Oviposition cues as a tool for developing a new malaria control strategy

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Akademisk avhandling som med tillstånd av Kungliga Tekniska Högskolan i Stockholm framlägges till offentlig granskning för avläggande av doktorsexamen i kemi med inriktning mot ekologisk kemi fredagen den 4 mars kl 13.00 i sal F3, KTH, Lindstedtsvägen 26, Stockholm. Avhandlingen försvaras på engelska. Opponent är Professor Willem Takken, Laboratory of Entomology, Wageningen University and Research Centre.

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Abstract

Anopheles gambiae sensu lato mosquitoes are among the dominant malaria vectors in sub-Saharan Africa. However, not much is known about the oviposition behaviour of these species. This knowledge is important for the development of malaria vector control strategies. New methods that can complement the currently used vector control methods: long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), are necessary due to the growing incidence of resistance to these methods.

With the aim of investigating cues associated with selected oviposition sites, artificial oviposition sites- ponds were set-up in an open field at Mbita, Western Kenya in 2012 and 2013. These ponds were plastic basin buried into the ground for concealment which contained 2kg of soil mixed with 30 L of water. They were allowed to be colonized by wild *An. gambiae s.l.*. The numbers of *Anopheles* early instar larvae were counted and used as a proxy for oviposition preference. Water samples were then analysed for physicochemical, bacterial and chemical profiles. The bacterial profiles were analysed using denaturing gradient gel electrophoresis (DGGE) and the chemical profiles with gas chromatography-mass spectrometry (GC-MS).

The detection of possible oviposition cues from oviposition substrates requires sensitive analytical methods. The detection of volatiles was improved seven times when 0.15 g/ml sodium chloride (NaCl) was added to the substrates and the volatiles trapped on Tenax traps for 20 h, were thermally desorbed compared to volatiles trapped on Porapak traps for 20 h that were eluted by liquid desorption with a solvent. Furthermore, to improve bacteria community profiling with denaturing gradient gel electrophoresis (DGGE), the detection of bacteria deoxyribonucleic acid (DNA) bands with DGGE was improved. This was achieved by pooling two replicate polymerase chain reactions and concentrating these to 10 µl which resulted in a minimum DNA concentration of 50 ng/µl. These two improved detection methods allowed for bacterial and chemical profiling of water samples taken from oviposition ponds.

Results showed that ponds were colonized differently. Fresh ponds were preferred over slightly older ponds and contained two times more *Anopheles* early instar larvae. Bacterial analysis revealed that the bacterial load may play a role in oviposition site selection where ponds with a low number of bacteria colony forming units (CFU) was preferred for oviposition. Furthermore, diverse groups of chemicals were associated with the preferred ponds. Taken together for all the data analysed, there was no volatile detected that was common in all the rounds within an experiment. However some volatiles, including: 6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) and 4-ethylbenzaldehyde, were associated with the oviposition preferred pond. In addition, physicochemical parameters such as low pH and high turbidity were associated with the ponds selected for oviposition.

Finally, fungi isolated from the rhizomes of nut grass that was collected from the same natural oviposition site as the soil used in the open field experiment, yielded a promising array of volatiles of which one is known to attract oviposition site seeking malaria mosquitoes. This finding opens the door for a cost effective and environmental friendly method of using fungi in an “attract and kill” strategy targeting malaria vectors.

Keywords: *Anopheles gambiae*, Malaria, Oviposition, Volatile compounds, GC-MS, DGGE, Sorption, Fungi, Bacteria, Physicochemical parameters, Nut grass, Bermuda grass

BOD	Biochemical oxygen demand
CFU	Colony forming unit
CIS	Cool injection system
DDT	Dichlorodiphenyltrichloroethane
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FMT	Fungi mixture
GC-MS	Gas Chromatography – Mass Spectrometry
GEE	Generalized estimating equation
GMM	Genetically modified mosquito
ID	Identification
IRS	Indoor residual spray
IVM	Integrated vector management
LLIN	Long lasting insecticide treated net
LSM	Larva source management
MTBE	Methyl-tert-butylether
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NTU	Nephelometric Turbidity Units
PCA	Principal component analysis
PCR	Polymerase chain reaction
PTV	Programmable temperature vaporization
RBM	Roll Back Malaria
SIT	Sterile insect technique
TAE	Tris/acetate-EDTA
TBV	Transmission blocking vaccine
TDU	Thermal desorption unit
UV	Ultra violet
WHO	World Health Organization
YEG	Yeast extract glucose

Anopheles gambiae sensu lato – A six sibling species complex consisting of *An. arabiensis*, *An. bwambae*, *An. merus*, *An. melas*, *An. quadriannulatus*, *and An. gambiae sensu stricto*
Cues – Factors including volatiles, physicochemical and microbial that may be influencing oviposition behavior of malaria mosquitoes
Endophagic – Indoor biting
Endophilic – Indoor resting
Exophagic – Outdoor biting

Exophilic – Outdoor resting
Oviposition – Egg laying
Pond – Artificial oviposition site consisting of a basin filled with an oviposition substrate of water mixed with soil
Post-colonization – Oviposition substrate after oviposition selection
Pre-colonization – Oviposition substrate at oviposition selection

This thesis is based on the following Papers, referred to in the text by their Roman numerals **I**-I**V**:

I. **Hay infusion and its volatiles are avoided for egg-laying by the malaria mosquito *Anopheles gambiae s.s.***
Lynda K. Eneh, Mike N. Okal, Anna-Karin Borg-Karlson, Ulrike Fillinger, Jenny M. Lindh
Submitted to Journal of Chemical Ecology

II. **Oviposition choice of malaria mosquitoes *Anopheles gambiae s.l.*: do they choose between similar habitats?**
Lynda K Eneh, Ulrike Fillinger, Gunaratna K Rajarao, Anna-Karin Borg-Karlson, Jenny M. Lindh
Submitted to Acta Tropica

III. **Factors associated with preferred *Anopheles gambiae s.l.* oviposition sites**
Lynda K. Eneh, Anna Karin Borg Karlson, Ulrike Fillinger, Gunaratna Kuttuva Rajarao, Jenny M. Lindh
Manuscript

IV. **Cedrol, a malaria mosquito oviposition attractant is produced by fungi isolated from rhizomes of the grass *Cyperus rotundus***
Lynda K. Eneh, Hiromi Saijo, Anna Karin Borg Karlson, Jenny M. Lindh, Gunaratna Kuttuva Rajarao
Submitted to Bioresource Technology

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To God be the Glory

1. Introduction

1.1. Malaria: Historical perspective

Malaria has been part of the human history since ancient times. It was mentioned in 4,000 years old Egyptian and Chinese text [1]. Besides, Egyptian mummies tissues was found to carry the parasite causing malaria [1]. It was Charles Laveran a French physician in 1880 that identified the malaria parasite and its connection to human blood [2]. Malaria is caused by *Plasmodium* parasites of which *Plasmodium falciparum* is the deadliest.

Ronald Ross through his experiments in the late 1890's confirmed the role of mosquitoes in human malaria transmission. He argued that since mosquito rest on wall after blood meal, targeting these mosquitoes at this point can reduce malaria transmission [2]. This knowledge launched the war on mosquitoes as means of controlling malaria starting from 1930's which included the use of the insecticide, dichlorodiphenyltrichloroethane (DDT) [2, 3]. DDT was the first persistent organic pesticide first synthesised in 1874 and with the insecticidal properties described by Paul Muller in 1939. This compound proved useful during World War 2 [2]. The global malaria eradication campaign of 1955–69 although successful in many parts of the world failed in Africa and in 1969, world health organization (WHO) formally abandoned the goal of global malaria eradication adjudging it not possible. In 1998, Roll Back Malaria (RBM) campaign was launched by WHO. The positive impact of RBM was not immediate which led some to think that the campaign was a failure [4]. However, a recent study showed that the current malaria interventions were estimated to have prevented more than 0.5 billion *P. falciparum* cases in the African

region between 2000- 2015 [5]. Moreover, the 2015 WHO malaria report stated that, in the last 15 years (2000-2015), 57 countries have reduced their malaria cases by 75% while a 66% decline in malaria mortality was recorded.

However, the fight against malaria is far from over. According to the 2015 WHO malaria report, about half of the world population (3.2 billion) is at risk of malaria infection and 214 million new malaria cases were reported in 2015 alone. According to the same report, 438,000 malaria related deaths were reported globally in 2015. This is despite the fact that malaria is a treatable and preventable disease.

1.2. *Anopheles* mosquitoes and *Plasmodium* parasites

Malaria parasites are transmitted only by female mosquitoes of the genus *Anopheles* [6]. This genus consists of about 430 species of which 30-40 can transmit malaria parasites. Species within the *Anopheles gambiae sensu lato* and *An. funestus* species complexes are the world's most efficient malaria vectors [7]. The *An. gambiae* complex consists of six sibling species including: *An. arabiensis*, *An. bwambae*, *An. merus*, *An. melas*, *An. quadriannulatus*, *and An. gambiae sensu stricto*. *An. quadriannulatus* is not a known malaria vector. There exist variation in host blood feeding preference within this complex. While *An. arabiensis* mosquitoes are both zoophilic and anthropophilic getting their blood meals both from animals and humans respectively, *An. gambiae s.s.* are strongly anthropophilic preferring human blood meals.

Malarial mosquitoes transmit malaria parasite inadvertently when taking a blood meal and they require at least two blood meals to

transmit malaria. In humans, malaria is caused by five *Plasmodium* species. These are *Plasmodium vivax*, *P. falciparum*, *P. malariae*, *P. knowlesi* and the sympatric *P. ovale* species: *P. ovale curtisi* and *P. ovale wallikeri*. The salivary gland of infected mosquito contains the parasite's sporozoites stage (Figure 1). Upon human infection via blood meal, the sporozoites migrate in the blood stream to the liver where they develop into merozoites. After some time, which varies between parasite species, the merozoites enter the blood circulation and infect red blood cells. In the red blood cells they go through an asexual proliferation. Some of the parasites in this blood stage develop into male and female gametocytes. When mosquitoes pick-up these gametocytes, the parasite lifecycle continues in the mosquito with a sexual proliferation (Figure 1). In the mosquito, the gametocytes goes through different life stages culminating in the sporozoites which migrates to the salivary gland from where they can be transmitted to another human during the next blood meal [8].

Female *Anopheles* mosquitoes take blood meals to develop their eggs. These eggs are laid in a suitable substrate [6] and majority hatch within the first two days after egg laying- oviposition. The eggs then develop through larvae, pupa and finally emerges as adult mosquitoes. Male *Anopheles* mosquitoes do not lay eggs and therefore do take blood meals or transmit malaria (Figure 1).

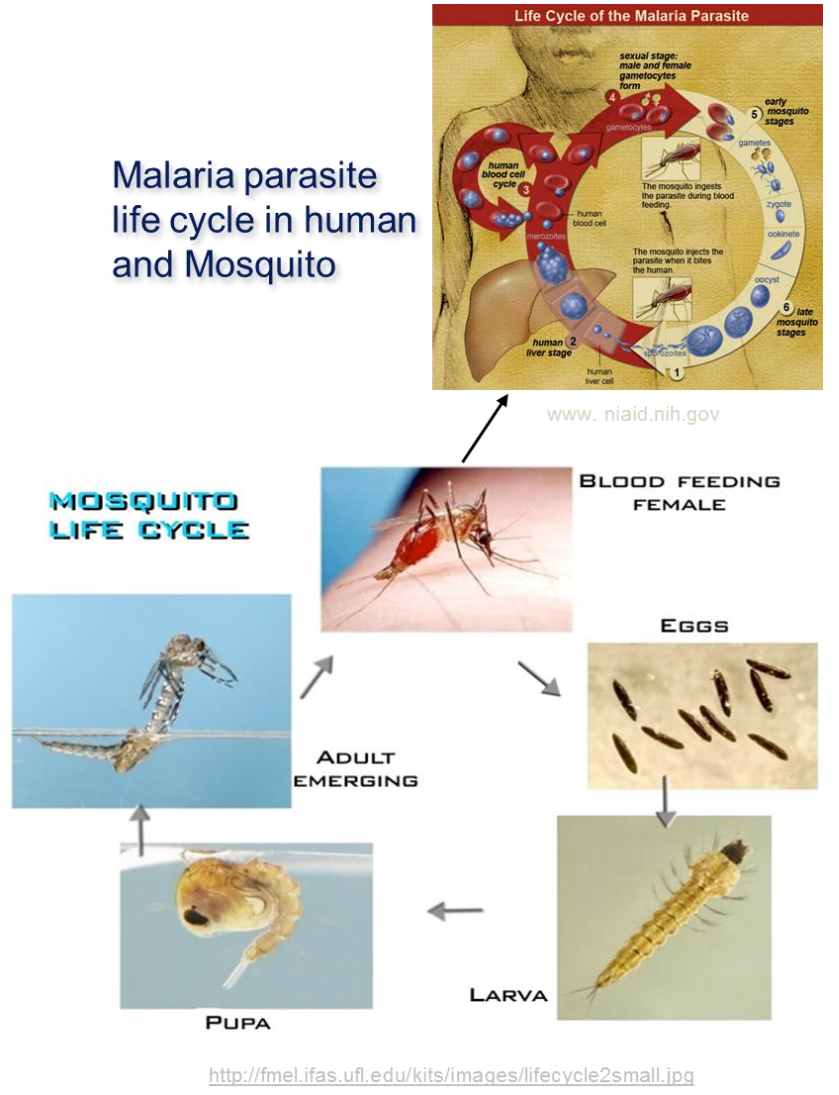


Figure 1. The life cycles of mosquito and malaria parasite in human and mosquito. Figure modified from http://fmel.ifas.ufl.edu/kits/images/lifecycle2small.jpg and www.niaid.nih.gov.

1.3. Currently used malaria vector control methods

Current malaria vector control measures rely heavily on long lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) both of which utilize insecticides [2, 5]. LLINs and IRS are malaria vector control measures with documented success rate. However, the continued success of these indoor based interventions is threatened by resistance [5, 9, 10], both to the commonly used insecticides in LLINs and IRS [9, 10] and behavioural resistance [11, 12] with more malaria mosquitoes biting outdoors away from the LLINs and IRS. Four classes of insecticides are approved for use in malaria vector control. These are carbamates, pyrethroids, organophosphates and organochlorides [13], out of which only pyrethroids are approved for LLINs. These insecticides have two modes of action: either acetylcholinesterase inhibition or an effect on the voltage-gated sodium channel [13]. Alteration in the sodium channel allows insects to tolerate high doses of insecticides without affecting the action of this channel. This alteration is known as knockdown resistance (kdr). The most studied mutation in sodium channel occurs in 1014 codon [14] where leucine is substituted with phenylalanine (1014F) or serine (1014S). Resistance to these modes of action has a huge negative effect on malaria vector control [14].

Furthermore, the observed change in biting behaviour could be attributed to the selection for outdoor biting (exophagic) and resting (exophilic) malaria mosquitoes through the extensive use of indoor control measures [11, 12, 15, 16]. Another issue deserving attention is the harmful effect of compounds as DDT used in control intervention [17, 18]. This compound remain in the environment up to 15 years after use and is banned in most countries of the world due to its harmful effect to the environment but approved for the control of malaria

vector. This emphasizes the need for application reduction of this harmful chemical in malaria mosquito control.

Integrated vector management (IVM) is a strategy to slow down resistance and increase control sustainability [13, 19, 20]. To ensure a continued reduction in malaria transmission rate, malaria mosquito must be controlled from different points of its life cycle [21] with emphasis on both the aquatic and non-aquatic stage. It has been observed that the control of mosquitoes at their aquatic stage, the so called larva source management (LSM), in combination with other control measures as long lasting insecticide treated nets (LLINs) and indoor residual spray (IRS) can reduce mosquito density and thereby the malaria burden [12].

1.4. Vector control methods focusing on adult mosquitoes

Other control measure includes improved housing. This can result in long term protection and does not affect mosquito diversity however it needs to be sustained for effectiveness [2, 22]. Research in the 60s raised hope about a malaria vaccine [23, 24] and in the late 1980, focus was on molecular technique. It was thought that genetically reducing vector competence will reduce the burden of the disease they transmit [2]. These two areas of research gave rise to other malaria vector control measures which include: use of genetically modified mosquitoes (GMMs) to reduce vector competence [25], sterile insect technique (SIT) [26]. Transmission blocking vaccines (TBVs) aim to prevent transmission of malaria parasite in mosquitoes and subsequently prevent transmission to human [27]. Other vector control measures are: toxic sugar baited traps [28] and zooprophylaxis [29].

1.5. Larval source management (LSM)

Prior to the extensive use of LLINs and IRS, source reduction was the main vector control strategy. These measures were utilized in malaria elimination in the Natal area of Brazil [2, 20] where the characteristic *Anopheles gambiae* habitat was identified and destroyed. This malaria elimination by source management was later repeated in Aswan, Egypt [2, 20]. Such environmental intervention as draining larval habitat could be expensive [2]. However, it may no longer be argued that LSM based control methods are too expensive to implement in poor countries. In fact, Worrall and Fillinger estimated that the cost for LSM using biological larval control is comparable to other presently used mosquito control measures [30].

1.5.1. Larval control

Larval control is effective in areas where larval sources are few and well defined [31]. This could imply that an effective larval control method needs to be coupled with other strategies such as attract and kill. Attracting an oviposition site seeking malaria mosquitoes to a pre-defined site could be valuable by reducing the number of larval habitats that need to be treated. Furthermore, it is necessary to observe that biological larvicides have been successful in mosquito larval control. These include use of fungi: *Aspergillus, Beauveria, Coelomomyces*, *Metarhizium, Fusarium* and bacteria: *Bacillus thuringiensis var. israelensis* (BTI) and *Lysinibacillus sphaericus* [32, 33]. A combination of a microbial larvicide such as BTI with oviposition attracting microbial odour source can occlude the need to use chemicals in larval control. Thus presenting an environmental friendly and cost effective

alternative for malaria vector control and at the same time, tackle the growing problem of insecticide resistance observed in vector control methods targeting adult mosquitoes. An oviposition based control method can thus be proposed as: 'microbial attract and kill strategy'

1.6. Need for oviposition study

Selecting an appropriate oviposition site is crucial for fitness of progeny, overall reproductive fitness and population dynamics [35]. Commonly used malaria vector control methods focus on indoor biting, endophagic mosquitoes. These methods fail to target the outdoor biting, exophagic malarial mosquitoes also capable of transmitting malaria. New control methods including exophagic mosquitoes have been suggested. This include the odour baited traps of the SolarMal project which uses attractive volatile odour blends mimicking human to mass trap mosquitoes before they enter the house [34]. A reminder that targeting malarial mosquitoes also outdoors can be important in reducing malaria transmission. Importantly, both endophagic and exophagic mosquitoes could be targeted equally at their aquatic stage. Thus, a complement to the adulticide based controls could be larval control [32].

1.6.1. Oviposition behaviour of Anopheles gambiae

Two species in the *Anopheles gambiae sensu lato* mosquito species complex namely: *An. gambiae s.s.* and *An. arabiensis* are among the dominant malaria vectors in sub-Saharan Africa. Although a lot is known about the host seeking behaviour of these mosquitoes, the knowledge about their oviposition behaviour is scares. In order to develop effective control measures targeting the aquatic stages of

malaria mosquitoes, it is crucial to understand how gravid female *Anopheles* mosquitoes' select oviposition site.

Anopheles gambiae s.l have been observed to prefer temporally puddles for oviposition, existing only for a given period compared with a more permanent habitat [35, 36]. Another study has shown that the age of an oviposition substrate affects its selection as an oviposition site [37]. However, other studies have shown that both temporal and permanent habitats are productive for adult mosquitoes and should equally be targeted in control programmes [38, 39].

In some studies, larval density, presence of sibling species and predators have been shown to affect larval development and mortality rates [40]. High larval density has for instance been linked to cannibalism [41]. All of these factors may invariably influence the oviposition behaviour of female *Anopheles gambiae* [42]. Rainfall increases the availability of aquatic habitats for oviposition. However, heavy rain may lead to loss of eggs and larvae by washing these away from their habitat [43].

In addition, one study observed that *An. gambiae* avoid substrates with culicine larvae when choosing oviposition substrates [44]. In contrast, *Anopheles* and *Culex* mosquitoes have been observed in a field study to colonize the same sites [45]. This habitat sharing maybe possible by the partitioning of food resources that was observed in another study [46].

Furthermore, the possible association between physicochemical profiles and larval density has been examined in some studies [36, 37, 47, 48]. For instance, turbid water was observed to have an arrestant property on *An. gambiae* in a study, with more eggs laid in turbid water from

natural breeding site compared to control water in a cage assay [49]. Furthermore, larval distribution was observed to be associated with turbidity [19, 48, 50, 51] and temperature [52]. Although, others observed that habitat characteristics are similar in both habitats with larvae and those without [35] and therefore no link between physicochemical parameters and larval distribution was observed [53].

It is known that mosquitoes can respond to olfactory cues when selecting oviposition sites [6, 54]. Bacteria and bacterial associated volatiles have also been observed to mediate oviposition selection [55, 56]. This oviposition preference was lost by autoclaving [45] thus suggesting a possible role of bacteria. In contrast, another study observe no oviposition preference by the addition of bacteria at different concentration to an oviposition substrate [57]. Water collected from a natural oviposition site was observed in laboratory oviposition assay to increase egg laying by *An. gambiae*. Volatile compounds as indole, 3-methylindole and 4-methylphenol were linked to this attraction by electro-antennogram (EAG) studies [58]. A number of volatiles have been suggested to elicit oviposition response in *An. gambiae s.l.*. However, except cedrol, their effect needs to be tested in bioassays in laboratory and field settings to confirm this response. These *An. gambiae* putative oviposition semiochemicals include (Figure 2): phenol, 4-methylphenol, 4-ethylphenol, indole, 3-methylindole, 3-methyl-1-butanol, cedrol, 4-methylcyclohexanol and ethyl acetate [56, 58-63].

Improvement of detection of volatiles that are emitted from the typical oviposition substrates should increase the likelihood of detecting putative semiochemicals. However volatiles from such substrates are typically emitted in low amounts and present in a complex water matrix

[64]. Moreover, insect olfactory systems are sensitive allowing them to detect and discriminate between ranges of odours [65]. This thus requires a sensitive method to detect such oviposition cues.

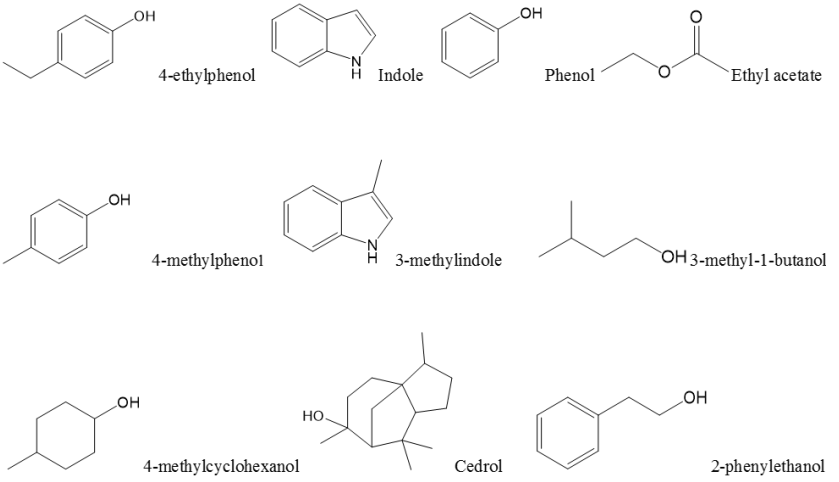


Figure 2. *An. gambiae* semiochemicals.

1.7. The aim of this thesis

The overall aim of this thesis was to identify oviposition cues that can be used in the development of control measures against *A. gambiae s.l.*

Specific aims:

1. Improve the detection of volatiles emanating from oviposition substrates.
2. Determine whether similar oviposition substrates vary in their attractiveness to wild oviposition site seeking malaria mosquitoes and to determine associated volatile cues.

3. Investigate oviposition cues associated with differently aged oviposition substrates while excluding larval based cues.
4. Investigate if fungi can produce oviposition attractant.

1.7.1. Hypotheses

In line with the four aims, four hypotheses were formulated:

(1) Is it possible to improve the detection of volatiles released from typical *Anopheles* oviposition substrates? (Paper I)

(2) Is similar ponds set-up in close proximity to each other colonized differently by wild malaria mosquitoes? (Paper II)

(3) Does the age of the oviposition substrate affect oviposition site selection and if so, which factors are involved? (Paper III)

(4) Can microbes, specifically fungi, produce volatiles that influence the oviposition behaviour of malaria mosquitoes? (Paper IV)

2.

Materials and methods.

Study experimental steps are summarized below (Figure 3)

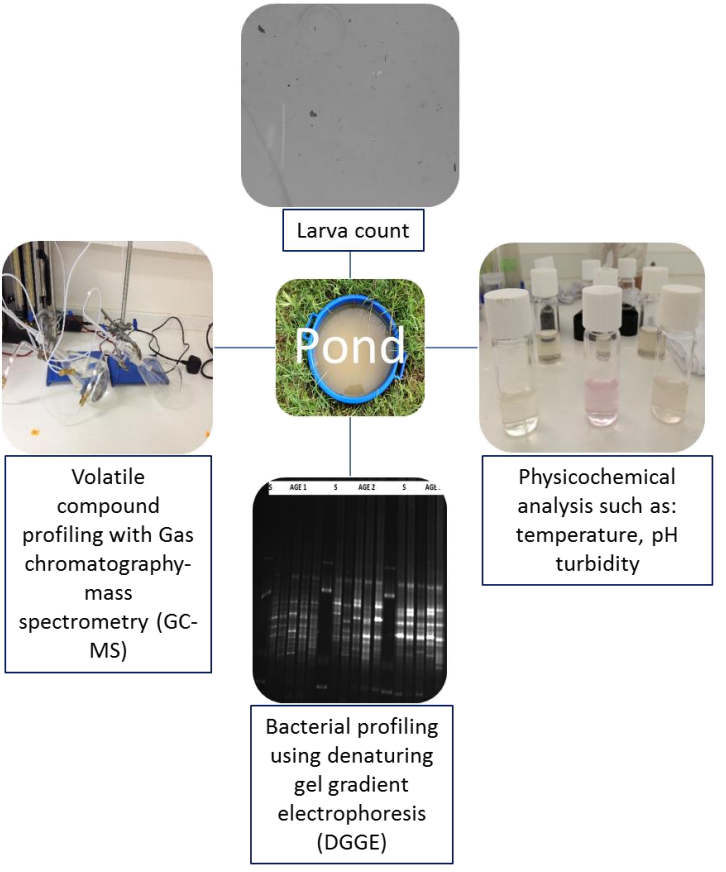


Figure 3. Summary of analysis performed on samples.

2.1. Study methods overview (Papers I-IV)

With the aim of (1) improving detection of volatile compounds, four volatile collection parameters were evaluated. These were: i) the addition of salt (NaCl) to the sample before headspace collection, ii) stirring the sample during collection, iii) varying collection time (1 h versus 20 h) and iv) thermal versus liquid desorption of the polymer traps which were analysed with gas chromatography – mass spectrometry (Paper I). (2) Identifying factors associated with oviposition site selection was achieved by setting-up open field studies during two consecutive years. It was investigated whether artificial oviposition habitats, ponds, varied in colonization by gravid *Anopheles* females. The number of early instar anopheline larvae was used as a proxy for oviposition preference. Ponds that were highly colonized were compared to ponds that were not. Factors that were compared included: age of pond substrate, physiochemical parameters, bacterial and chemical profiles (Paper II and III). (3) Finding an environmental friendly microbial odour source that can be utilized in development of an, attract and kill strategy was investigated by isolating fungi from rhizomes of *Cyperus rotundus* present in natural oviposition soil used in the open field experiments.

2.2. Oviposition substrates (Papers I-III)

2.2.1. Bermuda grass infusion preparation (Paper I)

Bermuda grass (*Cynodon dactylon (L.)*) infusions were prepared by mixing 90 g of the dried grass with 24 L of non-chlorinated tap water. The infusion was left to ferment at ambient temperature (25 – 28 °C) for three days outdoors in the shade under a roof and thereafter filtered

through a net (0.6 x 0.6 mm^2). The resulting infusions were utilized in analysis.

2.2.2. Artificial oviposition habitats (ponds) (Paper II-III)

Experiment location

All open-field experiments were conducted in a field at International Centre of Insect Physiology and Entomology -Thomas Odhiambo campus (icipe-TOC), Mbita, Western Kenya (Figure 4). The climate of Mbita is tropical with annual temperature of between 18-28 °C. The average annual rainfall was 1769 mm for 2010-2013 (data obtained from icipe's weather station). Experiments were conducted between March and June in 2012 and 2013, during the long rainy season.

Pond set-up

Artificial oviposition habitats, hereafter ponds, were made of plastic basin with a 55 cm diameter and 20 cm depth that were buried into the ground. The oviposition substrate in the ponds was made of 2 kg of dried soil from a natural oviposition site mixed with 30 L of non-chlorinated tap water pumped from Lake Victoria. An overflow point covered with a mesh was made at two sides of the basin to prevent loss of larvae with rain. Ponds were placed 4 m by 4 m apart in five rows with each row containing three (Paper III) or four ponds (Paper II). In the study where ponds were allowed to age differently (Paper III), the positions of ponds of different age were alternated in each row. All open field experiments were replicated over three rounds.

In Paper III colonization by wild mosquitoes were prevented by covering the ponds with a fine net. The net cover was removed and ponds opened simultaneously for colonization after aging 0, 4 and 17 days. Physicochemical profiling of the pond oviposition substrates was

performed on site and water samples were taken for bacterial and chemical profile analysis. Sampling were carried out between 8.00 and 11.00 a.m.

Ponds were monitored every morning (Paper II) or 4 days after set-up (Paper III) for early instar larvae using either a standard WHO's 350 ml dipper (Paper II) or with a sweep net (length= 40 cm, width = 5 cm, height = 10 cm; Paper III). The sweep net was passed over the surface of the water ten times to collect all mosquito larvae present. With the dipper, sampling was performed at four corners of each pond and the middle. The content was emptied into a white plastic bowl for larval counting. Anopheline larvae were differentiated from culicine larvae based on morphology. In Paper II, the experiment was performed over 12 days and extended for additional 14-15 days thereafter to observe the colonization profile. After larval counts, the water and contents were returned to the pond while pupae were removed daily and allowed to pupate in the laboratory. The emerged adult anopheline mosquitoes were further identified to *An. gambiae s.s.* and *Anopheles arabiensis* using polymerase chain reaction (PCR). For Paper III, the experiment was ended after larval count and physicochemical measurements by discarding the content of the ponds on the ground to kill the larvae.

Figure 4. Open-field experiment set-up for monitoring oviposition associated cues. Yellow arrows point to ponds- soil mixed with water mimicking natural oviposition sites.

2.3. Isolation of fungi from *Cyperus rotundus* grass rhizomes (Paper IV)

2.3.1. Collection of Cyperus rotundus rhizomes

Soil taken from a malaria mosquito breeding site in Western Kenya also used in open field experiments in this study was transported to Sweden. Subsequently, *Cyperus rotundus* rhizomes (Herbarium No. S15-29906; Naturhistoriska riksmuseet, Stockholm) present in this soil were harvested for fungi isolation. The harvested rhizomes were washed in sterile water to remove traces of soil and then mixed with sterile water at a concentration 70 g/L. This rhizome-water infusion was allowed to stand for 2 or 6 days at room temperature (about 22±2 °C).

2.3.2. Media preparation and fungi isolation

Yeast extract glucose (YEG) agar was prepared by adding 4 g of yeast extract to 1 L flasks containing 4 g of glucose, 15 g agar and 1 L of deionized water. This was mixed and then sterilized using an autoclave at 121 °C for 20 minutes. YEG broth was prepared the same way but without the agar. The sterile YEG agar media was poured into sterile petri dishes and allowed to cool. Thereafter, 100 µl of the rhizome infusion was spread evenly, or thin slices of the rhizome were inoculated directly on YEG agar plates. The plates were incubated for 2-6 days at room temperature. From the plates morphological single colonies were isolated. A loop full of each single isolate was transferred individually to sterile 50 ml Eppendorf tubes containing 15 ml of YEG broth and grown for 1-2 days. From each broth culture, 100 µl was transferred to individual 500 ml E-flasks containing 300 ml YEG broth for volatile collection on day 0, day 6 and day 12.

2.3.3. Fungal mixture (FMT) preparation

A mixture of all five fungal isolates (FMT) was prepared from YEG agar cultures of single isolates. A loop full of each isolate was transferred to a sterile 50 ml Eppendorf tube containing 15 ml of YEG broth. The mixture was then incubated in room temperature for 1-2 days. Subsequent steps were as described for single isolates.

2.4. Volatile collection procedures (Papers I-IV)

Polymer traps used for volatile collection were made of glass liners packed with 25 mg of either Tenax TA of mesh size 60/80 or Porapak Q of mesh size 50/80.

Volatiles were collected from the headspace above 300 ml of grass infusion samples (Paper I) and pond water samples (Papers II, III) and fungal cultures (Paper IV) in a 500 ml Erlenmeyer flask (E-flask). The E-flask was fitted with a gas wash bottle head and charcoal filtered air was pumped into the flask at 0.1 l/min through the inlet and drawn out through the Tenax (Paper I-IV) or Porapak trap (Paper I) at 0.1 l/min (Figure 5). Volatile collection time was for 1 h (Paper I) and 20 h (Papers I-IV).

In Paper I, four different volatile collection protocols were compared which were conducted in parallel. These protocols were: (1) without salt and without stirring and collection time of 20 h; (2) as in method 1 but with NaCl (0.15 g/ml infusion) added to the sample; (3) as in method 2 and with addition of stirring with a magnetic stirrer bar at 300 rpm during volatile collection; (4) as in method 2 but with 1 h collection time instead of 20 h.

During all volatile collections, empty E-flasks were sampled to determine background volatiles.

2.5. Gas chromatograpy-mass spectrometry (GC-MS) analysis (Papers I-IV)

GC-MS analysis was performed with an Agilent 7890A gas chromatograph connected to an Agilent 5975C inert MSD mass spectrometer detector. The GC system was fitted with GERSTEL Multi-Purpose Sampler. Agilent HP-5MS (5% phenyl and 95% dimethyl polysiloxane) column (30 m, 250 μm internal diameter and 0.25 μm film thickness) was connected to the GC.

Tenax traps were thermally desorbed in a thermal desorption unit (TDU) with heptyl actetate (3.16 ng / µl) added as standard in the TDU unit prior to analysis. The carrier gas was helium. The MS was at full scan and identified mass ranges from 30-400 m/z. Electron ionization was at 70 eV and ion source temperature at 230 °C. In Paper I, Porapak traps were eluted with 100 µl of methyl-tert butyl ether (MTBE) and 10 µl of the eluent was injected into the GC.

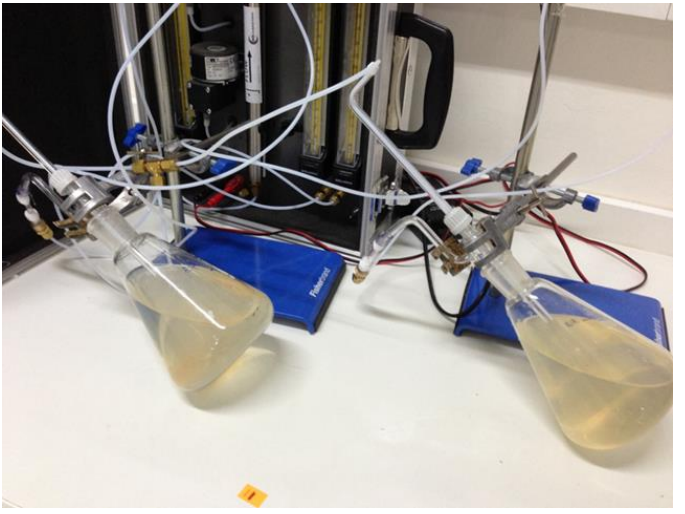


Figure 5. Volatile collection set-up with E-glass was slanted to increase surface area of the substrate.

2.5.1. Identification of volatile compounds

All standards were at >95% purity. These were diluted with MTBE and injected at a concentration of between 5-50 ng/µl into thermal desorption unit using same GCMS program as described above. Retention time and mass spectra of authentic standards were compared with those found in the samples to confirm compound identities.

2.5.2. Analysis of GC-MS Data

Chromatographic data was recorded and processed with Agilent's enhanced Chemstation software version E.02.01.1177. Chromatograms and mass spectral data were compared to empty bottle background controls. Sample peaks having different retention time and/or mass spectra compared to the control were manually integrated. Volatiles with a peak-area at least twice as large in the sample compared to the control were also included. For analysis of detected amounts the peak-area of the control was then subtracted from the peak-area of the sample and the area of each volatile was normalized against the area of the heptyl acetate standard to get the normalized peak area. All volatiles with the same retention time and mass spectra were given the same volatile identification (ID) number. Volatile ID numbers were assigned in increasing order of retention time. Mass spectra were compared to that in National Institute of Standards and Technology (NIST) library 2008 for matches.

2.6. Polymerase chain reaction (PCR) (Papers II, III)

To identify mosquitoes' species within the *An. gambiae* species complex, a part of mosquito leg was used as DNA template and together with species specific primers, part of the intergenic spacer region of ribosomal DNA was amplified [66] (Paper II).

For bacteria community studies, extracted DNA from oviposition substrate samples were used as template to amplify the V3 region of the 16s rDNA using bacteria universal primers. Forward primer 968F (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') with GC clamp and reverse primer

1601R (5'-CGG TGT GTA CAA GAC CC-3') were used (Paper III). Agarose 1.5% gel electrophoresis was used to separate DNA fragments and gelRed was used to stain DNA bands for visualization. The size of the DNA fragments were determined by comparing the migration distance of them with the migration distance of the bands in the GelPilot 100 bp - base pair ladder.

Due to a low DNA band intensity on gels after denaturing gradient gel electrophoresis (DGGE) a PCR concentration method was developed. PCR products were purified and concentrated using a commercially available purification kit: Qiagen MinElute PCR purification kit following the protocol by the manufacturers. This was achieved by using 50 µl of PCR products from two replicate PCR reactions which was concentrated to 10 µl after purification. The DNA concentration of the eluted sample was determined using nanodrop 1000 (Thermo Scientific, Mytogen Limited, Sweden) according to the manufacturer's instructions.

2.7. Denaturing gradient gel electrophoresis (DGGE) analysis (Paper III)

Bacteria profiling of water samples was performed with DGGE. Purified and concentrated PCR products were analysed using Dcode™ Universal Mutation Detection System. The DNA fragments were separated on polyacrylamide gels using a denaturing gradient that ranged from 30% to 60% denaturant (were 100% denaturant is defined as 7 M Urea and 40% wt/v deionized formamide). Each well was loaded with 400 ng of PCR product. Electrophoresis was performed in a 1 × TAE buffer using an isothermal temperature of 60 °C for 16.5 h. After electrophoresis, the gels were stained by gentle shaking in an aqueous buffer with 0.1 M

NaCl and 3x gelRed. Stained gel was photographed using ChemiDoc XRS with UV filter fitted. Gel image analysis was with ImageLab gel analysis software.

2.7.1. DGGE band identification

Unique DGGE bands were excised and allowed to elute overnight in 50 µl nuclease free water. The eluted DNA were templates for PCR amplification using the earlier described PCR protocol. Amplified and purified PCR product was sent for Sangers sequencing (GATC, Sweden). The resulting sequence data was compared to the sequences in the National Center for Biotechnology Information (NCBI) nucleotide database using BlastN.

2.8. Physicochemical analysis (Paper III)

The physicochemical parameters were measured pre- and post-colonization. These include: pH, conductivity (µS/cm) and dissolved oxygen (ppm) of ponds water using a hand-held multimeter. Others were phosphate (mg/l), Nitrate (mg/l), carbonate hardness (mmol/l), total hardness (mg/l) and nitrite (mg/l) measured using Aquamerck® test kits. Water turbidity (NTU) and chlorophyll (µg Chl/l) content were measured using a turbidity meter and AquaPen AP100, respectively. Biochemical oxygen demand (BOD) was calculated by collecting 50 ml water samples in brown bottles and measuring the dissolved oxygen before and after storing the samples at a constant temperature of 25 °C for 5 days. BOD (ppm) was calculated as the difference between the two measurements.

2.9. Statiscal analysis (Papers I-IV)

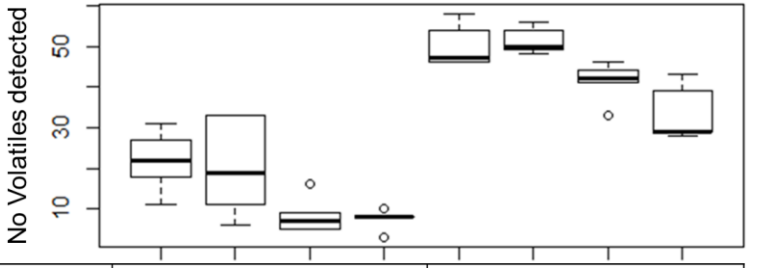
Larval count data and data on the number of volatiles detected with the volatile detection protocols were analysed using General Estimating Equation (GEE) with a negative binomial distribution fitted and round/day as repeated measure (Papers I, II). When larval count data were not repeated in the same pond generalized linear model with negative binomial distribution was used (Paper III). Mean and 95% confidence interval were derived from these models (Papers I-III). Test of significance between samples groups were with non-parametric methods using either Wilcoxon signed rank test or Kruskal-Wallis test depending on the number of test groups in IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp (Papers I, III). Principal component analysis (PCA) was used to visualize volatile, bacterial and physicochemical profile difference between sample groups. PCA were performed using Canoco multivariate statistical software version 5.02. The plots on effect of age and volatile collection methods were performed using SIMCA statistical software version 13.0.3.

3. Result

3.1. Improvement on detection of volatile compounds (Paper I)

The number of detected volatile was increased 7 times by adding salt to the infusion using thermal desorption of Tenax traps compared to liquid desorption of Porapak traps collected without salt added to the infusion (Figure 6). Overall, thermal desorption of Tenax traps significantly increased the number of volatiles detected after subtracting background volatiles compared to liquid desorption of Porapak traps ($p<0.001$).

To demonstrate the effect of the four volatile collection protocols on individual volatiles, the four most abundant volatiles from Bermuda grass infusion using Tenax trap were selected. These were volatile ID 19, 38, 53 and 83 (Figure 7). Method 2 without stirring collected relatively similar amount of the volatiles as method 3 with stirring but relatively lower for volatile ID 83. Indicating that for many but not all of the volatiles detected in this study, the stirring step can be excluded.



Desorption method	Liquid				Thermal			
Polymer type	Porapak				Tenax			
salt	Yes	Yes	No	Yes	Yes	Yes	No	Yes
stirring	No	Yes	No	No	No	Yes	No	No
Time	20 h	20 h	20 h	1 h	20 h	20 h	20 h	1 h

Figure 6. Summary of the effects of the different sampling and analysis methods on volatile detection. Thermal desorption methods collected significantly more volatiles ($p<0.001$) than liquid desorption methods.

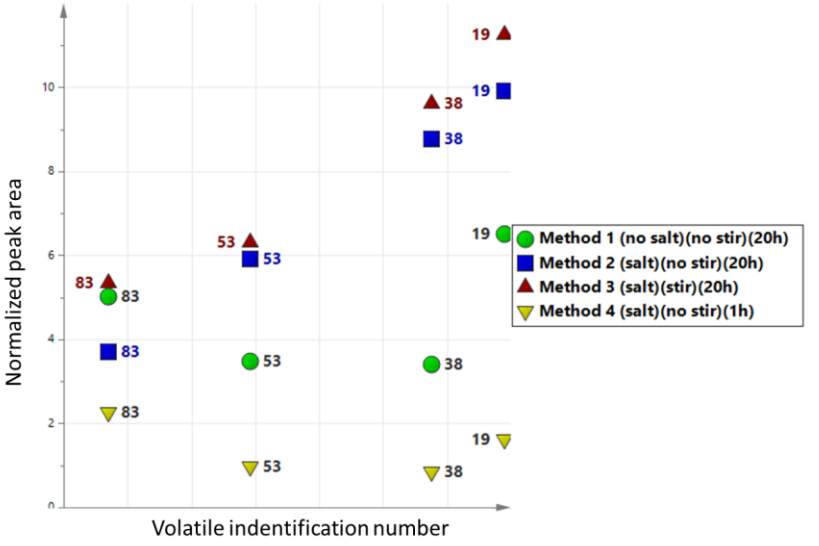


Figure 7. Comparison of normalized peak area of the four compounds (Volatile IDs: 19, 38, 53 and 83) detected in highest amount from Bermuda grass infusion using the

four protocols and thermal desorption of Tenax traps. The methods were: Method (1) volatile collection for 20 h (green circle); Method (2) as method 1 but with NaCl added to infusion (blue square); Method (3) as method 2 but with stirring of infusion (red triangle); Method (4) as method 2 but with 1 h volatile collection (yellow triangle upside down). The first compound (83) is an indole containing compound while the last (19) is an alcohol.

3.2. Similar ponds varied in larval density (Paper II)

Similar ponds set up in close proximity differed in the number of early instar *Anopheles* larvae present. To observe the colonization pattern the experiment was monitored for a total of 26-27 days (Figure 8). The preferred ponds contained twice as many early instar *Anopheles* larvae as the less preferred ponds of same age in all three rounds ($p<0.001$), Figure 9). The proportion of culicine first instar larvae were highest in the ponds during the first 6-8 days (except in round 2) and subsequently replaced by anopheline larvae (Figure 10). In all three rounds, ponds received rainfall on day 3. However in round 2, the rainfall was heavier than that of rounds 1 and 3. This may account for the different colonization pattern observed in round 2.

A total of 118 *Anopheles* mosquitoes emerged from the pupae removed from the ponds. Sixty-two of these were randomly selected for species identification using PCR and 57 out of them successfully amplified. Fifty-five (96%) were *An. arabiensis* while 2 (4%) were *An. gambiae s.s.*.

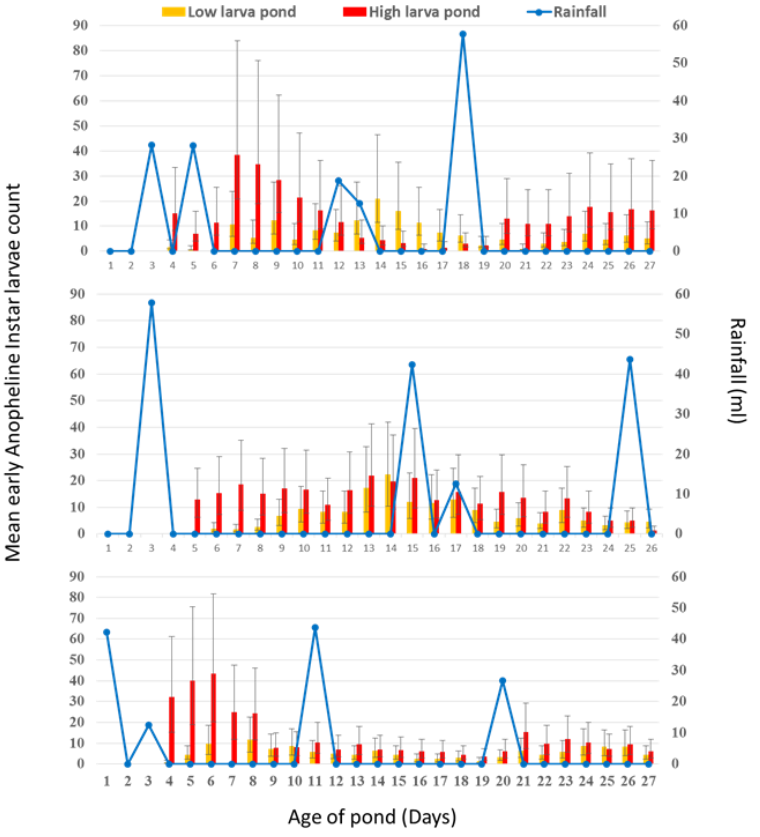


Figure 8. Similar ponds set up in close proximity to each other differed in number of early instar *Anopheles* larvae. The red bars represent the high larval density pond while the yellow bars represent low larval density pond. The error bar are at 95% confidence interval of mean.

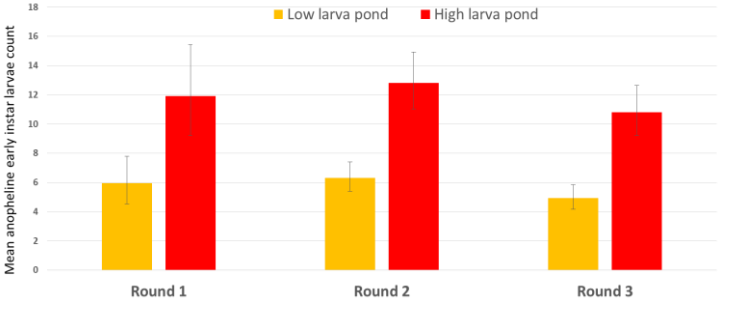


Figure 9. Colonization preferred ponds referred to as high larval density ponds (red) contained significantly more early instar *Anopheles* larvae compared to the low larval density ponds (yellow) of same age in all three rounds ($p<0.001$).

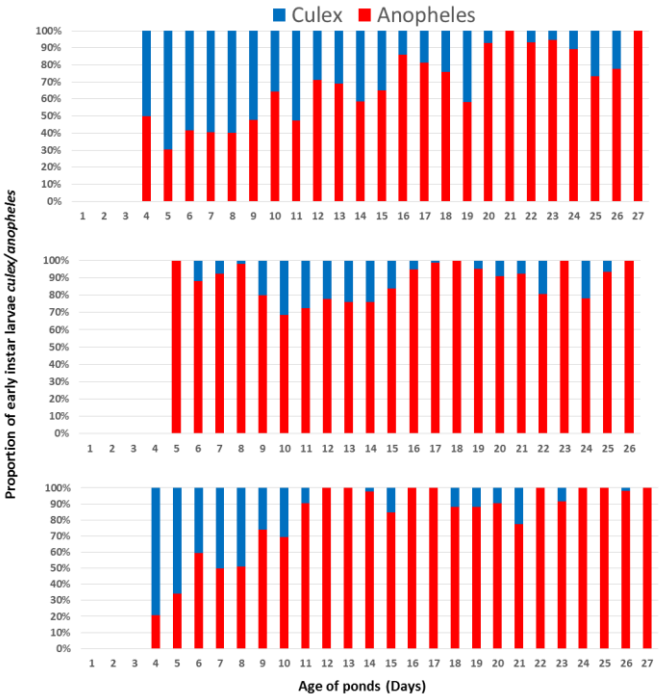


Figure 10. Proportions of early instar *Anopheles* (red) and *Culex* (blue) larvae. The proportions of early instar *Culex* larvae were highest in ponds within the first few days after colonization in round 1 and 3. The early instar *Culex* larvae were proportionately lower in round 2 than in the other two rounds.

3.2.1. Chemical profile of high and low larval density ponds

The chemical profile of high and low larval density ponds were compared in samples from day 6 after set-up of ponds. This coincides with the period of increased colonization of ponds using the number of *Anopheles* early instar larvae as a proxy. The PCA analysis (Figure 11) showed that high and low larval density ponds grouped differently based on their chemical profile. The chemical profiles varied between

rounds and ponds of the same round had a more similar volatile profile than ponds of different rounds. Some volatiles were detected in high larval density ponds and not in low larval density ponds. These include: one in round 1 (volatile ID 95), four in round 2 (volatile IDs: 7, 8, 31 and 38) and three in round 3 (volatile IDs: 41, 55, 83).

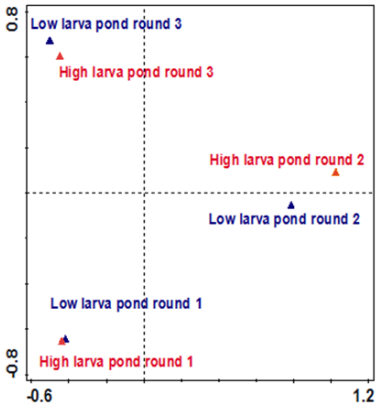


Figure 11. PCA analysis showed that high and low larval density ponds grouped differently based on their chemical profile. This is observed in rounds 2 and round 3 but not so much in round 1 with four, three and one volatiles respectively detected in difference between the two larval densities. However, the chemical profiles of the ponds were more similar within a round than between rounds.

3.3. Ponds of different age varied in *Anopheles* early instar larval densities (Paper III)

Colonization of ponds with oviposition substrates of different ages by wild mosquitoes was analysed by comparing the number of *Anopheles* early instar larvae present. Age 1 ponds contained two times more *An. gambiae s.l.* early instar larvae than age 2 and age 3 ponds opened for colonization on day 0, 4 and 17 respectively (Figure 12).

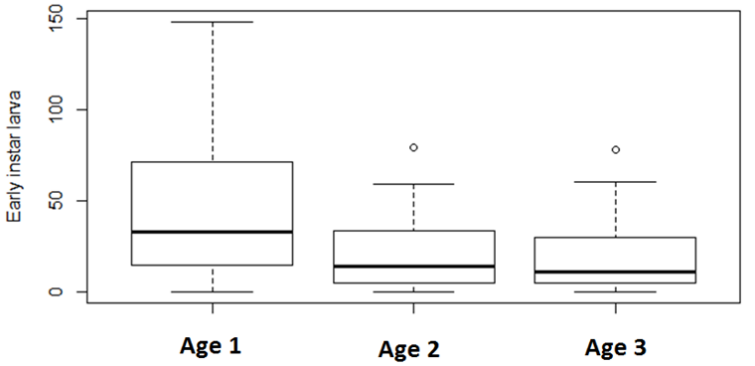


Figure 12. Ponds of different ages varied in larval densities. Age 1 ponds contained two times more *An. gambiae s.l.* early instar larvae compared to age 2 and age 3 ponds opened for colonization on day 0, 4 and 17 respectively.

3.3.1. Bacterial profiles of ponds

The bacterial profiles of the oviposition pond substrate of different ages at pre-colonization were analysed by a culture dependent method (colony forming unit/ml) and a culture independent method (DGGE). The average number of colony forming units (CFU) of age 1 pond water were two times lower than that of age 2 and similar to age 3 pond water samples.

The optimization of the DGGE-protocol allowed for detection of bacterial DNA bands present in pond water samples by increasing the DNA concentrations to a detectable 50 ng/µl. DGGE analysis of bacterial profile showed that no band was unique to any of the three age groups. However, the visual intensity of the bands varied between age groups with age 1 showing less intense bands than ages 2 and 3 (Figure 13).

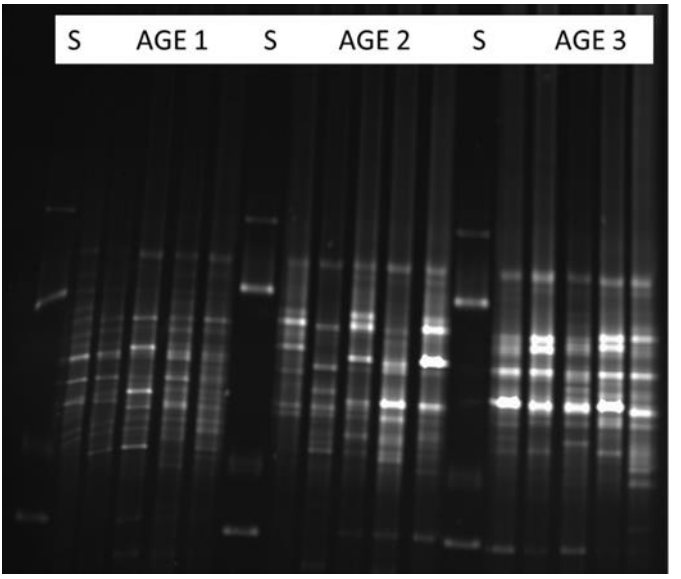


Figure 13. Denaturing gradient gel electrophoresis (DGGE) of pond substrates of ages: 1, 2 and 3. No band was unique to any of the three age groups however, the intensity of the bands varied between age groups. Age 3 had visually more intense bands compare to the other age groups. S indicate positions of the standard.

3.3.2. Ponds differed in physicochemical profile

Analysis of the physicochemical parameters showed that the oviposition preferred age 1 ponds have higher turbidity and temperature but lower pH values compared to the less preferred ages 2 and 3 ponds.

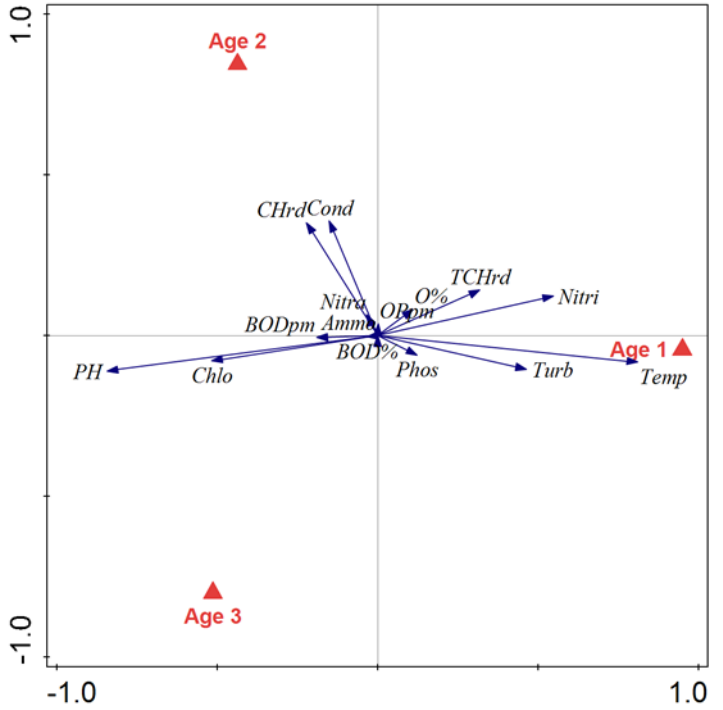


Figure 14. Principal component analysis shows the differences in physicochemical profiles of oviposition substrates of different ages. Age 1 were associated with higher temperatute and lower pH. Data included were: Cond=Conductivity (µS/cm); CHrd=Carbonate hardness (mmol/l); TCHrd=Total hardness (mg/l); Phos=Phosphate (mg/l); Turb=Turbidity (NTU); Chlo=Chlorophyll, Nitri=Nitrite (mg/l); Nitra=Nitrate (mg/l); Temp=Temperature (°C); pH; O%=Oxygen dissolved (%) and BODpm=Biochemical oxygen demand (ppm).

3.3.3. Volatile profile of ponds of different ages

Volatiles from pond water of different age groups varied in their chemical profiles. A range of volatiles were exclusively detected in the ponds that were preferred for oviposition. These include volatile identification numbers: C15, C44 and C90 (Figure 15).

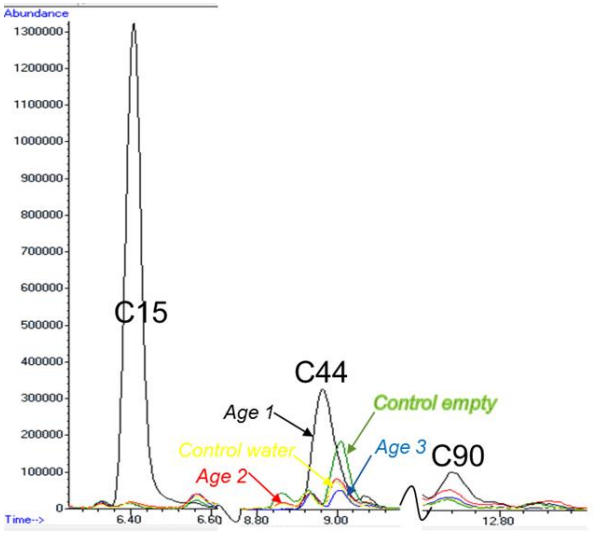


Figure 15. Chromatographic peaks of volatiles exclusively detected in the ponds that were preferred for oviposition: volatiles IDs C15, C44 and C90.

3.3.4. Analysis of pond substrates physicochemical parameters at post-colonization

To determine whether presence of larvae effect physicochemical parameters such as temperature and turbidity, the effect of age was excluded by comparing two groups of ponds of same age. One group was with larvae (post-colonization stage) while the other group was without larvae (pre-colonization stage). Turbidity and temperature was observed to increase when larvae were present compared to ponds of similar age without larvae.

3.4. Rhizome *Cyperus rotundus* grass from natural oviposition site soil (Paper IV)

Five fungi were isolated from rhizomes of *Cyperus rotundus* (Figure 16). Two of these: R2D and R6D, were isolated from the rhizome

infusion while the remaining three: DTB, DRB and DRNB were from direct rhizome cultures.

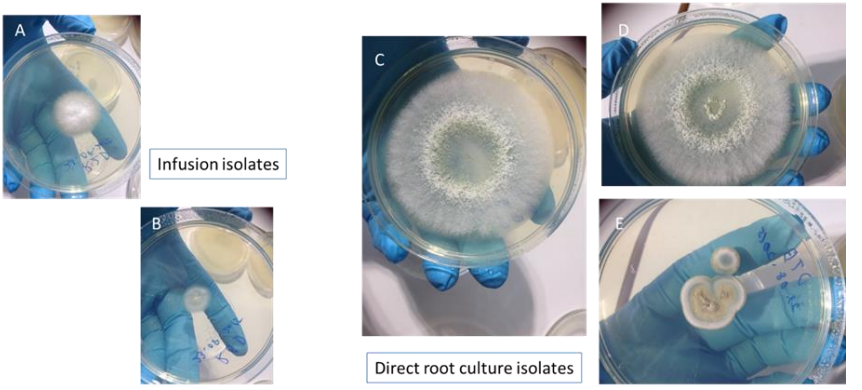


Figure 16. Five fungi isolated from rhizomes of the grass *Cyperus rotundus* grass. A= R2D; B =R6D, C = DTB, D =DRB and E = DRNB.

3.4.1. Volatiles detected from fungi headspace

The most abundant volatile detected in the fungi culture headspaces was volatile ID 11. This compound was detected in the headspace above individual cultures such as: DRB, DRNB and R6D as well as when the five fungi isolates were grown together (Figure 17).

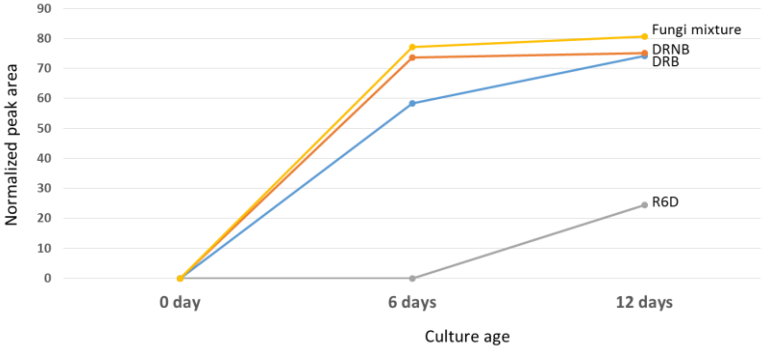


Figure 17. The normalized peak areas of volatile ID 11 detected in different fungi and fungi mixture overtime. Volatile ID 11 was the most abundant volatile in the headspace of cultures of these fungi.

Volatile ID 35, an *An. gambiae* oviposition attractant, was detected in the headspace of fungi R2D and R6D. The highest amount was detected above a 12 day old culture of R2D (Figure 18).

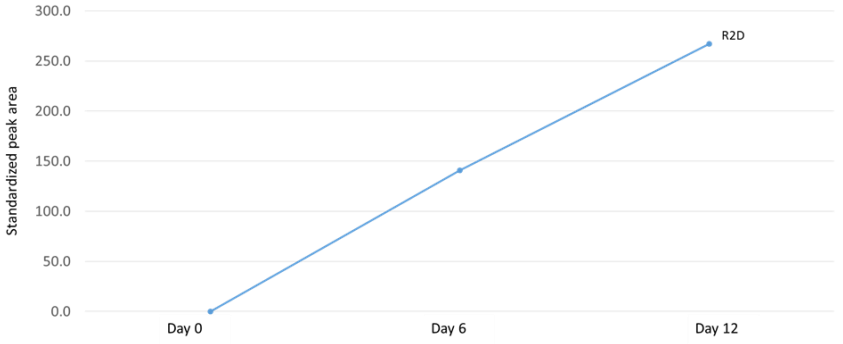


Figure 18. Volatile ID 35 was detected from the headspace of R2D. The highest amount of this volatile was detected from 12 day old culture.

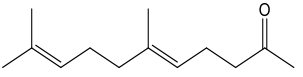
3.5. Summary of oviposition compounds

A diverse range of volatiles were detected in the oviposition studies performed in 2012 and 2013. In the 2012 study, in total 81 volatiles were detected from 18 samples. Fifty-two of these were detected in more than one sample. In total 284 volatiles were detected from 36 samples in the 2013 study. One hundred and forty-nine of these volatiles were detected in five samples or more.

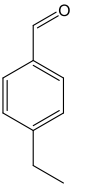
In the two open-field studies performed in 2012 and 2013, there were no volatile detected that was common to all three rounds. However, 12

volatiles were present in two rounds in 2012 of which two (6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) and 4-ethylbenzaldehyde had higher prevalence (75%) in the oviposition preferred high larval density ponds compared to low larval density ponds (Figure 19). In the 2013 study, 10 volatiles were unique to age 1 ponds which were preferred for oviposition in this study, four of these were detected in two out of the three rounds and three were detected in more than 50% of the samples from age 1 ponds.

In 2015 fungi study, 41 volatiles were detected in the headspace of individual fungal isolates and the fungal mixture including one compound previously described to influence oviposition behaviour of *An. gambiae*.



6,10-dimethyl-5,9-undecadien-2-one (geranylacetone)



4-ethylbenzaldehyde

Figure 19. Example of volatiles detected in higher prevalence in oviposition preferred ponds compared to low larval density ponds.

4.
Concluding Remarks

New vector control methods are needed to handle the increasing mosquito resistance to the currently used control measures. This thesis deals with detection of cues associated with oviposition preferred substrates by gravid female *Anopheles gambiae sensu lato* mosquitoes. Identification of these potential oviposition cues may facilitate the development of a malaria vector control strategy: an "attract and kill" strategy. This strategy could be based on attracting gravid females to traps or oviposition sites using these cues and then killing the ovipositing female or her larvae with preferably a microbial larvicide.

The detection of volatiles from oviposition substrates was improved 7 times by adding salt to the infusion and thermal desorption of Tenax traps compared to liquid desorption of Porapak traps. This improved method allowed for analysis of volatiles released in low quantities from the oviposition substrates. Furthermore, the optimization of the denaturing gradient gel electrophoresis (DGGE) method allowed for detection of bacterial DNA present in the oviposition substrates.

Although, none of the DGGE bands was uniquely detected from only oviposition selected ponds water, their intensity varied. We therefore speculate that varying their concentration may influence oviposition behaviour. This concentration effect is supported by the observed lower count of bacteria CFU and lower intensity of DGGE bands in the oviposition preferred water.

In two consecutive years of field work, factors affecting oviposition selection were detected. These include the age of the oviposition substrate, pH and turbidity.

By analysing the physicochemical profile of pond water before and after mosquito colonization, a difference in the measured values at these two colonization stages was observed. This could possibly be ascribed to the presence of larvae. It is particularly important as field studies analyse physicochemical parameters as a possible larval density marker due to ease of measurement using handheld devices. The presence of larvae and its associated by-products may explain the mixed research results in this area in other studies of the role of physicochemical parameter as marker of colonization status.

It is possible that malaria mosquitoes can use a diverse range of chemicals to select between oviposition sites. Different volatiles were detected in different rounds associated with oviposition preferred pond water. This has an implication in attract and kill strategy of malaria mosquito control where more than one compound may function as an oviposition attractant. Thus, it could be possible that a single oviposition attractant used in attract and kill strategy may have to compete with other such attractants present in nature. A blend of oviposition attractants may be a preferred option.

It is reported here a fungus producing an *An. gambiae* oviposition attractant. A fungal culture thus has the potential of serving as a producer of blends of oviposition attractants. The five fungi isolated in this study produced a range of compounds. Volatile production from fungi provides an interesting alternative for development of an “attract and kill” malaria vector control method. This is because fungi produce

not just a single but a range of compounds that could be optimized for the production of oviposition active compounds by modifying culture conditions. In combination with a biological larvicide, fungi volatiles could provide a cost effective and environmental friendly malaria vector control method of oviposition seeking malarial mosquitoes in an “attract and kill” strategy.

5.
Future prospects

1. In this study, we detected compounds associated with oviposition preferred water. It will therefore be interesting to test the effect of these compounds both separately and as a mixture in oviposition bioassays to investigate their possible effect on the oviposition behaviour of gravid female *Anopheles gambiae sensu lato* mosquitoes.
2. Physicochemical parameter such as pH was observed to be associated with oviposition. Elucidating the effect of pH on oviposition selection will be of interest. For example to investigate if pH affects the volatility of compounds by making them more or less volatile.
3. Two fungi isolated in this study were observed to produce volatiles previously documented to influence the oviposition behaviour of *An. gambiae s.s.* A nice follow-up study would be to test whether these fungal volatiles can be used as oviposition baits in gravid traps in "attract and kill" strategy.
4. A range of compounds were produced by the isolated fungi in this study; some of which are important in the industry. A study to optimize the production of these compounds could represent a cost effective production source.

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Appendix A

The following is a description of my contribution to Publications **I** to **IV**, as requested by KTH.

Paper I: I participated in the experimental design and implementation. Volatile analysis and cage bioassay were performed by myself and Mike Okal respectively with whom I also shared manuscript writing.

Paper II: I participated in the experimental design and performed data analysis and wrote the manuscript.

Paper III: I participated in the experimental design and implementation. Performed the experiment, data analysis and wrote the manuscript.

Paper IV: I participated in the experimental design and implementation. Performed fungi isolation, and part of the data collection and analysis and wrote the manuscript.