad air". The disease was historically associated with swamps and the putrid odours rising from them (Cox, 2002; Tuteja, 2007). The characteristic periodic fevers caused by malaria are recorded early in human history, as far as 3700 years ago (Cox, 2002). Not until the end of the 19th Century it was discovered that malaria is caused by *Plasmodium* parasites and that a mosquito acts as its vector (Cox, 2002). Due to the long history that man and mosquito share together, there is a close human-vector association and a very stable African population of *P. falciparum.* This results in a very high parasite inoculation rate in Africa, which is higher than in other malaria-endemic areas of the world (Touré, 2000).

1.1 Anopheles

There are many *Anopheles* species which feed almost exclusively on humans (anthropophily), but approximately 40 species are known to transmit malaria (Catteruccia, 2007). In the sub-Saharan region of Africa only three of these vector species are considered to be of major importance; *Anopheles gambiae sensu stricto*, *An. arabiensis* and *An. funestus* (Takken & Knols, 1999). *An. gambiae* s.s. is considered to be the world's most efficient vector for malaria and is a member of the *An. gambiae* complex which includes seven morphologically identical species. The efficiency of *An. gambiae* s.s. as a malaria vector is partly due to its high susceptibility to *Plasmodium* parasites. On top of that it has a high preference for resting and feeding indoors and is highly anthropophilic (Takken & Knols, 1999).

This species can be divided into two molecular forms, M and S, and five chromosomal forms, defined according to inversions on chromosome 2. In the lab interbreeding between the M and S form occurs and gives rise to fertile offspring, but in the field hybridisation is rare (Della Torre *et al*., 2002; Catteruccia, 2007). Apparently a strong pre-copulatory mating barrier exists in nature, and not in the laboratory, which prevents the different forms from hybridisation. Several possible mechanisms for this have been proposed, including auditory cues (Tripet *et al*., 2004) and the spatial and temporary differences of swarming (Lounibos *et al*., 1998). But until now no satisfactory explanation has been found for this phenomenon of reproductive isolation. This is further elaborated in paragraph 1.4.4.

1.2 Malaria control

The incidence of malaria is increasing and has been predicted to double over the next twenty years (Catteruccia, 2007) indicating that the currently used methods to control the disease are not sufficient (Jacobs-Lorena, 2006).

There are three major approaches used to contain insect-transmitted diseases; vaccination to prevent infection, medication to cure infected people and the control of vector populations to reduce disease transmission (Jacobs-Lorena, 2006). Due to the complex life cycle of the parasite there is no clinically proven vaccine available for malaria, except for the RTS,S vaccine which is currently been

tested to confirm its efficacy against malaria (Aponte *et al.*, 2007). Therefore the prevention and treatment of malaria infections depend mainly on medication. However, several *Plasmodium* strains have acquired resistance against antimalarial drugs contributing to the difficulties in malaria control. Progress has been made in understanding the molecular background of resistance against antimalarials giving rise to new classes of drugs used in combination against different targets in the metabolic pathway of the parasite (Hyde, 2007). For example artemisinin-based combination therapies (ACTs) are now generally accepted as the best treatments for uncomplicated falciparum malaria. ACT's are combinations of an artemisinin derivative and another structurally unrelated antimalarial (Nosten, 2007).

Despite of these new findings there is a continued need to manage drug resistance. This leaves the option of vector control for reduction of disease transmission.

1.3 Vector control

Malaria vector control is the most effective way to contain the disease (Catteruccia, 2007). Reducing the vector population and shortening of the life-span of the mosquito will give a reduction of the *Plasmodium* sporozoite rate, because the parasite does not get a chance to complete its sporogonic cycle (WHO, 2006). The reduction of mosquito populations can be accomplished in various ways; with insecticides, by altering the environment, by interfering with reproduction and by decreasing the competence of the vector to the parasite (Jacobs-Lorena, 2006).

1.3.1 Insecticides and larval control

The most important vector control methods at present are the use of persistent insecticides for indoor residual spraying (IRS) and for insecticide treated bednets (ITN) (Catteruccia, 2007). Since many anopheline vector species bite at night and rest on indoor surfaces, these are highly effective methods to reduce transmission rates (WHO, 2008). However, the use of insecticides is facing the problem of resistance by mosquitoes (WHO, 2008; Catteruccia, 2007).

Insecticides leave the environment where mosquitoes reproduce intact. Therefore, when insecticide treatment is stopped it is likely that the population will grow back to levels prior to the insecticide treatment (Jacobs-Lorena, 2006). To prevent this, mosquito breeding also needs to be controlled by applying larvicides to water surfaces, intermittent irrigation, sluicing and biological control (WHO, 2006). However, many of these aquatic breeding sites are created by agricultural irrigation or rainfall. This results in many, temporary puddles and pools and treatment of all these sites is simply not feasible. Nevertheless Killeen *et al*. (2002) gave an example of an exceptionally successful eradication of *An. gambiae* in Brazil in 1938, that was mainly targeted at larval control. Although very effective, the larvicide that was used (Paris green, an arsenic compound) could not be applied today because of its established toxicity (Killeen *et al*., 2002). Although suitable (biological) alternatives exist at present, contemporary vector control mainly focuses on adult mosquitoes (Collins *et al*., 2000).

1.3.2 Effectiveness of the control of adults

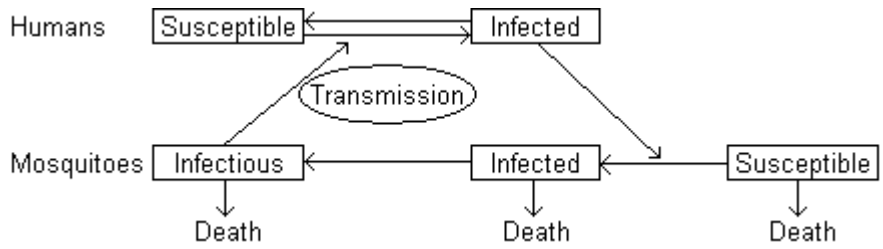


Figure 1 – The Ross-Macdonald model for malaria transmission (adapted from Koella, 1991)

The effectiveness of adult vector control can be found in a mathematical model. The Ross-Macdonald model (shown in figure 1) is the best-known and most widely used model for malaria transmission (Koella, 1991).

In 1911, Ross introduced the concept of a threshold density and concluded that “in order to counteract malaria anywhere we need not banish Anopheles there entirely—we need only to reduce their numbers below a certain figure”. This means that malaria can persist in a population only if the number of mosquitoes is larger than a given threshold (Ruan *et al.*, 2008). Macdonald extended Ross’ basic model and analyzed several factors contributing to malaria transmission resulting in the following mathematical equation:

$$R_0 = \frac{ma^2 b_1 b_2 e^{-\mu T}}{r\,\mu}$$

R_0 can be described as a measure of the intensity of malaria transmission. The equation includes the number of mosquitoes per human host (m), the biting rate of the mosquitoes on their human host (a), the susceptibility of humans (b_2), the infectiousness of humans to mosquitoes (b_1), the mortality of adult mosquitoes (μ), the incubation period of parasites within the mosquito vector (T), and the rate of recovery of infected humans (r) (Koella, 1991). The term $e^{-\mu T}$ indicates the proportion of mosquitoes surviving from the time of being infected through the incubation period of the parasites. The largest reduction of the intensity of malaria transmission is expected for increase in adult mosquito mortality (μ) because of the exponential correlation (Ruan *et al.*, 2008).

As mentioned in paragraph 1.3.1, the currently used methods directed towards the control of adult mosquitoes based on insecticides, IRS and ITN, are losing their efficacy by insecticide resistance (N’Guessan *et al.*, 2007). One very promising biological method for adult control is the use of entomopathogenic fungi. The fungus *Metarhizium anisopliae* was found to reduce longevity in adult *Anopheles* mosquitoes (Scholte *et al.*, 2005). Farenhorst *et al.* (2008) found a suitable delivery system for *M. anisopliae* in African water storage pots, which require only a small area to be impregnated. This method increases the mortality of adult mosquitoes (μ), which (according to the equation) can contribute to a great extent to the reduction of the intensity of malaria transmission. Furthermore, this method can decrease the number of mosquitoes per human host (m), the feeding propensity (a) and the daily survival rate (T). The decrease of each of these parameters can result into a decrease of malaria transmission, where decreasing parameter a has a larger effect than the other two because of the square of the biting rate. In order to transmit the parasite, mosquitoes must bite the human host twice: once to take up gametocytes and once to transfer sporozoites.

The use of medication for prevention and treatment of infections decrease parameter b_2 and increase parameter r, respectively. This can lead to the decrease of malaria transmission. However, due to the acquired resistance of several *Plasmodium* strains against antimalarial drugs, the efficacy of this method is limited.

1.3.3 Genetic vector control

The use of genetics in vector control, includes the use of transgenic technologies to reduce the transmission potential of insect vector populations and the Sterile Insect Technique (SIT). These are based on the release of large numbers of sexually active but genetically sterile or transformed insects, normally males, over large areas (Catteruccia, 2007). Sterilization can be induced by irradiation (Helinski *et al.*, 2008) or by chemical means. When wild females mate with these sterile males, the females will have fewer or no progeny. As a result the local population declines or disappears (Jacobs-Lorena, 2006; Catteruccia, 2007). This can lead to a smaller number of mosquitoes per human host (m), which contributes to the reduction of the intensity of malaria transmission.

One variant of SIT is the release of insects carrying a dominant lethal (RIDL) (Thomas *et al.*, 2000; Jacobs-Lorena, 2006). These males carry two copies of a dominant lethality gene that is only expressed in females and the gene is repressed by a compound that is not present in nature (i.e. tetracycline). The gene is only expressed in females because it is controlled by a promoter that is sex-specific (Thomas *et al.*, 2000). Therefore the female progeny of those males and wild females will die in normal breeding sites and 50% of their male progeny will carry this lethality gene (Curtis, 2006).

This will result in the elimination of local vector populations over time (Catteruccia, 2007). However, these transgenic males have to compete for females with normal males. Therefore, transgenic modification should have no influence on male fitness and survival and mating competitiveness (Jacobs-Lorena, 2006).

Another strategy is population replacement where the vector populations could be replaced by genetically modified mosquitoes refractory to *Plasmodium* (Kim, 2004; Jacobs-Lorena, 2006). However, to achieve this there are many challenges to overcome, including ethical and legal issues (Catteruccia, 2007; Knols *et al.*, 2007). For these molecular strategies to succeed, a thorough knowledge of the population structure and barriers of gene flow is needed to determine on what scale the modified mosquitoes have to be released to optimize the spread of the transgene(s) (Collins *et al*., 2000). Moreover, integration of an effector gene into a wild population requires normal mating behaviour, which can only be determined if this behaviour is properly understood (Takken *et al.*, 2006).

1.4 Mating behaviour

Mating of anopheline species usually takes place in swarms. Males aggregate during sunset and form swarms above markers, which can practically be any feature in the environment (Charlwood, 1980; Takken & Knols, 1999; Yuval, 2006). Females fly to these swarming males, however little is known of the factors that enables them to locate a swarm (Clements, 1999). When approaching the swarm she is recognised by her (lower) flight tone (Charlwood, 1979; Clements, 1999; Yuval, 2006). Several males may approach her and she will leave the swarm *in copula* with one of the males (Takken & Knols, 1999).

1.4.1 Swarming

Aggregation in swarms is an important first phase in the mating behaviour of most mosquito species, of which most reports from swarming involve species of *Anopheles, Culex and Aedes* (Clements, 1999). According to Clements (1999) all mosquito swarms have three common characteristics:

- The individuals in a swarm fly in species-characteristic patterns within a limited space corresponding to a certain feature in the environment.
- The swarms consist mainly of males and when females enter a swarm, males will approach and attempt to couple with them.
- Swarming is limited to times of day characteristic to the species, usually close to dusk and dawn.

The function of these swarms is to bring males and females of the same species together for mating (Clements, 1999). Other systems to aggregate males and females are described in paragraph 1.4.3.

1.4.2 The influence of physical factors on swarming

There are several factors that influence swarming, of which light intensity is very important. During field observations by Marchand (1984) swarming activity by *An. gambiae* was only observed during dusk and in a similar field study by Charlwood *et al*. (2002) swarming activity by *An. gambiae* was also observed during dusk and only on rare occasions small swarms were seen during dawn. This indicates that the time during and shortly after sunset is the most important for swarming and thus for mating. Yuval *et al*. (1993) also found, for a different *Anopheles* species, that swarms formed five to ten minutes after sunset and ended when it became completely dark.

Temperature and humidity may affect the swarming and mating behaviour of mosquitoes as for many insects, flight activity depends on body temperature (Clements, 1999). Under laboratory conditions these factors remains constant. However, daily fluctuations in physical factors will parallel some biological rhythms is nature, where they might be absent under artificial conditions.

1.4.3 Other mating systems

Next to the previous described station-keeping swarms, there are three more forms of assembly of males and females at a certain time or place, each with different methods of species recognition.

One of these assembly forms is pupal attendance. In some species, males emerge before the females and aggregate at emergence sites, gathering on or near the water surface waiting for sexually receptive females to emerge. The males respond to either the pupae, emerging females or newly emerged females, depending on the species (Clements, 1999). An example are the *Deinocerites* species (crab hole mosquitoes), which grasp a pupa of any sex. However, closer to emergence of the female, the time of attendance on female pupae increased exponentially (Clements, 1999; Yuval, 2006). When a female emerges, the tip of the male abdomen is inserted into the pupal case and coupling occurs even before the female has entirely emerged.

Another assembly form is one at which both sexes assemble near the vertebrate hosts on which the females feed. Males of these species respond to host odours. For example male *Aedes* species fly around (human) hosts and couple with females as soon as they approach for blood feeding. Copulation can take place in flight or while the female feeds (Clements, 1999). Species recognition occurs by contact sex pheromones (Nijhout, 1971).

The third assembly form is mating at resting sites. Males fly around areas where females of the same species rest. Mostly these are surfaces of trees. This substrate based mating system is exhibited by *Sabethes* species (Clements, 1999; Yuval, 2006). When a male locates a female on the vegetation, he displays a series of specialized behaviour patterns specific to the species (Hancock, 1990; Clements, 1999; Yuval, 2006). The male performs this courtship behaviour to overcome female unreceptiveness.

Each of these assembly forms has as function to bring conspecific males and females together for mating and reduce the risk of contact between males and females of different species. Consequently, hybridisation will be prevented. For *An. gambiae* the assembly form is the stationary swarm, which is formed mostly during dusk. The location and moment in time that swarms are formed are not specific for *An. gambiae.* Though rare, mixed swarms of *An. gambiae* with other species (like *An. arabiensis*) have been observed (Marchand, 1984), yet hybridisation is very rare in nature. Therefore, there must be other factors that prevent hybridisation, but it remains unknown what determines species recognition between and within members of the *An. gambiae* complex. In the next paragraph some factors that might determine mate-choice for *An. gambiae* are discussed.

1.4.4 Factors that can determine mate-choice

Many stimuli can be used to bring males and females of the same species together for mating. For example volatiles and sound, which can function over medium to large distances (Clements, 1999). These stimuli can be species-specific and therefore prevent hybridisation (Clements, 1999). For species in the *An. gambiae* complex several possibilities have been proposed like wing-beat frequency (Tripet *et al*., 2004) and female size (Okanda *et al*., 2002) but none have given a satisfactory explanation.

Females can accept or reject copulation by the males approaching her in the swarm (Charlwood, 1979), therefore it can be assumed that females can choose mates according to species or form (Tripet *et al*., 2004).

Ng'habi *et al*. (2005) found that male size can influence mate-choice in *An. gambiae*; males who successfully obtained a female were larger than those that did not. However, in a study by Charlwood *et al.* (2002) no correlation between male size and copulatory success was found for *An. gambiae.*

In most culicid species the specificity of the wing-beat frequencies is enough to distinguish between conspecific males and females. However, there is broad overlap between amplitudes of the wing-beat of members within the *An. gambiae* group (Tripet *et al.*, 2004; Yuval, 2006). Since hybridization has rarely been observed in nature between the different forms of the *An. gambiae* complex it is possible that mosquitoes use more information from flight tones than the wing-beat frequency to differentiate between the different forms (Tripet *et al.*, 2004). A second possibility is that

there is an additional mechanism that is specific for each of these mosquito forms and which functions as a final recognition cue before copulation (Tripet *et al.*, 2004; Yuval, 2006).

It has been suggested that sex pheromones are involved in specific mate recognition by mosquitoes (Takken & Knols, 1999), however this has not been confirmed yet for *An. gambiae*.

1.4.5 Male sexual responsiveness and female receptivity

For the majority of species, the male mosquito is not capable of inseminating a female directly after emergence. Several hours to days are required for sexual maturation, which includes a capability of erecting the antennal fibrillae and inversion of the terminalia, where ventral structures rotate to the dorsal side (Clements, 1999). Swarming males show erect antennal fibrillae, which allow them to recognize the frequency of the female's wing beat (Yuval, 2006).

For *An. gambiae* Verhoek and Takken (1994) found an optimal insemination rate when the males were 7 days old. When males of different age groups were placed with 7 day old females, for 24 hours in a 1:1 ratio, the insemination rate peaked (35%) when the males were seven days old. Moreover, males were not likely to inseminate females within the first four days of life. No clear influence for female age was found, although the insemination rate rose slightly (from 20% to 32%) after the first five days of life. They found a mean insemination rate of 25% when the male:female ratio was 2:1 and the insemination rate increased to 46% in a 3:1 ratio. Charlwood (1976) also found an increase in insemination rate when the male:female ratio increased from 1:1 to 2:1 to 4:1 (52% to 84% to 90% respectively). These insemination rates are much higher than the insemination rates found by Verhoek and Takken. Moreover, Charlwood did find that some females were inseminated within the first 24 hours after emergence. After 48 hours even as much as 50% of all females were inseminated at a male:female ratio of 2:1. The large differences between the two studies point out that results from different studies can not easily be compared and that the insemination rate might be largely dependent on the laboratory setting and the mosquito strain that is used.

An. gambiae males can inseminate multiple females during a short period. In the laboratory transfer of spermatozoa of a single male to five or more females during one night has been observed (Clements, 1999). However, in nature males can outnumber females in a swarm up to 600 times (Charlwood, 1980) and therefore it is very unlikely that a single male will mate more than once. Therefore the males developed a mechanism to ensure that the female will not mate again. In anophelines, a gelatinous mating plug is produced by the males' accessory glands which is transferred to the female after sperm has been transferred and it provides a short-term barrier (for 24 to 48 hours) against additional copulations. Long-term inhibition of female receptivity can be accomplished by a substantial quantity of ejaculate in the spermatheca (Yuval, 2006).

1.5 Olfaction

Olfaction plays an important role in mosquito behaviour such as reproductive and feeding behaviour (Catteruccia, 2007). Male anopheline mosquitoes mostly respond to plant odours and females respond to plant and host odours for sugar and bloodfeeding, respectively. It has been suggested that semiochemicals may serve as aggregation and sex pheromones (Takken & Knols, 1999) and that at close range contact pheromones could play a role in recognition between *An. gambiae* forms and female mate-choice (Tripet *et al.*, 2004).

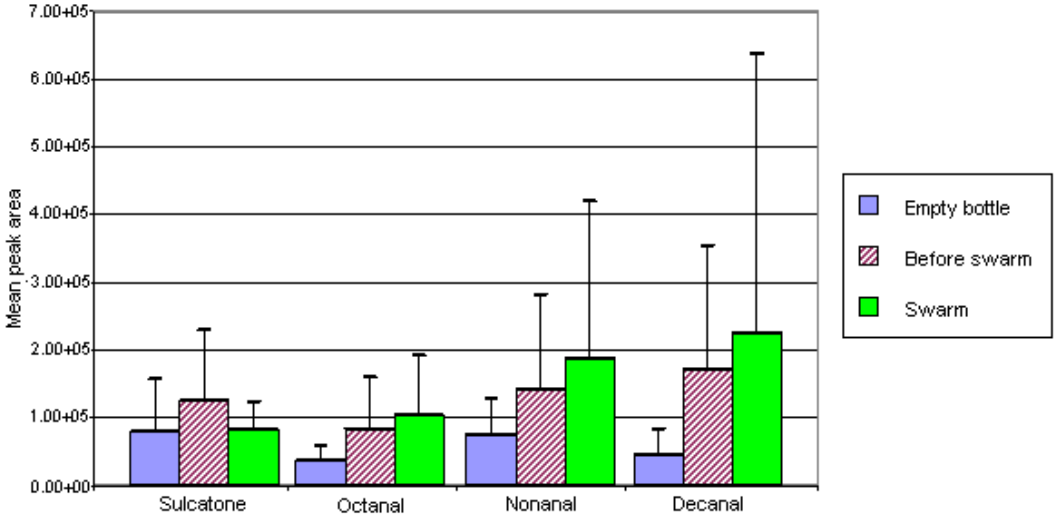


Figure 2 - Volatiles emitted from male *Anopheles arabiensis,* (KGB strain, n=50. 7 replicates) during dimming of light to simulate dusk and induce swarming. From Pålsson *et al.*, unpublished.

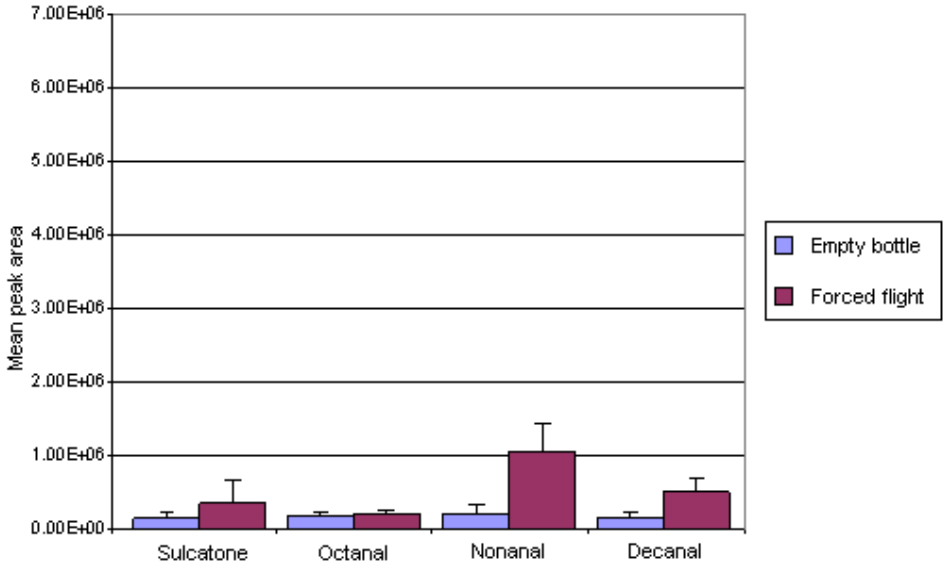


Figure 3 - Volatiles emitted from male *Anopheles arabiensis* (KGB strain, n=50. 3 replicates) during forced flight in daytime. From Pålsson *et al.*, unpublished.

Swarming male *An. arabiensis* mosquitoes produce different volatiles to which females respond, of which four have been identified with using the GC-MS technique: the aldehydes octanal (C8), nonanal (C9), decanal (C10) and the terpenoid 6-methyl-5-hepten-2-one (sulcatone). Figure 2 shows these compounds that were trapped from swarming *An. arabiensis* males. Increasing amounts of especially decanal were found by Pålsson *et al.* (unpublished). Males produce the volatiles during forced flight as well, however in significantly lower amounts (figure 3). The release of these odours by *An. gambiae* was tested as well. The four odours were found to be produced in low amounts and the ratio into which these compounds were produced was approximately C8:C9:C10:S = 1:1:1:1 (Pålsson *et al.*, unpublished data).

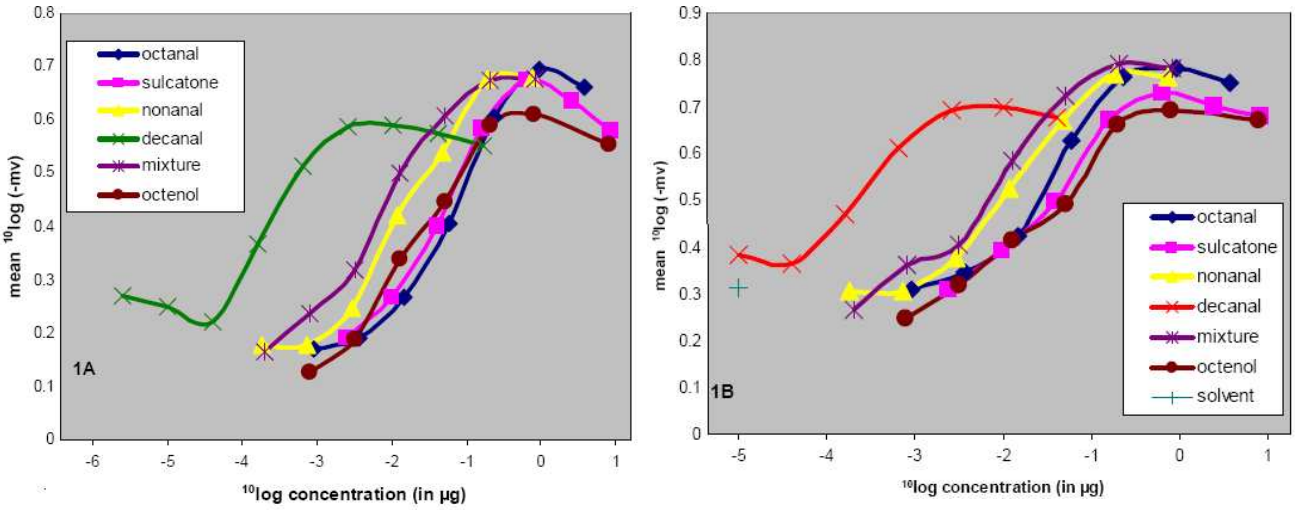


Figure 4 - EAG responses of virgin females (1A, n=36) and mated females (1B, n=18) of *An. arabiensis* to four putative male sex pheromone components, their mixture and a control compound, 1-octen-3-ol. From Qiu, unpublished.

Figure 4 shows the electroantennogram responses of virgin and mated female *An. arabiensis*. Especially to decanal a strong response was found and the next highest response was recorded to the mixture of these four compounds. The EAG response of males to these compounds was 4-10 times lower than the EAG response of females (Qiu, unpublished data). In this experiment the ratio of the four compounds in the mixture was adapted to the ratio of these compounds that was trapped by Pålsson *et al.* for *An. arabiensis.*

Behavioural responses of female *An. gambiae* to different odour stimuli have been tested in an olfactometer but no clear response was found to different concentrations of the odour combinations (Smallegange, unpublished data). However, in this experiment the compounds were present in the ratio similar to the ratio of these compounds that was trapped for *An. arabiensis.* When this mixture is tested for *An. gambiae* it cannot be concluded whether it is the wrong ratio of compounds or whether this species does not respond to the compounds at all. Although *An. arabiensis* was tested as well with the same mixture of the compounds, again no clear response was found. It was noted that the responses of virgin females was very different to those of inseminated females normally used for studying responses to human odours.

1.6 Methods for determining the mating status

The mating status of an *An. gambiae* female can be determined by several methods. One of those methods is direct observation of (tethered) females. Males approach these females, hold on to the females, will adopt an end-to-end position for mating and will fly off after several seconds (Charlwood, 1976). This is not a very efficient method since only one female can be observed and it does not necessarily mean that the female is inseminated. Copulation can take place without sperm being present in the spermatheca. To be able to determine the mating status of multiple females it could be an option to mark all males in the cage with a fluorescent powder. When a male holds
on to a female for mating, the powder will be transferred to the female and a mating attempt could be verified by the presence of the powder on the abdomen of the female. However, the accuracy of this method has not been tested and again, a mating attempt does not guarantee insemination.

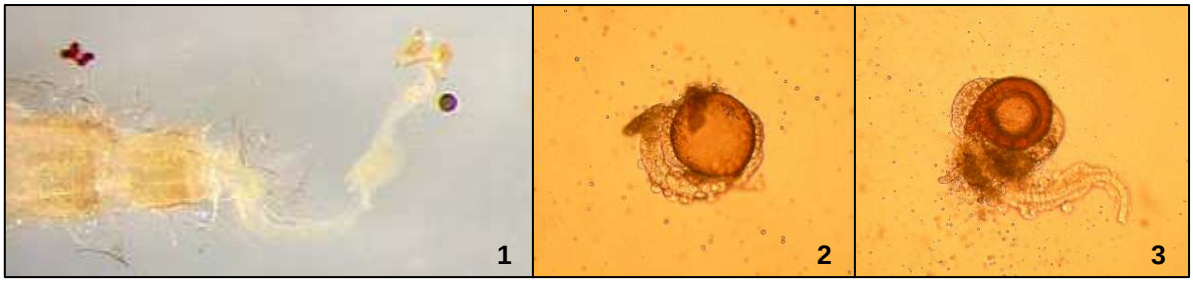


Figure 5 – Female *An. gambiae* mosquito with removed terminalia. The spermatheca is visible as a dark ball (1). Empty spermatheca under 100x magnification (2). Spermatheca containing spermatozoa (3). *Picture 5.1 obtained from MQ Benedict (2007) Methods in Anopheles research. Picture 5.2 and 5.3 by Annet van Swaay.*

To be certain that a female is inseminated she can be dissected to determine whether spermatozoa are present in the spermatheca. The spermatheca is located near the terminalia inside segment VIII and can often be seen through the cuticle. It is a spherical, transparent dark-brown organ as shown in figure 5.1. The spermatheca will be exposed after gently removing the terminalia and segment IX. The malpighian tubules, ovaries, and digestive tract are also often exposed. Then the spermatheca can be easily removed and observed under 100-400x magnification. Insemination status is usually clearly visible. The spermatheca of uninseminated females is fairly transparent (figure 5.2). In the spermatheca of inseminated females sometimes movement can be observed and the spermatozoa are visible as long thread-like structures within the spermatheca. Often, the spermatozoa are peripherically orientated (figure 5.3). Although this method is reliable, it is time consuming, labour intensive and requires fresh specimens. Therefore, a different method can be used.

Recently, a new method for determining the mating status of *An. gambiae* females has been developed by Ng'habi *et al.* (2007). In this molecular technique male DNA from inseminated females of *An. gambiae s.s.* can be amplified using Y chromosome–specific PCR markers. DNA from dried specimens amplified in a similar way as those from fresh specimens and no amplification was obtained from virgin females (Ng'habi *et al.* 2007). This method makes it possible to screen a larger number females and even dried mosquitoes can be tested for insemination. However, in the present study, the dissection method was chosen to determine the females' insemination status.

2. Aims of the study

The main aim of this study was to determine the influence of four male produced volatiles, C8, C9, C10 and sulcatone, on the insemination success of *An. gambiae*. Although it had been shown that males produce these volatiles, and that females show clear electrophysiological responses to these in a typical dose-response manner, no clear evidence at the behavioural level exists to date for the role of these chemicals. This study attempted to demonstrate a behavioural effect through the application of different concentrations of these male-produced volatiles on the abdomen of females to see whether this would distort normal sexual behaviour as measured through a decreased proportion of inseminated females.

Since many females had to be dissected to determine the mating status, a method for reducing the number of females to be dissected was examined. This method involved the application of a fluorescent dust marker on the males to determine male-female contact.

To test these main questions the following sub-questions were addressed:

1. What are the insemination rates for different ratio's of males:females:

- 1:4?

- 4:1?

This was tested by placing males and females together in these two ratio's and afterwards determine the insemination rate by dissection of the females.

The hypothesis here was that the insemination rate would increase when a higher proportion of males was present.

2. Does the application of fluorescent dust marking on male An. gambiae affect their ability to inseminate females?

The fraction of inseminated females that have been in the same cage with dust-marked males and the fraction of inseminated females that have been in the same cage with males that were not marked were determined. To test whether the dust influences the ability of males to inseminate females, these two proportions were compared.

The hypothesis was that application of the dust would not affect a male's ability to inseminate females.

3. Is the presence of fluorescent dust on female An. gambiae an indicator for male mating attempts?

This was tested by examining the females that have been in the same cage with dust-marked males for presence of the dust that might have been transferred from male to female during a mating attempt. Subsequently, all females were dissected to confirm insemination.

It was hypothesized that the presence of dust on females could be used as a proxy for insemination having taken place.

4. What is the effect of a mixture of male produced volatiles on the insemination rate?

- Does the insemination rate decrease when it is applied in different concentrations to females?

- Does the insemination rate change when it is applied to males?

- Does the insemination rate change when it is present in the cage?

The effect was tested by applying either different concentrations of the volatiles or the solvent (water with 15 mg/ml Tween (20) and 15 µl/ml methanol) to the females and the two groups were marked with different colours of fluorescent dust. Subsequently the two groups of females were placed in one cage with males in a 4♂:1♀ ratio and were dissected.

For the effect of application to the males, no fluorescent dust was used since the control group and the test group were separated over two cages in a 1♂:4♀ ratio.

The effect of the presence of the volatiles in the environment was tested by placing a source of these volatiles in the cage.

The hypotheses were that male volatiles, in a dose-dependent fashion, would reduce the insemination rate in females. Application of male-produced volatiles to males was assumed not to affect the proportion of females becoming inseminated.

5. What is the effect on the insemination rate when a mating stimulant of a different species is applied to the females?

This effect was tested by applying methyl eugenol (1,2-dimethoxy-4-(2-propenyl)benzene), a mating stimulant of *Ceratitis capitata,* to the females and placing the test group together with the control group in one cage with males in a 4♂:1♀ ratio. The distinction between the two groups was made by application of two different colours of fluorescent dust to the females.

The hypothesis was that even though this is a sexual stimulant in fruit flies that it would not affect mating in anophelines.

3. Materials and methods

3.1 Mosquitoes

Anopheles gambiae (Suakoko strain) originated from Liberia (courtesy Prof. M. Coluzzi) and has been reared on human blood since 1988. The mosquitoes are reared under controlled conditions. Adults are placed in standard 30x30x30 cm gauze-covered cages at 27 ± 1 ℃ and 80 ± 5% relative humidity, under a 12L:12D light regime with artificial dusk and dawn. The larvae are reared in plastic trays filled with tap water and are fed with Tetramin® baby fish food. Pupae are removed from the trays daily and transferred to the adult cages where these emerge.

For all experiments 5-7 day old mosquitoes (both sexes) were used that had not received a blood meal and were fed *ad libitum* on a 6% glucose solution.

3.1.1 Virgin mosquitoes

All mosquitoes used in the experiments were virgins. To make sure that the mosquitoes have never mated prior to the start of the experiment, the males and females were separated within 24 hrs after emergence.

Two different methods of separating sexes have been used and both were tested on efficiency and reliability. For the first method the pupae were placed individually in 2.0 ml Eppendorf tubes that contained 1 cm of water. After emergence, the adults were placed in cages separated by sex.

For the second method the males and females were separated within 24 hours after emergence. All mosquitoes were allowed to emerge in the same cage, were individually taken from that cage (with the use of an aspirator) and were placed into new cages separated by sex. Since previous studies show different results for the insemination rate within these 24 hours (paragraph 1.4.5), a number of 100 one-day old females was dissected to determine the insemination rate for these specific mosquitoes in this specific setting. These females were taken from three different cages on three different days.

3.2 Fluorescent marker tests

To test if fluorescent powder applied to the male terminalia does not affect the mating behaviour and whether the powder is suitable for indicating male mating attempts two different ratios of males and females were tested. The experiments were performed in standard rearing cages with small numbers of mosquitoes. It was positioned in a climate controlled room under a 12L:12D light regime. The lights were controlled with a dusk-dawn simulator. The light intensity increased or decreased gradually (simulated dawn or dusk, respectively) over a period of 30 minutes.

Table 1 – Setup for fluorescent marker test

Exp no.	♂	♀	Marker	Freezer	Replicates
1.1	40	10	-	-	6
1.2	10	40	-	-	5
1.3	40	10	++	+	5
1.4	10	40	++	+	5
1.5	40	10	-	+	3
1.6	10	40	-	+	3
1.7	40	10	+	+	3
1.8	10	40	+	+	3

This experiment was performed as outlined in table 1, where experiment 1.1, 1.2, 1.5 and 1.6 represent control treatments in which no marker was applied to the males. For treatment 1.5 and 1.6 the males were placed in the freezer before the start of the experiment to observe the effect of this

type of anaesthesia. For treatment 1.3 and 1.4 the fluorescent marker was applied on the lower half of the abdomen and terminalia and for 1.7 and 1.8 it was applied to the last two segments of the male's abdomen only.

Males and females were kept in the same cage for 24 hrs and during lights-on the females from treatment 1.3, 1.4, 1.7 and 1.8 were taken from the cage individually and observed under fluorescent light to check for traces of fluorescent powder on their body. After this the females from all groups were dissected to determine insemination rates. From every single female (and for some experiments from every male as well) one wing was taken and measured as a proxy for adult body weight (Nasci, 1990). For all experiments mosquito wings were removed under a dissecting microscope and measured under 32x magnification. The distance between the alular notch and the tip of the wing was measured and converted to millimetre.

3.2.1 Application of the fluorescent marker

The fluorescent marker was applied to the abdomen with a fine artists' brush (Royal Talens, no 1) to make sure the marker is present on the place where they make contact with the females during mating; the last few segments of the abdomen. To be able to apply the marker, the males were transferred from the cage to a petri dish and placed in a freezer for ± 90 seconds to decrease their activity. The petri dish was subsequently placed under a microscope to be able to apply the marker accurately. After application of the marker the males were allowed to recover and remove excess powder before placing them together with the females.

3.2.2 Fluorescent marker test in a larger setting

Since the standard experimental cages (30 x 30 x 30 cm) are rather small, the mosquitoes do not have any problem finding each other. In fact, there are many accidental encounters between individuals, which could disrupt a couple *in copula* and could give contamination with the marker. Therefore, the same experiment was performed in a larger cage with the sizes 75 x 75 x 115 cm. This experiment was performed according to table 2, where 2.3 represents the control.

Table 2 – Setup for fluorescent marker test in a larger setting

Exp no.	♂	♀	Marker	Freezer	Replicates
2.1	80	20	+	+	3
2.2	20	80	+	+	3
2.3	20	80	-	-	1

The experiments were performed under the same controlled conditions as in the previous experiments. However, the cages are too large to fit in the same climate controlled room. Therefore an old office room was set up as an experimental room. The temperature was controlled with an electrical heater and a humidifier controlled the humidity. The room was lit by three 100 Watt light bulbs and dusk and dawn periods were simulated .

3.2.3 Additional fluorescent marker tests

To determine if fluorescent powder was transferred to the females solely by the males or also via alternative routes, a series of additional tests was performed.

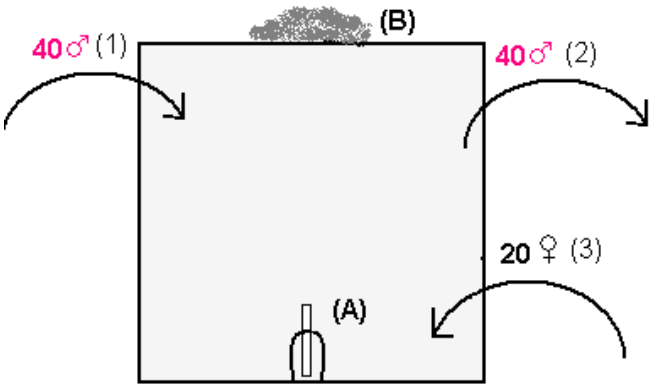


Figure 6 – Setup of the additional fluorescent marker tests. A= Bottle containing 6% glucose; B= Cotton wool saturated with 6% glucose.

To test if the powder is transferred through contamination of the cage, the following tests were performed. The setup is shown in figure 6. For the first test 40 male mosquitoes, containing fluorescent powder, were transferred to a standard cage (1). The males were allowed to fly around for a period of 24 hours and were then removed from the cage (2). Subsequently, 20 females (no treatment) were transferred to the same cage and left there for 24 hours (3). Finally these females were examined for the presence of fluorescent powder. This test was performed twice. In one experiment 6% glucose was present in a bottle containing a filter paper (A), in the other the glucose was offered in cotton wool on top of the cage (B).

The transfer of the powder by means of accidental encounters was tested. A total of 50 females was placed in a standard cage. To 40 of these females the fluorescent powder was applied and the other 10 females were left untreated. These females were left together for 24 hours. Glucose solution was available in cotton wool on top of the cage.

3.3 Odour tests

In this experiment the effect of different concentrations of a mixture of four putative sex pheromones were tested. In addition, the effect of methyl eugenol, a mating stimulant of *Ceratitis capitata*, the Mediterranean fruit fly, was tested to make sure that the observed results are not a consequence of a repellent effect (or the presence of just any chemical) on the males caused by the altered scent of the females.

The four compounds (C8, C9, C10 and S) were tested in a 1:1:1:1 ratio. The amount of compound trapped by Palsson (unpublished) ranged from 0.01 ng to 0.2 ng per mosquito. Therefore the initial concentrations of these compounds to be tested was: 0.01, 0.1, 1.0 and 10.0 ng/µl. Starting with the highest concentration. However, later was decided to increase the concentrations to 10 µg/µl instead of decreasing to 0.01 ng/µl.

Methyl eugenol was tested in the highest concentration that was tested for the mixture of the four compounds: 10 µg/µl. The control groups were treated with the solvent: 15 mg/ml Tween (20) and 15 µl/ml methanol in water.

3.3.1 Experimental setup

The experiments were performed according to table 3. In the first four experiments the control group (solvent treatment) and test group (odour treatment) were placed in the same cage. In other words, half of the females in the cage (n=15) was treated with the solvent and the other half (n=15) with the odour mixture. Both groups were marked with either pink or green fluorescent powder, as can be seen in figure 7, to be able to distinguish between the two groups at the end of the experiment.

Table 3 – Setup for odour tests

Exp.	♂	♀	Concentration	Odour applied to	Replicates
C	120	30	10 ng/µl	♀	8
D	120	30	100 ng/µl	♀	8
A	120	30	1 µg/µl	♀	10
B	120	30	10 µg/µl	♀	8
M	15	60	10 µg/µl	♂	2 x 4
E	120	30	10 µg/µl	♀	8

To be able to apply the mixture and the marker, the mosquitoes were transferred from the cage to a petri dish and placed in a freezer for ± 90 seconds to decrease their activity. Per mosquito 0.5 µl of the mixture was applied with a syringe on the lower half of the abdomen and at the same time the fluorescent powder was applied by dusting (as described above).

Figure 7 – The two colours of fluorescent marker. *By Annet van Swaay*

After application of the mixture and the marker, the females were placed into a standard rearing cage where 120 males were already present for approximately one hour. They were allowed to mate for 24 hours. After that, the females were removed from the cage, separated by colour and dissected to determine their insemination status. Then, from every single female one wing was taken and measured to determine body size. For some tests this was done for the males as well.

In experiment M, the males were treated with the mixture. It was not possible to place the control group and test group into the same cage (as the presence of sperm could not determine from which male it originated) and therefore each group was placed into a separate cage. In table 3 it can be seen that there were 2 x 4 repeats of this experiment. This means that this experiment was repeated four times and since the control and test group were split into two different cages, there were two times four repeats. For this experiment, it was not necessary to apply the fluorescent powder to distinguish between the two groups because they were separated. The experiments for both groups ran simultaneously for 24 hours.

An additional experiment was performed to test the influence of the odour mixture when it was present in the cage but not applied directly onto females. A 0.5 ml Eppendorf tube was filled with 10 µg/µl of the odour mixture and placed in a small glass bottle to make sure it would stay upright in the cage. This construction was covered with gauze to allow the odour to enter the cage and to prevent the mosquitoes from entering the bottle. The same setup filled with the solvent was placed in a different cage. Each cage contained 20 females and 80 males and none of the mosquitoes were treated. The two cages were placed as far apart in the room as possible to prevent the volatiles from entering the other cage. The experiment ran for 24 hours and after that the females were dissected to determine the insemination rate. Again, from all females and from some males one wing was taken and measured.

3.3.2 Preparation of the mixtures

Both the control mixture and the different concentrations of the odour mixture had to be prepared. The concentration of the stock of each compound was 10.0 mg/ml. The solvent was water to which methanol and Tween (20) were added (amounts are stated below).

C8 is available in a 99% solution. The density of this compound is 0.821 mg/µl. To get 10.0 mg/ml, the volume in which 10.0 mg of C8 is present has to be calculated.
1µl (99%) contains 0.824 mg of C8. So 10 mg is present in (10/0.821)/ 0.99= 12.30µl.

C9 is available in a 95% solution. The density of this compound is 0.827 mg/µl
1µl (95%) contains 0.827 mg of C9. So 10 mg is present in (10/0.827)/ 0.95= 12.73µl.
C10 is available in a 95% solution. The density of this compound is 0.830 mg/µl.
1µl (95%) contains 0.830 mg of C10. So 10 mg is present in (10/0.830)/ 0.95= 12.68µl.

S is available in a 98% solution. The density of this compound is 0.852 mg/µl.
1µl (98%) contains 0.852 mg of S. So 10 mg is present in (10/0.852)/ 0.98= 11.98µl.

Methyl eugenol is available in a 99% solution. The density of this compound is 1.036 mg/µl.
1µl (99%) contains 1.036 mg of S. So 10 mg is present in (10/1.036)/ 0.99= 9.750µl.

The above calculated volumes of the compounds were added to 15 mg Tween (20) and 15 µl methanol. Tween (20) helps to properly attach the solution to the cuticula of the mosquitoes (Qiu, personal communication) and methanol improves the solubility of the compounds in water. Then milli Q was added to a volume of 1ml. Four stock solutions were made, one for each compound. The control solution was made in the same manner, but it does not contain the compounds.

To make the mixture, equal amounts of the stock of each compound were mixed and 0.1% Tween (20) was added to make the desired concentration.

3.4 Data analysis

From all experiments mosquito wings were taken and measured. Using SPSS 15.0 software, the mean wing size of inseminated females and uninseminated females was determined, for each treatment and replicate separately.

For all experiments the fraction of inseminated females was determined. Using SPSS 15.0 software, the fraction of inseminated females of two different populations, control and marker, were compared. This comparison was done by performing an independent samples t-test. The spread of the results among the different replicates of each treatment was visualised in a boxplot made in SPSS 15.0 as well.

4. Results

4.1 Virgin mosquitoes

To be certain that all mosquitoes to be used in the experiments were virgins, the males and females had to be separated. Initially, this was done by placing pupae individually in one cm of water in Eppendorf tubes. After emergence, the adults were placed in cages separated by sex. With this method, there was never any contact between males and females and therefore 100% of all mosquitoes were virgins. However, this method is very labour intensive and time consuming. Moreover, approximately 15% of the pupae never emerged and a considerable number of adults drowned in the water that was present in the tubes. Therefore, a different method was used.

As mentioned in paragraph 3.1.1, the males and females were separated within 24 hours after emergence. Since previous studies show different results for the insemination rate within these 24 hours (paragraph 1.4.5), a number of 100 one-day old females was dissected to determine the insemination rate for these specific mosquitoes in this specific setting.

The 100 mosquitoes were collected on three different days from three different cages. For none of these females insemination has been observed. This indicates that the insemination rate within the first 24 hours is 0% for these specific mosquitoes in this specific setting.

4.2 Mosquito size

The mean wing size and standard deviation of inseminated females and uninseminated females was determined, for each treatment and replicate separately. The SPSS output of these analyses can be found in appendix II. The mean wing length for all females together was 2.77±0,16 mm. With a minimum of 2.34 mm and a maximum of 3.34 mm. With the difference of mean wing lengths between the treatments being almost 0.3 mm (minimum is 2.64 and maximum is 2.92), this could influence the outcome of the experiments.

4.3 Fluorescent marker test

The mean insemination rate in the used setting with a 4♂:1♀ ratio and with a total of 50 mosquitoes was 82±7.0% (treatment 1.1). The mean insemination rate in the used setting with a 1♂:4♀ ratio and with a total of 50 mosquitoes was 30±4.2% (treatment 1.2) (figure 8). When a fluorescent marker was applied to the males, the insemination rate decreased, for both ratios, to 56±10.6% and 10±4.8%

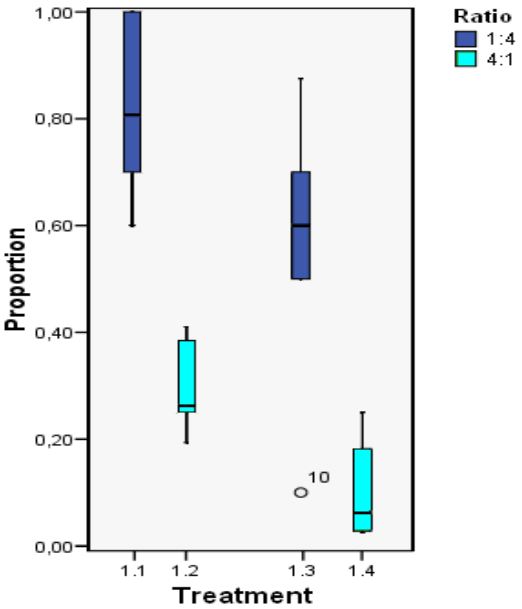


Figure 8 – Boxplots of proportion of inseminated females in a male: female ratio of 4:1 and 1:4 in absence (1.1 and 1.2) and presence (1.3 and 1.4) of a fluorescent marker.

respectively (treatment 1.3 and 1.4) (figure 8). The decrease in insemination rate when fluorescent powder was applied to the males was significant (P= 0.003) for the 1♂:4♀ ratio as can be seen in table 4. For the 4♂:1♀ ratio, the decrease is significant (P= 0.036) as well. The proportion of females that had a mark can be found in table 5.

Table 4 – Comparisons of two treatments. Mean fractions of inseminated females per treatment, mean difference between treatments ± SE and the α (1-tailed) for each of the marker tests

Compare treatment	Ratio ♂:♀	Mean per treatment	Mean difference ± SE	α (1-tailed)
1.1	4:1	0.819	0.257 ± 0.127	0.036
1.3		0.563		
1.2	1:4	0.300	0.199 ± 0.056	0.003
1.4		0.102		
1.3	4:1	0.563	0.338 ± 0.155	0.033
1.5		0.900		
1.4	1:4	0.102	0.164 ± 0.061	0.016
1.6		0.266		
1.1	4:1	0.819	0.415 ± 0.169	0.022
1.7		0.404		
1.2	1:4	0.300	0.073 ± 0.059	0.129
1.8		0.227		

There were two additional control groups where the males were placed in the freezer before the experiment (treatment 1.5 and 1.6) to test the influence of this treatment without the fluorescent powder. The results of these treatments were compared to the results from treatments 1.1 and 1.2. There was no difference observed between these two treatments for either ratio of males and females (see appendix I for the comparison). Therefore, only treatment 1.1 and 1.2 were compared to treatment 1.7 and 1.8 respectively. As can be seen in table 4 the application of fluorescent powder to the final two segments of the abdomen (instead of to the lower half) increased the mean insemination rate within the 1♂:4♀ group (treatment 1.8). The significant (P= 0.036) decrease in insemination rate for this ratio, observed when half of the abdomen is marked, is not observed when only the last two segments are marked (P= 0.129). For the 4♂:1♀ group (treatment 1.7) applying less marker had no influence on the mean insemination rate compared to marking half of the abdomen. Therefore there still is a significant decrease (P= 0.022) in the insemination rate in the marked group (treatment 1.7) compared to the group without any treatment (treatment 1.1). An overview of the different treatments together with the mean fractions of inseminated females can be found in appendix I.

Table 5 shows the proportions of females on which fluorescent marker was found. In treatment 1.3 all females had (traces of) the fluorescent marker on their abdomen, however only half of these were inseminated. In treatment 1.4 approximately 80% of the females had fluorescent marker on the abdomen, although only 10% was inseminated. When less fluorescent marker was applied to the males, fewer females had the marker on the abdomen (treatment 1.7 and 1.8). Moreover, a larger part of the females in the 1:4 ratio that did contain the powder were inseminated. For the 4:1 ratio there was no considerable difference. However, the chance that a male does not transfer the powder to a female during mating is larger when less powder is applied. For treatment 1.8 can be seen in the table that a few females were inseminated, but did not have the powder on the abdomen.

The results from these experiments show that marking leads to reduced insemination. This could be caused by obstruction of the terminalia or decreased mobility.

Table 5 – Proportion of females per test: without marker/ not inseminated, with marker/ not inseminated, with marker/ inseminated, without marker/ inseminated. In experiment 1.3 and 1.4 the quantity of applied marker was higher than in exp 1.7 and 1.8.

Exp no.	♂:♀	Quantity of marker	No Marker / No Ins.	Marker / No Ins.	Marker / Ins.	No Marker / Ins
1.3	4:1	++	0	0.45	0.55	
1.4	1:4	++	0.21	0.69	0.10	
1.7	4:1	+	0.07	0.52	0.41	
1.8	1:4	+	0.40	0.37	0.18	0.04

4.3.1 Fluorescent marker test in a larger setting

When the mosquitoes were placed in a larger cage the mean insemination rate decreased, although the total number of mosquitoes was doubled.

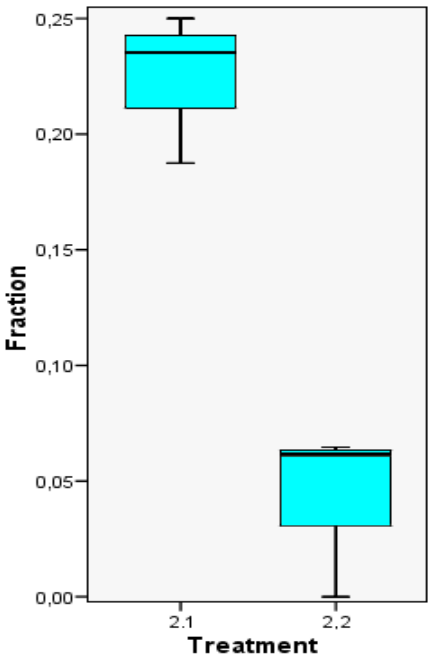


Figure 9 – Proportion of inseminated females in a 75x75x115 cm cage with a male: female ratio of 4:1 (2.1) and 1:4 (2.2).

When the males were treated with the fluorescent powder, the mean insemination rate for the 4♂:1♀ ratio was 22% and the mean insemination rate for the 1♂:4♀ ratio was 4% (figure 9). For the first ratio almost all females had the fluorescent powder somewhere on their abdomen, although the insemination rate was only 22%. For the second ratio more than half of the females had the powder on their abdomen, however only just a few females were inseminated. In a larger setting, the insemination rate decreases even further when the powder is applied. This could be caused by a negative influence of the powder on the mobility of the males.

Due to the low insemination rates for these experiments it was chosen not to proceed with the tests in this larger setting. These results would not provide a good basis for the subsequent odour tests. There were too many additional variables to consider due to the change in the setting as compared to the previous fluorescent marker tests. The mortality in this room was higher than in the room used for the previous marker tests. Out of one hundred mosquitoes the number of dead individuals varied from five to twenty per test. Moreover, many mosquitoes were lost in the larger cage or escaped from it.

In this large setting there was one test performed in a 1♂:4♀ ratio where the males were not treated with the powder. In this specific experiment 32% of the females was inseminated. This was

approximately the same as observed in the standard cages. It seems that the larger setting does not negatively influence insemination when the males did not receive any treatment.

4.3.2 Additional fluorescent marker tests

To determine whether the fluorescent powder was transferred to the females solely by the males a series of additional tests was performed.

The transfer of the powder via the cage and via the bottle that contained 6% glucose was tested. A number of 20 females were released into a cage where 40 powder-treated males had been present one day before. Almost all of these females contained the fluorescent powder somewhere on the abdomen after 24 hours in the cage. When the bottle with glucose solution was replaced by cotton wool on top of the cage, a number of 16 females (80%) contained the fluorescent powder on the abdomen.

As a result of this outcome another test was performed. The setup was the same as for the previous two experiments, except that the sleeve of the cage was covered with tape and paper, so that none of the mosquitoes could fly in there. In the end, a number of 4 females (20%) contained the powder somewhere on the abdomen. Powder transfer must therefore also take place through the walls of the cage

Finally, the transfer of the powder through accidental encounters between mosquitoes was tested. When 10 untreated females were released into a cage where 40 powder-treated females were present, 5 of these females (50%) had the powder somewhere on their abdomen after 24 hours. For this test cotton wool soaked with 6% glucose was present on top of the cage and the sleeve of the cage was covered.

4.4 Odour tests

Before performing the actual odour test, some pre-tests to assess the potential toxicity of the chemicals and solvent were performed. Stock solutions of the four different compounds were prepared as described in paragraph 3.3.2. From each compound separately an amount of 1µl of a 10µg/µl solution was applied to male mosquitoes. Males were used because females were not available at that time (it was assumed that the impact of the chemicals would be similar for females). These males were kept in a cage for 24 hours and the survival rate was determined. For C8 and S all of six tested males had survived. For C10 only one male of six survived and for C9 none of the six males survived.

When this was repeated with 1µg/µl solutions again for C8 and S all tested males survived after 24 hours. All males from the C10 treatment survived as well and for C9 all but one survived. When a 1µg/µl mixture of these four compounds was applied to mosquitoes, all of them survived.

Table 6 – Mean difference of proportions of inseminated females between control groups and test groups ± SE and the P-value (* = 2-tailed) for each of the odour tests

Exp	Concentration	Mean difference ± SE	P
C	10ng/µl	0.099 ± 0.090	0.144
D	100ng/µl	0.086 ± 0.074	0.137
A	1µg/µl	0.188 ± 0.089	0.025
B	10µg/µl	0.231 ± 0.109	0.027
M	10µg/µl	0.031 ± 0.049	0.550*
E	10µg/µl	0.124 ± 0.059	0.053*

4.4.1 Application of the odours to the females

The first concentration of the mixture to be tested was 1µg/µl since, as mentioned above, when this concentration was applied to the mosquitoes all of them survived. As can be seen in figure 10 the mean proportion of inseminated females for this concentration is nearly 0.3 and the proportion of inseminated females in the solvent treated group was nearly 0.5. These two groups were present in

the same cage and were thus exposed to the same males and identical environmental factors. The only difference was the content of the solution they were exposed to. Therefore these two groups can be directly compared. The difference between the insemination rates of the two groups was significant (P= 0.025). The mean differences and the significance between the odour and control groups for all tested concentrations can be found in table 6. What also can be seen in figure 10 is that the higher the concentration of the odour mixture, the lower the mean insemination rate for the odour group will be.

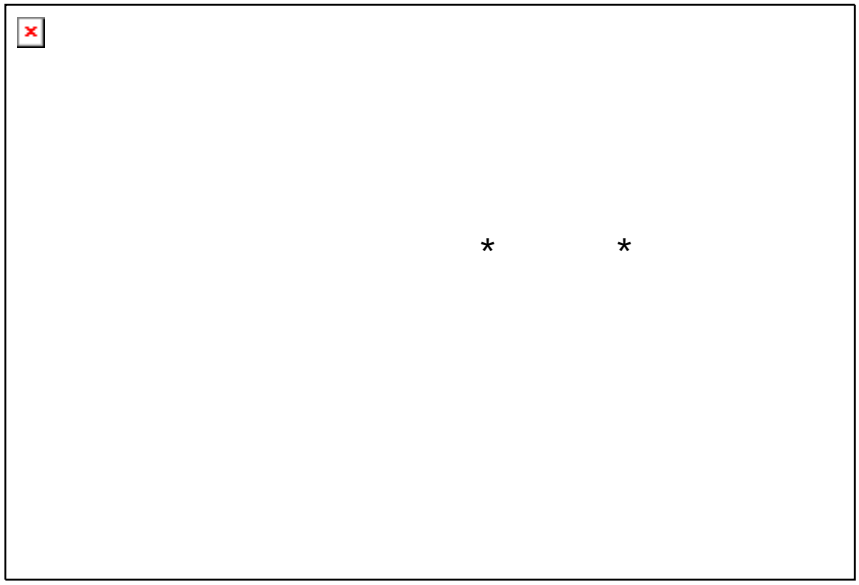


Figure 10 – Mean proportions of inseminated females for each tested concentration of the mixture together with their control group. * = significant difference as compared to solvent group (P< 0.05). Error bars represent SEM.

Although there is a significant difference for the first concentration that was tested, it is just slightly significant. Therefore, it was chosen to increase the concentration with a factor 10. Hence, the next concentration to be tested was 10µg/µl. As can be seen in the table, the results for this concentration of the mixture are very similar to the previous concentration. Consequently, the significance of the difference is comparable as well (P= 0.027).

The next step was to decrease the concentration of the mixture. The third concentration to be tested was 100ng/µl. This lower concentration did result in a different insemination rate for the odour group. The mean insemination rate for this treatment was higher than the insemination rate for the two previous tested concentrations. The mean insemination rate for the solvent group was again comparable to the previous two solvent groups. The difference between the odour group and the solvent group was smaller and not significant (P= 0.137). In table III in appendix I the mean insemination rates for all odour tests can be found.

When an even lower concentration was tested (10ng/µl), the results were approximately the same as for the 100ng/µl mixture. Again, there was no significant difference between both treatments (P= 0.144). However, the overall insemination rate was higher, for both the odour group and the solvent group.

4.4.2 Application of the odours to the males

Since the compounds used in these experiments are produced by males, their effect when applied to males was tested as well. The highest concentration of the mixture (10µg/µl) was applied to the males. Although this concentration of the individual compounds was lethal to the males (as mentioned above), the same concentration of these compounds in a mixture did not cause any mortality.

From four replicates there was no significant difference (P= 0.550) in insemination rate between the odour treated group and the solvent group (“Experiment M” in table 6). There was however a decrease in the mean insemination rate within both groups as compared to the tests where the females were treated (as can be seen in table III in appendix I).

4.4.3 Treatment of females with methyl eugenol, mating stimulant of *Ceratitis capitata*

These experiments were performed to test whether the decreases insemination rate in the odour groups was caused by a repellent effect of the females' altered scent. Methyl eugenol was tested in the concentration 10µg/µl, which was the highest concentration used in the experiments where the mixture was applied to the females.

The results for this experiment are based on eight replicates and can be found in table 6 as "Experiment E" (more details can be found in table III in appendix I). What can be seen is that the mean insemination rate in the solvent group is lower than in the odour group. The difference was almost significant (P= 0.053). For all other experiments the mean insemination is higher in the solvent group.

4.4.4 Presence of the odours in the cage

The mean insemination rate of the solvent groups in these odour tests is by far lower than the 82% found in the first fluorescent marker test for the 4♂:1♀ group. The highest insemination rate for the odour tests is 55%. This was found for the solvent group in the cage where the lowest concentration of the mixture was tested. Therefore the influence of the odour mixture was tested when it was present in the cage and not on the mosquitoes.

Both the solvent treatment and the odour treatment were performed just once. The results were exactly the same for both treatments: of 20 females mosquitoes 18 were inseminated. The insemination rate for both treatments is therefore exactly 90%. This is in fact higher than the 82% that was found in the fluorescent marker tests for this 4♂:1♀ ratio.

5. Discussion

5.1 Environmental conditions

All major tests were performed in the same room under the same standard conditions. The different environmental factors were kept as constant as possible throughout the experiments, since these could influence the swarming (and therefore mating) behaviour. Unfortunately, during several periods other persons had to use the same room for their experiments and could have influenced the mosquitoes' mating behaviour, especially in the cages close to the door. The importance of constant environmental factors can be specified by a certain incident where the humidifier in the room failed. The humidity in the room decreased from 80% to 40% during four days, resulting into death of nearly half of all mosquitoes present in that room.

5.2 Ensuring virginity

All mosquitoes used in the experiments needed to be virgins. Two different methods were used to separate the males from the females. The first method, the most reliable, was placing the pupae into separate tubes and placing the emerged males and females into separate cages. Virginity can be guaranteed by the use of this method since adult males and females have ever been into contact with each other. However, many pupae were needed since a large portion of the mosquitoes never emerged or drowned in the water. Besides, the used ratios of males and females requested for at least twice the number of pupae as the highest number of one of the sexes that was needed. For example, when for one test 10 females and 40 males were needed, it was necessary to take at least 80 pupae to make sure that there were enough males among the emerged mosquitoes. In addition the loss of adults in the six days prior to the start of the experiments had to be compensated. This means that even more pupae had to be taken and a large proportion of the females in this example could not even be used since just 10 were needed. Moreover, it is a very labour intensive and time consuming method. Therefore, the choice was made to use a second method for separating the males from the females. Within 24 hours after emergence the males and females were separated. The right proportion of males and females can already be taken and all mosquitoes will be used. It is not very likely that the mosquitoes will mate within this time period, less that 1%. However, this second method is less accurate than the first because there is a chance, albeit very small, that a female was inseminated prior to the start of the experiment.

5.3 Rearing conditions and availability of mosquitoes

In the time period that the experiments were performed, there were times that there were not enough females to start an experiment. Especially the rearing problems following the Christmas break caused a delay in the scheduled experiments. There were no mosquitoes to perform experiments for the first six weeks of 2008. Therefore an extra rearing was started. The excess pupae that no one else needed could be used for these experiments. However, these mosquitoes were reared under slightly different conditions than the mosquitoes from the main rearing. In the second room the humidity was not very constant and there was no red light present in the room. Therefore, when someone entered the room in the mornings, the main lights were switched on and could have influenced the larvae's and pupae's circadian rhythm. Moreover, some of the pupae came from a larval-feeding experiment. The influence of the amount of food on larval growth was tested, which resulted into adults of different sizes. As was found in this research, the size of females in an experiment can influence the insemination rate. However, when the pupae were needed, they were taken to the climate controlled room where all experiments were performed and were kept as adults in that room for six days to acclimatize. There were days that even from this rearing not enough pupae could be used to perform an experiment. On these days some pupae from the original rearing were taken to compensate for the shortage.

Therefore, for some experiments mosquitoes from different batches were used. The mosquitoes from each batch might have responded differently to the treatment because of the different rearing methods.

5.4 Fluorescent marker tests

As mentioned in paragraph 1.4.5, there are large differences in insemination rates found in literature for different ratio's of males and females. Here, the insemination rate found for a ratio of 1♂:4♀ with a total of 50 individuals in a standard rearing cage was 30%. Charlwood (1976) found for the same ratio in a cage with the same size an insemination rate of 6%. However, the number of individuals was as high as 250. This might point towards a negative and density-dependent effect of crowding on the insemination rate. However, for the opposite ratio of 4♂:1♀ Charlwood found an insemination rate of 90% for the number of 250 mosquitoes. In this research, the mean insemination rate for that ratio was found to be 82% for a number of 50 mosquitoes. The suggested negative influence of crowding on the insemination rate does not add up for this ratio. When these results are compared to what Verhoek and Takken (1994) found, an even lower insemination rate was observed. Although for a slightly different ratio (3♂:1♀), the mean insemination rate for a number of 200 mosquitoes was found to be less than 50%. This difference could be due to the fiercer competition between males when more males have to compete for the same number of females. However, for the 2♂:1♀ ratio, Charlwood (1976) found an insemination rate that was higher (84%) than the 50% found by Verhoek and Takken for the 3♂:1♀ ratio. What can be said is that the results from one specific test can never be applied to another specific situation.

When fluorescent powder is applied to the males under similar circumstances, the mean insemination rate decreases significantly. When the powder is applied on the lower half of the males' abdomen, the insemination rate decreases from 80% to 60% and from 35% to 10% (for 4♂:1♀ and 1♂:4♀, respectively). This means that the powder influences the male's ability to inseminate females. A possible explanation could be an obstruction of the terminalia which the male needs to hold on to the female during copulation. When the male is not able to do so, insemination will not take place. This also can be an explanation for the fact that the powder was found on many different females. When the male approaches a female for mating, he tries to seize her and powder is transferred. However, the male is not able to hold on to her because the terminalia are obstructed and would do another mating attempt, perhaps with a different female. The powder can therefore lead to more mating attempts. Another cause of the decreased insemination rate could be a decrease in the males' mobility caused by the extra weight the males are carrying.

When the experiments where the males were treated with the powder were performed in a larger cage, the insemination rate would decrease even further. Although twice the number of mosquitoes was used, the mean insemination rate for the 4♂:1♀ ratio was only 22%, whereas 60% was observed for the same ratio and the same treatment in the standard cage. This very large difference could have been caused by several factors. First, the increased size of the cage. If the males' mobility is decreased, this probably has a larger influence over larger distances. Second, the fluorescent powder on the males decreases their capability to fly, of which the effect will be greater when the males need to fly larger distances. Third, these experiments were performed in a different room in which the conditions were not as constant as in the room where the standard cage experiments were performed. However, this should also result into a larger standard deviation, which was not recorded. Therefore, the influence of the second two factors on the decrease of the insemination rate is probably more important. When the other ratio (1♂:4♀) was tested in the larger setting, the mean insemination rate decreased as well: 4% against the 10% observed for the standard cage. When for this same ratio no fluorescent powder was applied to the males in the same large setting, the observed insemination rate was 32%. This is equal to the 30% that was observed for the standard cage. It can be concluded that for this ratio the size of the cage does not influence the males' capability to find females for mating. It appears that the presence of the powder has a large influence on the males' capability to fly over larger distances and inseminate females. However, this 32% was obtained from one single test. Therefore, to get reliable results, more replicates of this test should be performed.

To be able to apply the powder, the males were anaesthetized by cooling. This could have a negative influence on their activity when they are used for an experiment several minutes after treatment. However, the groups that experienced the “freezer treatment” did not show a lower mean insemination rate as compared to the groups without any treatment. Therefore, it can be concluded that this treatment does not influence the insemination success and any effect observed in these experiments can be ascribed to the fluorescent powder.

The influence of the quantity of the applied fluorescent powder was tested. The insemination rate decreased, as compared to the control group, when a large amount of powder was applied. When only the final two segments of the male’s abdomen were marked, the insemination rate for the 4♂:1♀ ratio did not change. However, for the 1♂:4♀ ratio the insemination rate increased slightly as compared to the group that received a large amount of marker. When there is a low number of males against a high number of females, the quantity of the applied marker influences the insemination rate. A higher amount of marker results in a lower insemination rate. When there are more males in the cage, it may be that these males can stand the burden of the extra marker by their high number of individuals.

Although decreasing the quantity of the marker within the 1♂:4♀ ratio seems to give an insemination rate close to the control group, there is a negative aspect. When a small amount of marker is applied to the male, the probability that the male will transfer the marker to the female during mating decreases. This is exactly what was found. Inseminated females came out of that experiment without any fluorescent dust on their abdomen (nor anywhere else). Considering the goal of this test, which was to test whether the fluorescent marker was suitable for indicating male mating attempts, it can be said that a small amount is not suitable. It can result in false negatives. A large amount of marker on the other hand can prevent false negatives, but can give false positives. It was found that a large amount of marker on the males can contaminate the cage which can result in transfer of the dust from the cage to the females. Another cause of false positives is the chance of accidental encounters. This can result into fluorescent dust transfer even when the applied amount was low. Therefore, if the use of this method is chosen to give an indication for mating attempts (including the risk of having false negatives), all females that contain the fluorescent marker should be dissected to confirm insemination.

It is also concluded that although this method may not be ideal for tests in laboratory cages, that it may be worthwhile to use it in much larger settings, such as semi-field systems (Ferguson *et al.*, 2008) and for mark-release-recapture procedures in the field to study mosquito population ecology and dispersal (Service, 1993; Reisen *et al.*, 2003). This technique is not only applied to mosquitoes. Dust has been used for over 75 years and is probably the most commonly used material for externally marking a variety of insect species (Service, 1993). Despite the advantages of dust marking Hagler and Jackson (2001) reported several drawbacks for the use of this method. It can increase mortality, decrease mobility, interfere with sensory organs and cause adverse behavioural effects. Moreover, the dust particles can be transferred to unmarked insects. Adverse effects were observed in this study as a decreased insemination rate, probably due to obstruction of the terminalia and decreased mobility, and as a contamination of the dust which can lead to false positives.

5.5 Odour tests

In these laboratory settings a mixture of the four identified male produced compounds showed to have an effect on the insemination success of *An. gambiae.* When different concentrations of this mixture were applied to the abdomen of females a dose-dependent effect was observed: the higher the concentration, the lower the insemination rate. A comparable technique is used in moths where mating disruption systems by applying pheromones are used to control moth infestations. Male moths become contaminated with female pheromones which negatively influences their sexual behaviour on various levels (Nansen, 2007).

There probably would be a maximum effect of this treatment. There are many other characteristics (like the difference in auditory signal and morphological features), besides scent, that distinguish females from males. Therefore the application of the odour mixture to females will never completely prevent insemination, since the other female characteristics are still perceptible by the

males and will reveal her sex, despite the fact that she smells like males. However, to be able to conclude that there is a maximum effect, an even higher concentration of this mixture should be tested.

A decreasing effect of the compounds on the insemination success has been found, indicating a function of these male-produced compounds in the mosquitoes' sexual behaviour. These compounds may play a role in aggregation of males at specific swarm sites and in females using these cues to locate swarms consisting of males of their own species. Different *Anopheles* species could produce these volatiles in different compositions and concentrations to mediate species recognition for mating. The volatiles produced by different *Anopheles* males have to be trapped, analysed and compared, and these compounds have to be further tested in behavioural essays and in (semi-)field conditions to be able to fully understand their function in sexual behaviour and mating barriers between *Anopheles* mosquitoes.

When the experiments for odour treatment D (100ng/µl) were running, other experiments were performed in the same room. This means that there was more human activity in the room that could have influenced the mosquitoes' behaviour. When the room is used more often in the mornings, while the lights are off, the mosquitoes' circadian rhythm might be disturbed by daylight leaking into the room. Since mating occurs at specific time intervals during the day, especially during dusk, disturbing the mosquitoes' rhythm can influence the results. This probably is the cause of the lower observed insemination rate within this tested concentration. The insemination rate within the control group decreased as compared to the control groups from the other tested concentrations (figure 10). Since the control and test group were in the same cage at the same time, the decreased insemination rate that was observed for the test group could also be a result of this human activity. The mean difference between the test and control group of odour treatment C (10ng/µl) is comparable to the mean difference of treatment D. This indicates that the observed decrease in insemination rate of odour group D is indeed a result of the increased activity in the room and to lesser extent a result of the increased concentration of the odour mixture. This information combined with the possible maximum decreasing effect of the mixture points towards a strong bend in the curve between 100ng/µl and 1µg/µl. This means that within the concentration range of 100ng/µl to 1µg/µl the influence on the insemination rate of the mixture could go from no effect to a maximum. Although to be certain, experiments have to be performed within this concentration range.

When the odour mixture was applied to the males, no influence on the insemination rate was observed. This is a rather logical result since the compounds are male-produced. Even the solvent group produces the compounds which means that the two groups are comparable. Naturally, two comparable groups give comparable results. However, the quantity of the applied compounds (5 µg) is higher than the quantity of those compounds that a male produces. Pålsson *et al.* (unpublished) trapped 0.01 to 0.2 ng of the compounds per swarming mosquito. Yet, these compounds are volatiles which means that the compounds will not remain on the mosquito for the complete 24 hours in the same quantity in which they were applied. The compounds were applied when the light intensity was 100% (just after dawn), however the probability of mating is the highest during dusk, approximately 12 hours later. The quantity of the compounds present on the males would have been decreased during mating. The same counts for the experiments where the females were treated with the compounds. Therefore, to know the exact quantity of the compounds that gives the effect, the amount of the compounds still present on the mosquitoes after 12 hours should be analyzed. This can be done by using the Solid Phase Micro Extraction (SPME) technique as undertaken by Pålsson *et al*.

It is possible that the decreasing effects on the insemination rate that were found when the mixture was applied to females are not specific for the compounds that were used. It is imaginable that the males are repelled by the altered scent of the females which causes the insemination rate to decrease. Therefore the effect of another compound was tested. A mating stimulant of a different species (*C. capitata*) should not have any influence on the insemination rate when it is applied to a female mosquito. Although in a study by Kiran and Devi (2007) methyl eugenol has been found to be a component of mosquito repellent oils with mosquitocidal activity, from leaves of *Chloroxylon swietenia* DC (Rutaceae), no effect has been found for the compound on its own. Therefore, the mosquitocidal activity of the oil is probably caused by other components and methyl eugenol alone is not expected to

give a repellent effect. As the results show, there is indeed no repellent nor mosquitocidal effect of methyl eugenol, since the insemination rate did not decrease as compared to the solvent group. Therefore, it can be concluded that the results that were found for the mixture of male-produced compounds are in fact specific for these compounds.

Although not significant, there was a trend observed towards an increasing effect of methyl eugenol on the insemination rate. For this compound there could also be a dose dependent effect e.g. when the applied concentration increases, the insemination rate could increase even further. Since just one concentration of this compound was tested, it cannot be concluded that it has no increasing effect on the insemination rate. If indeed an increasing effect will be found, it can be concluded that methyl eugenol stimulates mating in *An. gambiae* as well and therefore is not suitable for testing whether the observed effects are specific for the male-produced compounds. Consequently, a different odour has to be chosen to test the compound specific effect.

The insemination rate of both the test groups and control groups in the odour experiments was not as high as the proportions that were found for the fluorescent marker experiments. Therefore it was thought that the presence of the odours in the cage also influenced the insemination rate of the control group. Therefore, an odour source was placed in the cage and the insemination rate was determined. However, no influence on the insemination rate was observed for the odours present in the cage. This was observed from just one single replicate. No additional replicates were carried out since the setup of the experiment was not suitable to obtain good results. The surface of the odour source was too small in order to release a significant amount of odours into the cage. The best situation would be if there was a constant flow of a certain concentration of the odour mixture through a sealed cage. Then, the odours are spread through the cage and the concentration present in the cage is known.

Moreover, an additional olfactometer or wind tunnel experiment would be interesting to test the females' response to the compounds. An olfactometer experiment has already been performed (paragraph 1.5) however, the odours were present in the proportion as produced by *An. arabiensis* males. Different results can be obtained if the odours are present in the proportion as produced by *An. gambiae* (C8:C9:C10:S = 1:1:1:1).

6. Conclusions

The main aim of this study was to determine whether four putative male-produced sex pheromones could influence the insemination success of *An. gambiae* mosquitoes. The results allowed answering the following sub-questions:

1. What are the insemination rates for different ratio's of males:females:

- 1:4?

- 4:1?

When 10 males are placed in a cage together with 40 females, the insemination rate is approximately 80%. When 40 males are place in a cage together with 10 females, the insemination rate is approximately 30%. The total amount of mosquitoes in all cages at the start of the experiments is 50. It is concluded therefore that the insemination rate increases when a higher proportion of males is present. However, these figures were only found for this specific situation with this specific number of mosquitoes. When the mosquitoes, male or female, were given any treatment the insemination rate would change.

2. Does the application of fluorescent dust marking on male An. gambiae affect their ability to inseminate females?

As compared to the control groups where 80% and 35% of the females were inseminated, the insemination rate decreased significantly to respectively 60% and 10% when half of the male's abdomen was marked with fluorescent dust. However, when a small amount of fluorescent dust was applied to the males, the 1♂:4♀ ratio groups showed no significant decrease as compared to the control groups. Therefore, it can be concluded that the application of fluorescent dust marker decreases the males' ability to inseminate females, dependent on the applied quantity. Moreover, the experiments in the larger cage revealed an even more negative influence of the dust on the males' ability to inseminate females. This was probably due to the fact that the dust decreased the males' flying capabilities over larger distances.

3. Is the presence of fluorescent dust on female An. gambiae an indicator for male mating attempts?

For all fluorescent marker tests where the males were marked, the majority of the females contained the fluorescent dust on their abdomen at the end of the 24 hours. For treatment 1.3 (4♂:1♀ ratio) even 100% of the females had the dust somewhere on their abdomen, though no more than half of these females were inseminated. For the other treatments even less than half of these marked females were inseminated (table 5). If the presence of fluorescent dust on females would be an indicator for male mating attempts this would mean that most of these attempts do not result into insemination. However, additional experiments indicated that contamination occurs by presence of the dust on the walls and in the sleeve of the cage and by accidental encounters. It must be pointed out that not all females that contain the dust were approached by a marked male for mating and therefore, the presence of fluorescent dust on female *An. gambiae* is not a good indicator for male mating attempts. To conclude, the presence of dust on females cannot be used as a proxy for insemination having taken place.

4. What is the effect of male produced volatiles on the insemination rate:

- when applied in different concentrations to females?

As compared to the control groups in the same cage, the application of a mixture of the male produced volatiles decreases the insemination rate. The observed effect is dose dependent: when the concentration of the applied mixture is higher, the number of inseminated females is lower.

- when applied to males?

The application of the odour mixture to males did not have an effect on the insemination rate, as compared to the solvent. Both groups of males, test and solvent group, produced the compounds

(since the compounds are produced by the males during swarming) and therefore both groups were comparable and gave comparable results.

- when present in the cage?

From the tests that were performed no effect was observed when the odour mixture was present in the cage. This was probably due to the weak setup of the experiment.

5. What is the effect on the insemination rate when a mating stimulant of a different species is applied to the females?

When methyl eugenol was applied to females, no decreasing effect on the insemination rate was observed. This indicates that the effect observed for the male-produced compounds were specific to these compounds and were not a cause of a repellent effect of their altered scent. However, there was a trend towards a increasing effect on the insemination rate. This effect might be stronger when the applied concentration increases. When a stimulating effect of methyl eugenol on the mating behaviour is found for *An. gambiae,* the conclusion that the observed effects are compound-specific should be reconsidered and tested with a different odour.

An. gambiae mosquitoes were found to respond to the four male-produced compounds, C8, C9, C10 and S, in a behavioural experiment. It was found that it is possible to use these compounds to (partially) 'transform' females into males, which reduces the insemination success. This points towards a function of these compounds in the sexual behaviour of *An gambiae*.

From these experiments it can be concluded that males are less attracted by females that received a mixture of male-produced compounds on their abdomen. In other words, when a female smells similar to a male, the chance that a male will mate with her becomes smaller. The stronger the female smells like a male, the smaller the probability of insemination. It is however not likely that the smell of the female alone will mask all other female characteristics, for example the wing beat. Therefore, there will always be a chance for a odour-treated female to become inseminated.

Since the compounds have shown to play a role in the mosquitoes' mating behaviour, several applications for these compounds can be established. Probably these compounds can also be used to increase the insemination success. This would be very suitable for mass rearing purposes needed for genetic control trials. In mass rearing facilities for *C. capitata* methyl eugenol is dispensed to increase mating and hence mass production of males for SIT releases. When the efficiency of modified mosquito production can be increased, it would result into a large reduction in production costs. Moreover, these compounds can be found to play a role in the pre-mating barriers between the different forms within the *An. gambiae* complex. Finally, odour based traps can be developed to attract females (and perhaps males) for sampling and control methods. The advantage of using these odours over using host odour based traps is that the mosquitoes can be collected earlier in life. Mosquitoes attracted to host odours could have been biting humans for days whereas mosquitoes attracted to odours involved in sexual behaviour had less time to bite humans, since mating occurs in an early stage of adult life.

7. Acknowledgements

First of all I would like to thank my supervisor, Bart Knols for all suggestions, ideas and great enthusiasm. That really motivated me during this thesis, although there were certain periods that I had other priorities. I appreciate your understanding.

Many thanks to Marit Farenhorst for showing me the mosquito rearing and places where I could get my materials. Thank you for your help with transforming a dirty, old office into an experimental room.

I would like to express my gratitude to Jeroen Spitzen for providing the fluorescent powder, explaining several techniques for application and for showing me how to dissect the females.

Thanks to YuTong Qiu for proposing an appropriate solvent for the compounds and a good concentration of these compounds to start testing with. Also for guiding me in finding suitable application methods.

Many people helped out in the IPO rearing during their weekends. Thanks to these people there were more mosquitoes available and without the effort of these people it wouldn't be possible for me to perform experiments in certain periods.

Finally, I would like to thank Yuri Stevens for being there for me to talk to, for his patience and for helping me with SPSS.

8. References

Alnwick D (2000) Roll Back Malaria - what are the prospects? *Bulletin of the World Health Organization* 78 (12): 1377-1377

Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, Sacarlal J, Manaca MN, Lafuente S, Barbosa A, Leach A, Lievens M, Vekemans J, Sigaugue B, Dubois MC, Demoitie MA, Sillman M, Savarese B, McNeil JG, Macete E, Ballou WR, Cohen J, Alonso PL (2007) Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. *The Lancet* 370 (9598): 1543-1551

Catteruccia S (2007) Malaria vector control in the third millennium: progress and perspectives of molecular approaches. *Pest Management Science* 63: 634–640

Charlwood JD (1976) The mating behaviour of mosquitoes. *PhD thesis*

Charlwood JD, Jones MDR (1979) Mating behaviour in the mosquito, *Anopheles gambiae* s.l. I. Close range and contact behaviour. *Physiological Entomology* 4: 111-120

Charlwood JD, Jones MDR (1980) Mating behaviour in the mosquito, *Anopheles gambiae* s.l. II. Swarming behaviour. *Physiological Entomology* 5: 315-320

Charlwood JD, Pinto J, Sousa CA, Ferreira C, do Rosario VE (2002) Male size does not affect mating success (of *Anopheles gambiae* in Sao Tome). *Medical and Veterinary Entomology* 16: 109–11

Clements AN (1999) *The biology of mosquitoes. Vol. II Sensory reception and behaviour.* CABI Publishing, Wallingford (UK)

Collins FH, Kamau L, Ranson HA, Vulule JM (2000) Molecular entomology and prospects for malaria control. *Bulletin of the World Health Organization* 78 (12): 1376-1376

Cox FEG (2002) History of human parasitology. *Clinical Microbiology Reviews* 15(4): 595–612.

Curtis CF (2006) Review of previous applications of genetics to vector control. *In: BGJ Knols, C Louis. Bridging Laboratory and Field Research for Genetic Control of Disease Vectors.* Wageningen UR Frontis Series 11, Springer, Dordrecht, pp 33-44

Della Torre A, Costantini C, Besansky NJ, Caccone A, Petrarca V, Powell JR, Coluzzi M (2002) Speciation within Anopheles gambiae - the glass is half full. *Science* 298 (5591): 115-117

Farenhorst M, Farina D, Scholte EJ, Takken W, Hunt WH, Coetzee M, Knols BGJ (2008) African water storage pots for the delivery of the entomopathogenic fungus Metarhizium anisopliae to the malaria vectors *Anopheles gambiae* s.s. and *Anopheles funestus. American Journal of Tropical Medicine and Hygene* 78(6): 910–916

Ferguson HM, Ng'habi KR, Walder T, Kadungula D, Moore SJ, Lyimo I, Russell TL, Urassa H, Mshinda H, Killeen GF, Knols BGJ (2008) Establishment of a large semi-field system for experimental study of African malaria vector ecology and control in Tanzania. Malaria Journal 7:158-173

Guerra CA, Snow RW, Hay SI (2006) Mapping the global extent of malaria in 2005. *Trends in Parasitology* 22 (8): 353-358

Hagler JR, Jackson CG (2001) Methods for marking insects: current techniques and future prospects. *Annual Review Entomology* 46:511–43

Hancock RG, Foster WA, Yee WL (1990) Courtship behavior of the mosquito *Sabethes cyaneus* (Diptera: Culicidae). *Journal of Insect Behavior* 3 (3): 401-416

Helinski MEH, Hassan MM, El-Motasim WM, Malcolm CA, Knols BGJ, El-Sayed B (2008)Towards a sterile insect technique field release of *Anopheles arabiensis* mosquitoes in Sudan: Irradiation, transportation, and field cage experimentation. *Malaria Journal* 7: 65

Hyde JE (2007) Drug-resistant malaria- an insight. *FEBS Journal* 274 (18): 4688-4698

Jacobs-Lorena M (2006) Genetic approaches for malaria control. *In: BGJ Knols, C Louis. Bridging Laboratory and Field Research for Genetic Control of Disease Vectors.* Wageningen UR Frontis Series 11, Springer, Dordrecht, pp 53-66

Killeen GF, Fillinger U, Kiche I, Gouagna LC, Knols BGJ (2002) Eradication of *Anopheles gambiae* from Brazil: lessons for malaria control in Africa? *The Lancet Infectious Diseases* 2: 618–627

Koella JC (1991) On the use of mathematical models of malaria transmission. *Acta Tropica* 49: 1-25

Kim W, Koo H, Richman AM, Seeley D, Vizioli J, Klocko AD, O'Brochta DA (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera : Culicidae): Effects on susceptibility to *Plasmodium*. *Journal of Medical Entomology* 41 (3): 447-455

Kiran SR, Devi PS (2007) Evaluation of mosquitocidal activity of essential oil and sesquiterpenes from leaves of Chloroxylon swietenia DC. *Parasitology Research* 101 (2): 413-418

Knols BGJ, Bossin HC, Mukabana WR, Robinson AS (2007) Transgenic mosquitoes and the fight against malaria: managing technology push in a turbulent GMO world. *American Journal of Tropical Medicine and Hygene* 77 (Suppl 6): 232–242

Lounibos LP, Couto Lima D, Lourenco-de-Oliveira R (1998) Prompt mating of released *Anophels darlingi* in Western Amazonian Brazil. *Journal of the American Mosquito Control Association* 14 (2): 210-213

Marchand RP (1984) Field observations ons warming and mating in *Anopheles gambiae* mosquitoes in Tanzania. *Netherlands Journal of Zoology* 34 (3): 367-387

Nansen C, MacDonald KM, Rogers CD, Thomas M, Poppy GM, Baxter IH (2007) Effects of sex pheromone in electrostatic powder on mating behaviour by *Lobesia botrana* males. *Journal of Applied Entomology* 131(5): 303–310

Nasci RS (1990) Relationship of wing length to adult dry weight in several mosquito species (Diptera: Culicidae). Journal of Medical Entomology 27: 716–719

Ng'habi KR, John B, Nkwengulila G, Knols BGJ, Killeen GF, Ferguson HM (2005) Effect of larval crowding on mating competitiveness of *Anopheles gambiae* mosquitoes. *Malaria Journal* 4: 49

Ng'habi KR, Horton A, Knols BGJ, Lanzaro GC (2007) A new robust diagnostic polymerase chain reaction for determining the mating status of female *Anopheles gambiae* mosquitoes. *American Journal of Tropical Medicine and Hygene* 77 (3): 485–487

N'Guessan R, Corbel V, Akogbeto M, Rowland M, (2007) Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area. *Benin Emerging Infectious Diseases 13:* 199–206.

Nijhout HF, Craig GB (1971) Reproductive isolation in *Stegomyia* mosquitoes. III. Evidence for a sexual pheromone. *Entomologica Experimentalis et Applicata* 14: 399–412

Nosten F, White NJ (2007) Artemisinin-based combination treatment of falciparum malaria. *American Journal of Tropical Medicine and Hygiene* 77 (Suppl 6): 181–192

Okanda FM, Dao A, Njiru BN, Arija J, Akelo HA *et al.* (2002) Behavioural determinants of gene flow in malaria vector populations: *Anopheles gambiae* males select large females as mates. *Malaria Journal* 1:10

Okech BA, Gouagna LC, Killeen GF, Knols BGJ, Kabiru EW, Beier JC, YanG, Githure JI (2003) Influence of sugar availability and indoor microclimate on survival of *Anopheles gambiae* (Diptera: Culicidae) under semifield conditions in Western Kenya. *Journal of Medical Entomology* 40 (5): 657-663

Reisen WK, Lothrop HD, Lothrop B (2003) Factors influencing the outcome of mark-release-recapture studies with *Culex tarsalis* (Diptera : Culicidae). *Journal of Medical Entomology* 40 (6):820–829

Ruan S, Xiao D, Beier JC (2008) On the delayed Ross–Macdonald model for malaria transmission. *Bulletin of Mathematical Biology* 70: 1098–1114

Service MW (1993) *Mosquito Ecology: field sampling methods*. New York: Elsevier. 988 pp

Scholte EJ, Ng'habi K, Kihonda J, Takken W, Paaijmans K, Abdulla S, Killeen GF, Knols BGJ, (2005) An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science* 308: 1641–1642

Takken W, Knols BGJ (1999) Odour mediated behaviour of Afrotropical malaria mosquitoes. *Annual Review Entomology* 44: 131–57

Takken W, Costantini C, Dolo G, Hassanali A, Sagnon N, Osir E (2006) Mosquito mating behaviour. *In: BGJ Knols, C Louis. Bridging Laboratory and Field Research for Genetic Control of Disease Vectors.* Wageningen UR Frontis Series11, Springer, Dordrecht, pp 183-188

Thomas DD, Donnelly CA, Wood RJ, Alphey LS (2000) Insect population control using a dominant, repressible, lethal genetic system. *Science* 287: 2474-2476

Touré YT, Coluzzi M (2000) The challenges of doing more against malaria, particularly in Africa. *Bulletin of the World Health Organization* 78 (12): 1376

Tripet F, Dolo G, Traore S, Lanzaro GC (2004) The "Wingbeat Hypothesis" of reproductive isolation between member of the *Anopheles gambiae* complex (Diptera: Culicidae) does not fly. *Journal of Medical Entomology* 41 (3): 375-384

Tuteja R (2007) Malaria - an overview. *FEBS Journal* 274: 4670–4679

Verhoek BA, Takken W (1994) Age effects on the insemination rate *of Anopheles gambiae s.l.* in the laboratory. *Entomologia Experimentalis et Applicata* 72: 167-172

WHO (2006) Malaria vector control and personal protection: report of a WHO study group. WHO technical report series; no. 936

WHO (2008) World malaria report 2008. WHO/HTM/GMP/2008.1

Yuval B, Wekesa JW, Washino RK (1993) Effect of body size on swarming behavior and mating success of male *Anopheles freeborni* (Diptera: Culicidae). *Journal of Insect Behavior* 6 (3): 333-342

Yuval B (2006) Mating systems of blood-feeding flies. *Annual Review of Entomology* 51:413–440

Appendix

Appendix I – Overview of different tests

Table I – Comparison of fractions of inseminated females with and without freezer treatment

Compare tests	Mean	Mean difference ± SE	α (2-tailed)
1.1	0.819	0.081 ± 0.070	0.297
1.5	0.900		
1.2	0.300	0.035 ± 0.063	0.601
1.6	0.266		
1.3	0.563	0.159 ± 0.205	0.465
1.7	0.404		
1.4	0.102	0.125 ± 0.058	0.068
1.8	0.227		

Table II – Overview fluorescent marker tests

Exp no.	♂	♀	Marker	Freezer	N	Mean fraction ins.	SE
1.1	40	10	-	-	6	0.819	0.070
1.2	10	40	-	-	5	0.300	0.042
1.3	40	10	++	+	5	0.563	0.106
1.4	10	40	++	+	5	0.102	0.038
1.5	40	10	-	+	3	0.900	0.000
1.6	10	40	-	+	3	0.266	0.040
1.7	40	10	+	+	3	0.404	0.206
1.8	10	40	+	+	3	0.227	0.027

Table III – Overview odour tests

Concentration	Treatment	Mean	N	Std. Deviation
10 ng/µl	Odour	,4553	8	,15238
	Solvent	,5542	8	,20232
	Total	,5047	16	,18040
100ng/µl	Odour	,3760	8	,07503
	Solvent	,4621	8	,19554
	Total	,4190	16	,14983
1µg/µl	Odour	,2954	10	,19562
	Solvent	,4831	10	,20195
	Total	,3892	20	,21615
10 µg/µl	Odour	,2893	8	,18405
	Solvent	,5201	8	,24760
	Total	,4047	16	,24213
Total	Odour	,3505	34	,16850
	Solvent	,5036	34	,20531
	Total	,4271	68	,20172
Male 10 µg/µl	Odour	,2640	4	,04671
	Solvent	,2954	4	,08566
Methyl eugenol 10 µg/µl	Odour	,5754	8	,05374
	Sovent	,4508	8	,02312

Appendix II – SPSS output wing size

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
Proportion	1349	2,34	3,34	2,7651	,16162
Valid N (listwise)	1349				

Report

Proportion

Experiment	Insemination	Mean	N	Std. Deviation
1ug/ul 1+	no	2,7500	6	,24922
	yes	2,8237	7	,17416
	Total	2,7897	13	,20617
1ug/ul 1-	no	2,8139	11	,22245
	yes	2,8984	4	,25178
	Total	2,8365	15	,22456
1ug/ul 2+	no	2,6891	10	,21581
	yes	2,7656	2	,41984
	Total	2,7018	12	,23456
1ug/ul 2-	no	2,7386	11	,15279
	yes	2,7852	4	,25012
	Total	2,7510	15	,17474
1ug/ul 3+	no	2,8906	8	,16620
	yes	2,8359	4	,14063
	Total	2,8724	12	,15394
1ug/ul 3-	no	2,7891	4	,16011
	yes	2,9091	11	,15716
	Total	2,877	15	,16172
1ug/ul 4+	no	2,8138	12	,17264
	yes	2,6172	2	,05524
	Total	2,7857	14	,17479
1ug/ul 4-	no	2,8219	5	,10689
	yes	2,8490	9	,17574
	Total	2,8393	14	,15067
1ug/ul 5+	no	2,7813	9	,13711
	yes	2,8958	3	,15415
	Total	2,8099	12	,14380
1ug/ul 5-	no	2,9063	5	,24382
	yes	2,8724	6	,17128
	Total	2,8878	11	,19688
1ug/ul 6+	no	2,7522	7	,29473
	yes	2,7109	6	,14913
	Total	2,7332	13	,23056
1ug/ul 6-	no	2,8203	8	,20963
	yes	2,8951	7	,19531
	Total	2,8552	15	,19953
1ug/ul 7+	no	2,9219	4	,07864
	yes	2,7743	9	,16813

Mean wing lengths of all females from the odour experiments, divided by Insemination status.

Experiment description represents: Concentration of applied odour, number of replicate, treatment with odour or solvent (+ or -, respectively).

	Total	2,8197	13	,15942
1ug/ul 7_	no	2,8036	7	,14202
	yes	2,7991	7	,13634
	Total	2,8013	14	,13377
1ug/ul 8+	no	2,8438	7	,09841
	yes	2,8594	5	,05063
	Total	2,8503	12	,07924
1ug/ul 8-	no	2,7734	4	,06929
	yes	2,7361	9	,13876
	Total	2,7476	13	,11982
1ug/ul 9+	no	2,8248	14	,18151
	Total	2,8248	14	,18151
1ug/ul 9-	no	2,7839	12	,13087
	yes	2,8229	3	,11727
	Total	2,7917	15	,12523
1ug/ul 10+	no	2,7188	13	,13197
	yes	2,7656	2	,11049
	Total	2,7250	15	,12677
1ug/ul 10-	no	2,7098	7	,13913
	yes	2,6938	5	,15373
	Total	2,7031	12	,13864
10ug/ul 1+	no	2,6901	12	,14955
	yes	2,7188	1	.
	Total	2,6923	13	,14340
10ug/ul 1-	no	2,6491	11	,23649
	yes	2,9010	3	,11727
	Total	2,7031	14	,23799
10ug/ul 2+	no	2,6506	11	,14205
	yes	2,7109	2	,03315
	Total	2,6599	13	,13199
10ug/ul 2-	no	2,6776	11	,17319
	yes	2,6523	4	,17787
	Total	2,6708	15	,16833
10ug/ul 3+	no	2,8535	8	,16972
	yes	2,7938	5	,23861
	Total	2,8305	13	,19156
10ug/ul 3-	no	2,7750	5	,13514
	yes	2,8047	8	,15891
	Total	2,7933	13	,14506
10ug/ul 4+	no	2,7676	8	,19126
	yes	2,9512	8	,21962
	Total	2,8594	16	,22038
10ug/ul 4-	no	2,8425	13	,16822
	Total	2,8425	13	,16822
10ug/ul 5+	no	2,6753	9	,09400
	yes	2,7969	2	,17678
	Total	2,6974	11	,11230
10ug/ul 5-	no	2,6027	7	,14819
	yes	2,6763	7	,13762
	Total	2,6395	14	,14261
10ug/ul 6+	no	2,7202	11	,14994
	yes	2,6680	4	,09995

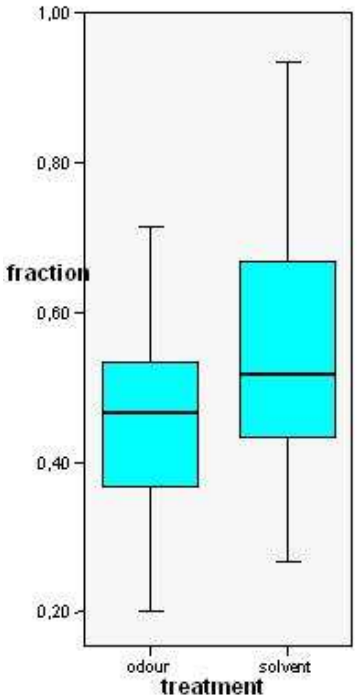
	Total	2,7063	15	,13701
10ug/ul 6-	no	2,7207	8	,08244
	yes	2,6901	6	,06204
	Total	2,7076	14	,07340
10ug/ul 7+	no	2,6578	10	,09359
	yes	2,7188	4	,08069
	Total	2,6752	14	,09156
10ug/ul 7-	no	2,6344	5	,08089
	yes	2,7719	10	,14698
	Total	2,7260	15	,14233
10ug/ul 8+	no	2,8698	6	,30517
	yes	2,7746	7	,20330
	Total	2,8185	13	,24882
10ug/ul 8-	no	2,8880	6	,18233
	yes	2,9349	6	,17578
	Total	2,9115	12	,17250
10ng/ul 1+	no	2,7688	5	,12568
	yes	2,7292	6	,11078
	Total	2,7472	11	,11350
10ng/ul 1-	no	2,8301	8	,13296
	yes	2,7924	7	,06912
	Total	2,8125	15	,10614
10ng/ul 2+	no	2,7344	4	,09375
	yes	2,7012	8	,09279
	Total	2,7122	12	,09024
10ng/ul 2-	no	2,7474	6	,22106
	yes	2,7254	7	,03592
	Total	2,7356	13	,14539
10ng/ul 3+	no	2,7214	6	,15386
	yes	2,6094	5	,17188
	Total	2,6705	11	,16454
10ng/ul 3-	no	2,6797	6	,13432
	yes	2,7902	7	,15091
	Total	2,7392	13	,14897
10ng/ul 4+	no	2,8094	5	,09530
	yes	2,8125	3	,10938
	Total	2,8105	8	,09279
10ng/ul 4-	no	2,8125	5	,11693
	yes	2,7520	8	,05813
	Total	2,7752	13	,08642
10ng/ul 5+	no	2,7455	8	,07195
	yes	2,7719	5	,08672
	Total	2,7556	13	,07553
10ng/ul 5-	no	2,7305	8	,11072
	yes	2,8250	5	,19991
	Total	2,7668	13	,15088
10ng/ul 6+	no	2,7969	6	,07329
	yes	2,9004	8	,11571
	Total	2,8560	14	,11000
10ng/ul 6-	no	2,8625	5	,07279

	yes	2,8724	6	,08582
	Total	2,8679	11	,07634
10ng/ul 7+	no	2,7930	8	,10720
	yes	2,7813	5	,05299
	Total	2,7885	13	,08761
10ng/ul 7-	no	2,9375	1	.
	yes	2,8034	12	,09665
	Total	2,8137	13	,09974
10ng/ul 8+	no	2,6953	6	,08714
	yes	2,7910	8	,10694
	Total	2,7500	14	,10721
10ng/ul 8-	no	2,7940	11	,07422
	yes	2,7930	4	,03465
	Total	2,7938	15	,06475
100ng/ul 1+	no	2,7472	11	,09025
	yes	2,7344	5	,07967
	Total	2,7432	16	,08462
100ng/ul 1-	no	2,7500	10	,12325
	yes	2,7656	4	,05413
	Total	2,7545	14	,10605
100ng/ul 2+	no	2,8307	6	,09080
	yes	2,8393	7	,07478
	Total	2,8353	13	,07906
100ng/ul 2-	no	2,9000	5	,24447
	yes	2,8047	8	,09073
	Total	2,8413	13	,16448
100ng/ul 3+	no	2,8663	9	,16371
	yes	2,9036	6	,09080
	Total	2,8813	15	,13645
100ng/ul 3-	no	2,9091	11	,16175
	yes	2,9453	4	,08219
	Total	2,9188	15	,14286
100ng/ul 4+	no	2,7634	7	,16098
	yes	2,7535	9	,14101
	Total	2,7578	16	,14490
100ng/ul 4-	no	2,6797	8	,13724
	yes	2,7943	6	,11372
	Total	2,7288	14	,13630
100ng/ul 5+	no	2,6875	11	,09244
	yes	2,8047	4	,12853
	Total	2,7188	15	,11190
100ng/ul 5-	no	2,7776	13	,11765
	yes	2,8242	4	,11509
	Total	2,7886	17	,11523
100ng/ul 6+	no	2,8003	9	,14401
	yes	2,8359	4	,16997
	Total	2,8113	13	,14608
100ng/ul 6-	no	2,8203	6	,09670
	yes	2,7168	8	,10446
	Total	2,7612	14	,11090

100ng/ul 7+	no	2,7594	10	,13485
	yes	2,7969	5	,15703
	Total	2,7719	15	,13810
100ng/ul 7-	no	2,7170	9	,15555
	yes	2,8021	6	,17751
	Total	2,7510	15	,16414
100ng/ul 8+	no	2,8086	8	,14118
	yes	2,7567	7	,21742
	Total	2,7844	15	,17591
100ng/ul 8-	no	2,7240	3	,20807
	yes	2,7784	11	,08778
	Total	2,7667	14	,11457
M1	no	2,6811	34	,14537
	yes	2,8052	15	,15900
	Total	2,7191	49	,15888
M2	no	2,6453	40	,18257
	yes	2,8151	12	,14746
	Total	2,6845	52	,18814
M3	no	2,7133	37	,15790
	yes	2,7684	17	,11216
	Total	2,7306	54	,14629
M4	no	2,7630	41	,15357
	yes	2,8047	16	,18974
	Total	2,7747	57	,16385
M5	no	2,7205	35	,17220
	yes	2,7699	22	,14988
	Total	2,7396	57	,16436
M6	no	2,7093	33	,19350
	yes	2,6815	21	,14273
	Total	2,6985	54	,17459
M7	no	2,7385	38	,17519
	yes	2,7472	11	,18353
	Total	2,7404	49	,17518
M8	no	2,7873	41	,16613
	yes	2,8245	13	,11449
	Total	2,7963	54	,15509
Total	no	2,7507	838	,16684
	yes	2,7887	511	,14986
	Total	2,7651	1349	,16162

Appendix III – SPSS output odour tests

Concentration: 10ng/µl



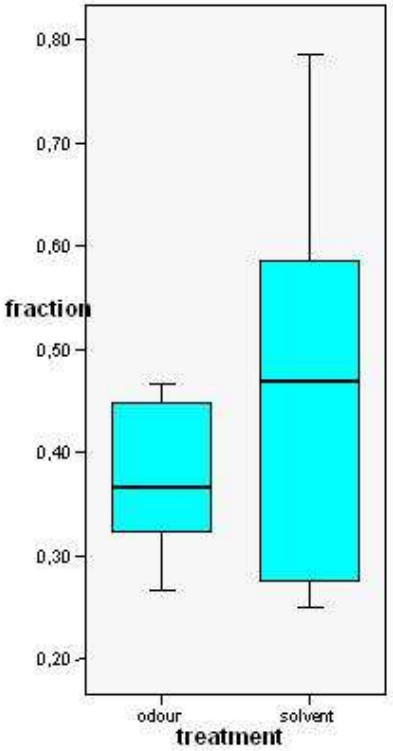
Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Fraction	3,00	8	,4553	,15238	,05387
	4,00	8	,5542	,20232	,07153

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
Fraction	Equal variances assumed	,596	,453	-1,104	14	,288	-,09886	,08955	-,29092	,09321
	Equal variances not assumed			-1,104	13,008	,290	-,09886	,08955	-,29230	,09459

Concentration: 100ng/µl



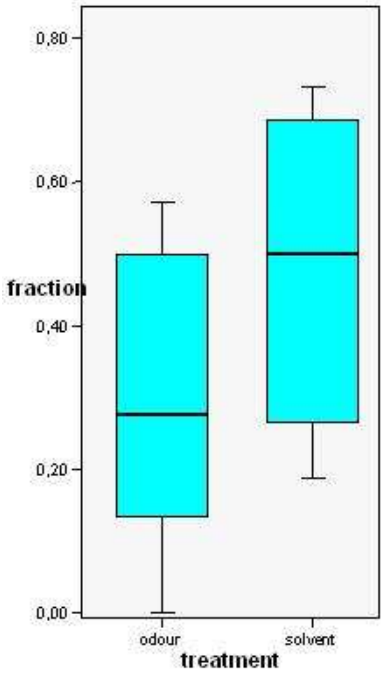
Group Statistics

	treatment	N	Mean	Std. Deviation	Std. Error Mean
fraction	odour	8	,3760	,07503	,02653
	solvent	8	,4621	,19554	,06913

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
fraction	Equal variances assumed	12,299	,003	-1,164	14	,264	-,08616	,07405	-,24498	,07266
	Equal variances not assumed			-1,164	9,018	,274	-,08616	,07405	-,25362	,08130

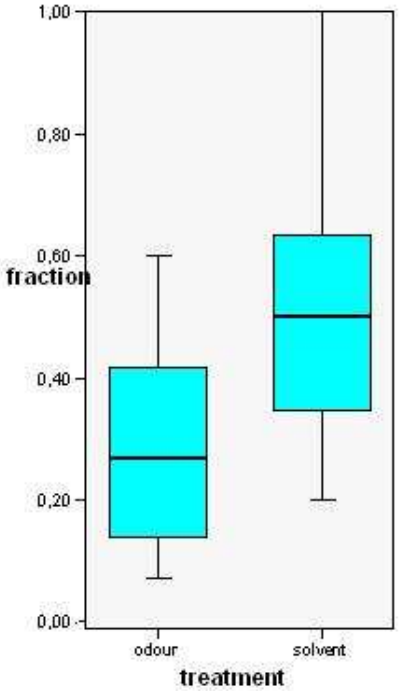
Concentration: 1µg/µl



Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
fraction	Equal variances assumed	,028	,869	-2,112	18	,049	-,18774	,08891	-,37453	-,00094
	Equal variances not assumed			-2,112	17,982	,049	-,18774	,08891	-,37455	-,00093

Concentration: 10µg/µl



Group Statistics

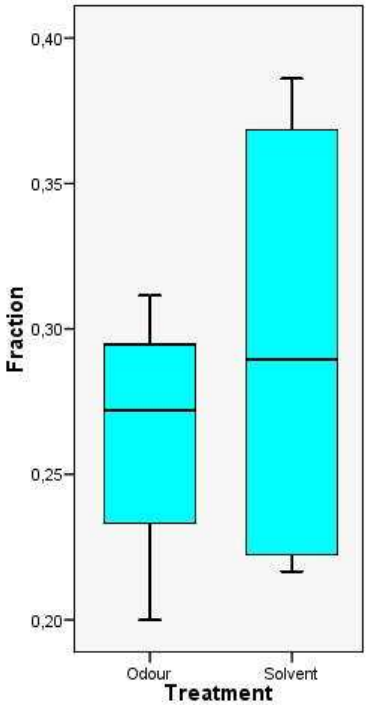
	treatment	N	Mean	Std. Deviation	Std. Error Mean
fraction	odour	8	,2893	,18405	,06507
	solvent	8	,5201	,24760	,08754

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
fraction	Equal variances assumed	,336	,571	-2,116	14	,053	-,23081	,10908	-,46476	,00314
	Equal variances not assumed			-2,116	12,926	,054	-,23081	,10908	-,46660	,00497

x

Males – Concentration: 10µg/µl



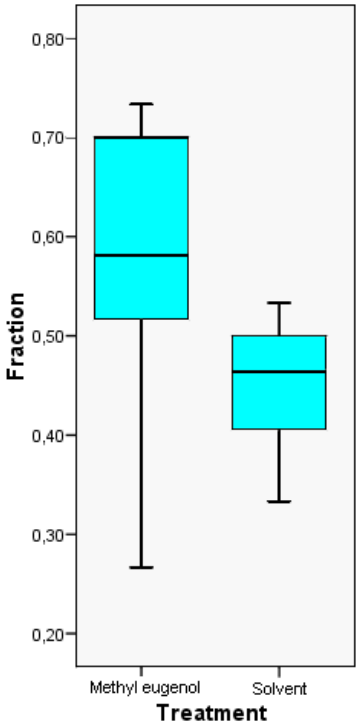
Group Statistics

	treatment	N	Mean	Std. Deviation	Std. Error Mean
fraction	odour	4	,2640	,04671	,02336
	solvent	4	,2954	,08566	,04283

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
fraction	Equal variances assumed	6,448	,044	-,644	6	,543	-,03141	,04878	-,15079	,08796
	Equal variances not assumed			-,644	4,640	,550	-,03141	,04878	-,15981	,09698

Methyl eugenol – Concentration: 10µg/µl



Group Statistics

	Treatment	N	Mean	Std. Deviation	Std. Error Mean
Fraction	1,00	8	,5745	,15201	,05374
	2,00	8	,4508	,06538	,02312

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Upper	Lower
Fraction	Equal variances assumed	2,414	,143	2,114	14	,053	,12365	,05850	-,00183	,24913
	Equal variances not assumed			2,114	9,504	,062	,12365	,05850	-,00763	,25493