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**icipe**

*African Insect Science for Food and Health*

Analysing the role of semiochemicals in the  
oviposition substrate choices of the malaria vector  
mosquito *Anopheles gambiae sensu lato*

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(Supervisor: Dr Ulrike Fillinger)

Thesis submitted in accordance with the requirements for the degree of  
Doctor in Philosophy of the University of London

Department of Disease control - Faculty of Infectious Diseases

**London School of Hygiene and Tropical Medicine**

2015

Funded by the National Institute of Health (NIH), USA

## Dedication

To the Okals .....

I know they will never read this thesis, but I dedicate it to their hopes and ambitions, struggling against great odds that they may collectively amount to something.

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## Abstract

The search for tools that target malaria vector that resist insecticides and bite outdoors has become a research priority. Such tools will be necessary for managing residual malaria transmission and hastening the eradication of this devastating disease. This study investigated chemicals that potentially affect the oviposition substrate choices of *Anopheles gambiae sensu lato (s.l.)*. It is foreseen that increased knowledge of the oviposition behaviour of this major malaria vectors and chemicals cues that mediate oviposition site-selection can be applied in the development of additional sampling methods and alternative interventions that to trap gravid malaria mosquitoes outdoors.

To achieve a reproducible high egg-laying success of *An. gambiae sensu stricto (s.s.)* and *An. arabiensis* four factors were evaluated: (1) the time provided for mating; (2) the impact of cage size, mosquito age and female body size on insemination; (3) the peak oviposition time; and, (4) the host source of blood meals. Then four bioassays were optimised for studying oviposition responses of *An. gambiae s.s.* in the laboratory and semi-field conditions: a WHO-tube bioassay and a wind-tunnel that detected short-range attraction in the laboratory; a two-tier choice egg-count bioassay that compared the relative proportion of eggs laid in substrates in the laboratory; and a modified BG Sentinel mosquito gravid trap that evaluated long-range attraction of gravid females to olfactory cues in the semi-field. Finally, the oviposition responses of gravid *An. gambiae s.s.* mosquitoes to water vapour, Bermuda grass hay infusion (hay infusion), and putative semiochemicals identified from the hay infusion and a soil infusion previously shown to elicit higher egg deposition compared to filtered Lake Victoria water (lake water) in two choice egg-count bioassays (Herrera-Varela *et al.* 2014), were evaluated.

High oviposition rates [84%, 95% confidence interval (CI) 77-89%] were achieved when 300 male and 300 blood-fed female *An. gambiae s.s.* were held together in a cage for four days. The chance of oviposition in the mosquitoes dropped when human host source of blood-meal was substituted with a rabbit (Odds ratio (OR) 0.30, 95% CI 0.14-0.66) but egg-numbers per female were not affected. All four optimised oviposition bioassays effectively showed between 15-20% shifts in oviposition substrate choices of mosquitoes with 80% statistical power and 5% significance. Using the WHO-tube bioassay, gravid *An. gambiae s.s.* were shown to be 2.4 times (95% CI 1.3-4.7 times) more likely to move towards high humidity in still air compared to non-gravid

mosquitoes. This was more pronounced in the airflow olfactometer where the gravid mosquitoes were 10.6 times (95% CI 5.4-20.8 times) more likely to fly into a chamber with water than a dry chamber.

Two-choice egg-count bioassays showed that *An. gambiae s.s.* were less likely to lay eggs in six-day old hay infusion (OR 0.10, 95% CI 0.03-0.33) compared to lake water. Ten putative semiochemicals were identified from the hay infusion using mass spectrometry and published electrophysiology data: 4-hepten-1-ol, 4-ethylphenol, phenylmethanol, 2-phenylethanol, indole, phenol, 3-methylindole, 3-methyl-1-butanol, 4-ethylphenol, and nonanal. Tested in two-choice egg-count bioassays, the first four listed compounds had no effect on egg deposition at the tested concentrations (between 0.01-5 parts per million) but mosquitoes were less likely to lay eggs in at least one concentration of 3-methylindole (OR 0.39, 95% CI 0.21-0.71), indole (OR 0.57, 95% CI 0.37-0.87), 3-methyl-1-butanol (OR 0.32, 95% CI 0.22-0.47), phenol (OR 0.55, 0.32-0.95), 4-methylphenol (OR 0.32, 0.18-0.57) and nonanal (OR 0.66, 95% CI 0.47-0.91) compared to lake water. In contrast to the hay infusion and hay infusion volatiles, *An. gambiae s.s.* were about two times more likely to lay eggs in cedrol, a sesquiterpene alcohol identified from the soil infusion, compared to lake water (OR 1.84, 95% CI 1.16-2.91). Cedrol attracted twice as many gravid mosquitoes in the semi-field also (OR 1.92, 95% CI 1.63-2.27). In the field, modified BG-Sentinel traps, electrocuting nets and OviART gravid traps with lake water and cedrol were three times more likely to trap malaria mosquitoes compared to traps with water only (OR 3.3, 95% CI 1.4-7.9).

In conclusion, water vapour was shown to be a strong, non-specific pre-oviposition attractant for gravid *An. gambiae s.s.* in still air and moving air. It is probably the long range cue that gravid *An. gambiae s.l.* use to detect the presence aquatic habitats beyond the range of chemical cues. Evidence showed that *An. gambiae s.s.* discriminate between potential oviposition substrates and that this selective process is in-part mediated by volatile organic compounds originating from the site. Water vapour leads gravid mosquitoes to aquatic sites but semiochemicals enable the mosquitoes to discriminate and select between potential habitats. It was demonstrated that synthetic equivalents of semiochemicals found to attract gravid mosquitoes such as cedrol can be used to trap malaria mosquitoes outdoors.

## Acknowledgements

*"..... I know the plans I have for you," declares the Lord, "plans to prosper you and not to harm you, plans to give you hope and a future....." Jeremiah 29:11. New International Version*

If someone had said to me in 2011 as I went about my job as a research assistant that in four years I'd be a PhD candidate I'd have laughed it away. However, if they also told me that above that I would find a beautiful wife and have a daughter and son; that my new supportive wife Evelyn Olang-Okal (my strongest appreciation reserved for her) would during that same time start and complete her PhD while taking care of Imani Okal and Baraka Okal; that as a family we would meet many great scientists and make many great friends across all continents; I would have considered him out-right crazy. This is a crazy dream come true and I thank God for it. It has been challenging journey, one that without any of these men and women mentioned here would not have been completed.

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## Foreword

Insects constitute the vast majority of the eukaryote biodiversity on planet earth (Gaston 1991). Estimated conservatively at five million species, with many not yet described, (Mora *et al.* 2011; Costello *et al.* 2013), this taxonomic community should command significant interest and research. However, insects are largely ignored, especially in Africa, except for those species that intimately interact with humans causing nuisance or impacting health, feed and economy.

Tropical countries bear the major brunt of many infectious diseases caused by viruses, bacteria and parasites that are spread by insects. In Africa, species of the two-winged flies (Diptera) predominate as biological vectors for such pathogens. These include the sandflies (Psychodidae) that spread cutaneous, mucocutaneous and visceral leishmaniasis (Killick-Kendrick 1990; Killick-Kendrick 1999; Sharma & Singh 2008; Ready 2013); the tsetse flies (Glossinidae) that transmit the African trypanosomiasis (Brun *et al.* 2010; Malvy & Chappuis 2011); and the blackflies (Simuliidae) that are responsible for infection with onchocerciasis and mansonellosis (McCall *et al.* 1998). However, mosquitoes (Culicidae) are far more important insect vectors of human diseases in Africa and beyond.

Mosquitoes spread the largest diversity of diseases including Malaria, lymphatic filariasis, dengue, rift valley fever, yellow fever, chikungunya, encephalitis, and west Nile fever to over 700 million people annually (Caraballo & King 2014). Compared to all these diseases, Malaria caused by a single-celled protozoan of the genus *Plasmodium* and vectored by the *Anopheles* mosquito is the most widespread and imposes the greatest toll and burden on human life. This thesis focuses on the oviposition substrate choices of the most prolific vector for malaria; *Anopheles gambiae sensu stricto (s.s.)*. By exploring and describing chemical factors that determine their oviposition sites choices, it is hoped that this combination of researches will promote a better understanding of chemical factors that determine the spatial distribution of the species and provide for new ways of monitoring and controlling malaria transmission in Africa.

# Chapter 1. General introduction

## 1.1. Malaria

Malaria is a mosquito-borne disease with an incomparable claim on human life and suffering (Witty 2004; Warburg *et al.* 2011). It imposes an insurmountable health, economic and social burden on human population across the tropics and subtropics, spreading throughout Africa into Asia and the Americas. Malaria is endemic in nearly 100 countries worldwide and is a risk for the rest the world through travel. In 2014, the World Health Organisation (WHO) estimated that an average of 198 million people suffered from malaria with about 584,000 deaths (WHO 2014). The vast majority of the dead (90%) were residents of sub-Saharan Africa, most (78%) children below the age of five (WHO 2013).

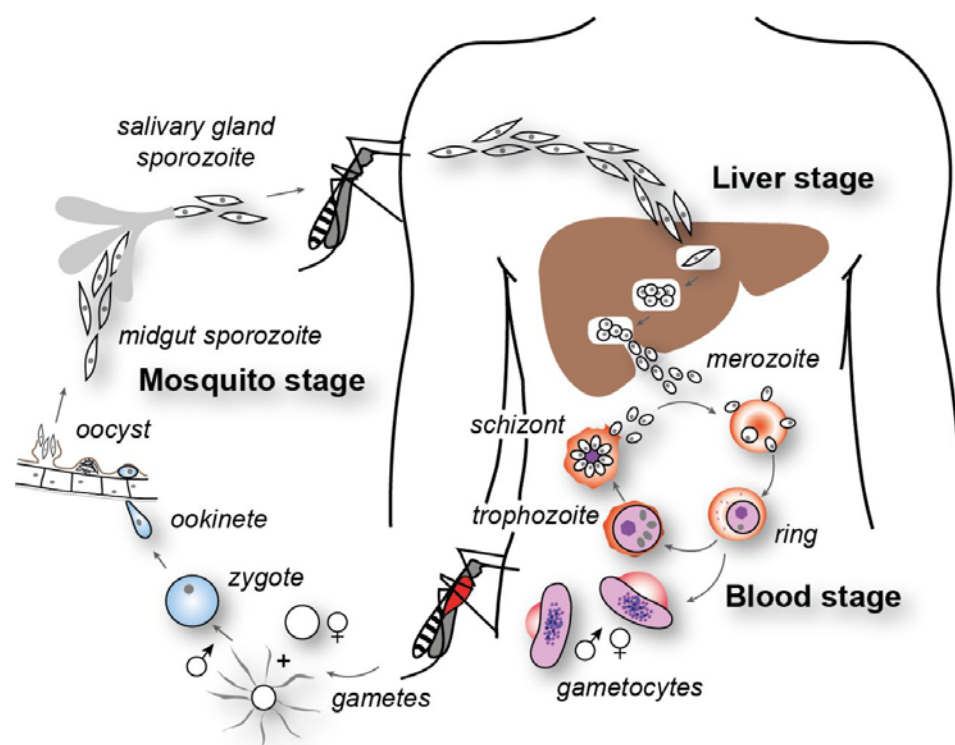
### 1.1.1. The biology of malaria transmission

Malaria is comprised of a group of closely related illnesses caused by multiple species of protozoan parasite *Plasmodium* (Haemosporida: Plasmodiidae). At least five pathogenic species have so far been described for human malaria: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Kantele & Jokiranta 2011; White *et al.* 2014). Most malaria cases are due to infection with *P. falciparum* or the less virulent *P. vivax*. *Plasmodium knowlesi* is largely restricted to Southeast Asia and exhibits a unique zoonotic cycle through the macaque monkey (Kantele & Jokiranta 2011).

Malarial parasites are spread by female *Anopheles* (Diptera: Culicidae) mosquitoes when they forage for vertebrate blood protein that they require to develop eggs (Beier 1998). At least forty-one species of *Anopheles* have been incriminated as vectors of human malaria (Harbach 2013). The most competent of these vectors are found in Africa and belong to the *Anopheles gambiae* and the *Anopheles funestus* species complexes (Edmondson 1959; Gillies & Coetzee 1987). Each of these species complexes comprises nine distinct species unique in behaviour, blood-meal host preferences, larval habitat requirements and vectorial capacity for malaria transmission. An understanding of these distinctive characteristics underpins the approach to vector control (Gillies & De Meillon 1968; Gillies & Coetzee 1987). *Anopheles gambiae* s.s. (Giles) and *An. arabiensis* (Patton) of the *An. gambiae* complex and *An. funestus* s.s. of

the *An. funestus* complex are the most effective vectors for malaria worldwide. This is because *An. gambiae s.s.* and *An. funestus* prefer human blood-meal hosts over any alternative host and together with *An. arabiensis* have a strong affinity to the peridomestic environment. They can also adapt to a wide range of environments and live relatively long and easily proliferate in the tropical climate (Gillies & De Meillon 1968; Gillies & Coetzee 1987; Sinka *et al.* 2012).

*Plasmodium* exhibit a complex life cycle including two or more hosts; a vertebrate host (human or macaque monkey) and an invertebrate host (*Anopheles* mosquito).



**Figure 1.1** Life cycle of the malaria-causing parasite *Plasmodium falciparum*  
source: (Cowman *et al.* 2012)

The sporogonic cycle commences when the female *Anopheles* mosquito bites an infected human. Gametocyte blood-stage malaria parasites are picked up with the red blood cells. The gametocytes differentiate into micro- and macro-gametes within the gut which fuse to form a diploid zygote. The zygote develops into a motile ookinete that actively burrows into the midgut wall and forms an oocyst which grows between the basal lamina and the epithelium. The oocyst undergoes sporogony through repeated



nuclear division to form tens of thousands of active haploid sporozoite-stage parasites. The sporozoites burst the oocyst and migrate to the haemocoel and salivary glands of the mosquito (Beier 1998; Roberts *et al.* 2008).

The development of the parasite in the mosquito lasts between 10 – 18 days depending on many factors including external temperatures (Beier 1998). This time period could be punctuated by more than one oviposition cycle when the mosquito becomes gravid and searches for suitable oviposition sites. Targeting the gravid mosquito at this time could prevent subsequent infective bites and transmission of the disease or help in monitoring the disease vectors.

Malaria is transmitted when humans are bitten by mosquitoes that survive beyond the sexual sporogonic cycle of plasmodium (Warrell *et al.* 2002) or more rarely through transfusion with infected blood. Sporozoites are introduced into the bloodstream when the mosquitoes inject salivary fluid to prevent the blood from clotting as it blood-feeds (Figure 1.1.). The sporozoites are transported with the blood to the liver cells (Hepatocytes) which they penetrate and develop into exo-erythrocytic schizonts. The cells rupture in 6 – 15 days releasing merozoites that invade the red blood cells (Erythrocytes) and multiply rapidly through schizogony to re-infect new red blood cells. This stage is characterised by the manifestation of malaria symptoms. As the infection progresses, some merozoites leave asexual multiplication and differentiate into sexual stages of the parasite, the gametocytes. The gametocytes are again picked up by mosquitoes that bite the host. Thus the transmission cycle is continued. (Beier 1998; Warrell *et al.* 2002; Roberts *et al.* 2008).

## 1.1.2. Malaria vector control and surveillance

### *1.1.1.1. The history of malaria control highlights the dangers of overdependence on few interventions and illustrates the importance of vector ecology studies*

There is evidence that humans had recognized ‘malarious areas’ in prehistoric times and learned to avoid such risky areas; this appears to be the earliest attempts to control the spread of malaria (Najera 2001). However, at the turn of the nineteenth century discoveries of the malaria parasite in the human blood by Alphonse Laveran and the role of the mosquito in its transmission by Sir Ronald Ross marked the beginning of systematic attempts to control vectors of malaria in many households, countries and

regions with the intention of eliminating and eventually eradicating the disease altogether (Nájera 2001).

A little over a decade later, Malcolm Watson noticed that not all *Anopheles* mosquitoes in lowland Malaysia carried and transmitted the malarial parasites. He showed that the chief vector for malaria in Malaysia was *An. umbrosus* and went ahead to demonstrate that by eliminating stagnant water pools that were characteristic breeding sites of this species it was possible to reduce the incidence of malaria. This provided early evidence that vector control was effective against malaria. It also highlighted the importance of understanding the ecology of incriminated malaria vectors as a prerequisite for controlling the disease (Watson 1913).

The strategy of attacking the main malaria vectors guided by a detailed understanding of anopheline ecology was later developed by Swellengrebel and termed “species sanitation” (Kawada *et al.* 2012). He and others believed this to be the gold standard approach; the only way to effectively control vectors (Bradley 1994). They later discovered that the principle of species sanitation was not universally applicable and that the successes witnessed by Watson in Malaysia would not be repeatable in North Holland. There was a need to explore additional approaches to vector control. Integrated vector management approaches were thereafter commonplace with many early malaria control programmes aiming to drain swamps, treat stagnant water bodies with oil or Paris Green, screen houses or spray pyrethrum extracts (Nájera 2001; Pinault & Hunter 2012). However, all these early successes and tools were forgotten with the arrival of Dichloro-Diphenyl-Trichloroethane (DDT) during the Second World War and so was the research into the various malaria vectors’ ecology (Litsios 1996).

The discovery that DDT could be used to control malaria vectors led to renewed efforts to eradicate malaria. DDT provided long lasting protection by killing mosquitoes resting on the walls of houses for more than six months after it was sprayed (Litsios 1996). This was a great improvement over insecticidal extracts that required frequent reapplication. It greatly reduced the effort and costs required for vector control interventions improving the sustainability of programmes. The insecticide DDT also helped extend vector control to rural areas. Interest and funding in vector ecology waned. Most vector control experts believed erroneously that they could finally eradicate malaria only using DDT (Nájera *et al.* 2011).

In 1955, the World Health Organisation (WHO) launched a Global Malaria Eradication plan that relied heavily on spraying using DDT and using Chloroquine for clinical case management (WHO 1957) with extraordinary successes. In temperate countries especially those that also had a stable economy, malaria transmission was reduced to zero and eliminated. However, this global effort excluded many tropical countries and failed in most that implemented it. In India and Sri Lanka, malaria transmission continued right after the intervention was stopped (WHO 1959). Drug resistance in man and insecticide resistance in mosquitoes began to emerge in some areas; coupled with lack of community participation these challenges made elimination using DDT impossible in many areas (WHO 1978). The goal of eradication was abandoned in 1977 and a more subtle approach of control favoured ever since. Once again, it was acknowledged that it was not possible to effectively control malaria using single approaches and that a profound knowledge of malaria epidemiology and vector ecology must precede elimination of the disease (Sharma 2012).

#### *1.1.1.2. Vector control in sub-Saharan Africa now hinges on targeting malaria mosquitoes in the indoor environment*

Following the failed attempt to eradicate malaria, global efforts to manage the vectors of the disease were abandoned. Research instead focussed primarily on finding a vaccine for malaria or developing effective drugs to treat the disease.

The Roll Back Malaria (RBM) initiative of WHO was launched in 1998 with the aim of reducing malaria levels to 50% by the year 2000 and by 75% fifteen years later (WHO 1999). RBM advocates for prompt diagnosis and treatment of malaria with artemisinin combination therapies (ACT's) and vector control using long-lasting insecticidal Nets (LLINs) and indoor residual spraying (IRS). These vector control tools are hinged on the ecology of primary malaria vectors in Africa which have a high affinity for the indoor and peridomestic environment and have been very successful with malaria control. The burden of malaria has been reduced in many tropical and sub-tropical countries by many orders of magnitude due to these (Lengeler 2004; Pluess *et al.* 2010; WHO 2014) inviting prospects for eliminating the diseases. Unfortunately, progress towards elimination of the disease has been slow. Countries that have the highest burden of malaria have made the least progress implementing the frontline interventions

(WHO 2014). Importantly, Malaria transmission has been sustained by mosquitoes that bite outdoors as well as those resistant to the small class of insecticides used with LLINs and IRS (Enayati & Hemingway 2010; Govella *et al.* 2013; Killeen 2014). Unless new complementary strategies are developed and added to the vector control strategies, malaria transmission will continue in Africa (Killeen 2014).

*1.1.1.3. Need for more interventions, especially those that target malaria vectors outdoors and at different behavioural stages*

Vector control is a critical and pivotal component of malaria control today. Two interventions, long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) have been used extensively to prevent deaths of hundreds of thousands due to Malaria in the last decade especially in Africa (WHO 2014). These two strategies use insecticides to target mosquitoes in the indoor environment. Unfortunately, these leave mosquitoes that resist the insecticides and those that bite outdoors unchallenged and spreading malaria (Killeen 2014).

All active ingredients recommended by WHO for safe use with IRS interventions fall in four classes of insecticides: pyrethroids, organophosphates, organochlorides and carbamates. Of these only pyrethroids are so far approved for use with LLINs (WHO 2006b). Unfortunately insecticide resistance is now widespread affecting nearly two thirds of countries with ongoing malaria transmission and reported in all major vector species to all four classes of insecticides permitted for use in public health (WHO 2006b). Widespread use of insecticide-based strategies within the last ten years has intensified selection pressure on resistance genes in mosquitoes. As a results, insecticide resistance has now been reported in about 64 countries. Importantly, resistance to pyrethroids - the safest effective insecticides for public health use and only group of permitted for use with LLINs - is the most widespread (Ranson *et al.* 2011).

Resistance management is very complex because there are several mechanisms of insecticide resistance, the most common being metabolic resistance where there is an increased detoxification of the active ingredient following contact and target-site resistance where structural genes of the central nervous system mutate and confer decreased sensitivity of the target site proteins (Ranson *et al.* 2011; Liu 2015). The

WHO therefore recommends four approaches to insecticide resistance management: (a) rotation of insecticides having different modes of action, (b) use of combinations of insecticides with different modes of action, (c) mosaic spraying of different insecticides in different geographical areas and (d) use of mixtures of insecticide with different modes of action (WHO 2012). However, since only pyrethroids can be used on LLINs it is impossible to manage resistance with this tool that is presently more focal to operational malaria control programs compared to blanket spraying of household interiors. Resistance management is further complicated by the development of cross-resistance and multiple mechanism resistance that limit the use of alternative insecticides (Ranson *et al.* 2009; Liu 2015).

Moreover, vector populations that bite outdoors including *An. arabiensis* and other vectors that play a secondary role in malaria transmission including *An. rivulorum* and *An. coustani* (Tirados *et al.* 2006; Bayoh *et al.* 2010). Such vector populations are little affected by current frontline interventions (Kawada *et al.* 2012; Mwangangi *et al.* 2013a; Mwangangi *et al.* 2013b) and maintain residual malaria transmission in many areas (Killeen 2014). Malaria vectors also show considerable plasticity in their behaviour in response to vector control measures. Cryptic exophagic and diurnal subgroups of vector species historically known to be strongly endophilic and nocturnal have been reported in areas with intensive vector control (Riehle *et al.* 2011; Sougoufara *et al.* 2014). Also, there is an increase in the relative proportion of mosquitoes that feed outdoor and in the early evening before people retreat to protected locations (Reddy *et al.* 2007; Ferguson *et al.* 2010; Govella *et al.* 2010; Riehle *et al.* 2011). New interventions that deal with the exophilic and exophagic vectors are required.

Eliminating malaria will take the addition of new tools that can target mosquitoes at different stages in their life cycle and beyond the peridomestic environment (Figure 1.2). Extensive ecological research must precede the development of such tools to understand how malaria mosquitoes forage for oviposition sites, mates, sugar meal and resting sites (Ferguson *et al.* 2010). A series of potentially targetable behaviours and alternative tools are highlighted by Killeen (Killeen 2014) and Ferguson (Ferguson *et al.* 2010). Infochemicals for these mosquito habits if found could be used as baits to improve larval source management, create toxic sugar baits or develop repellents.



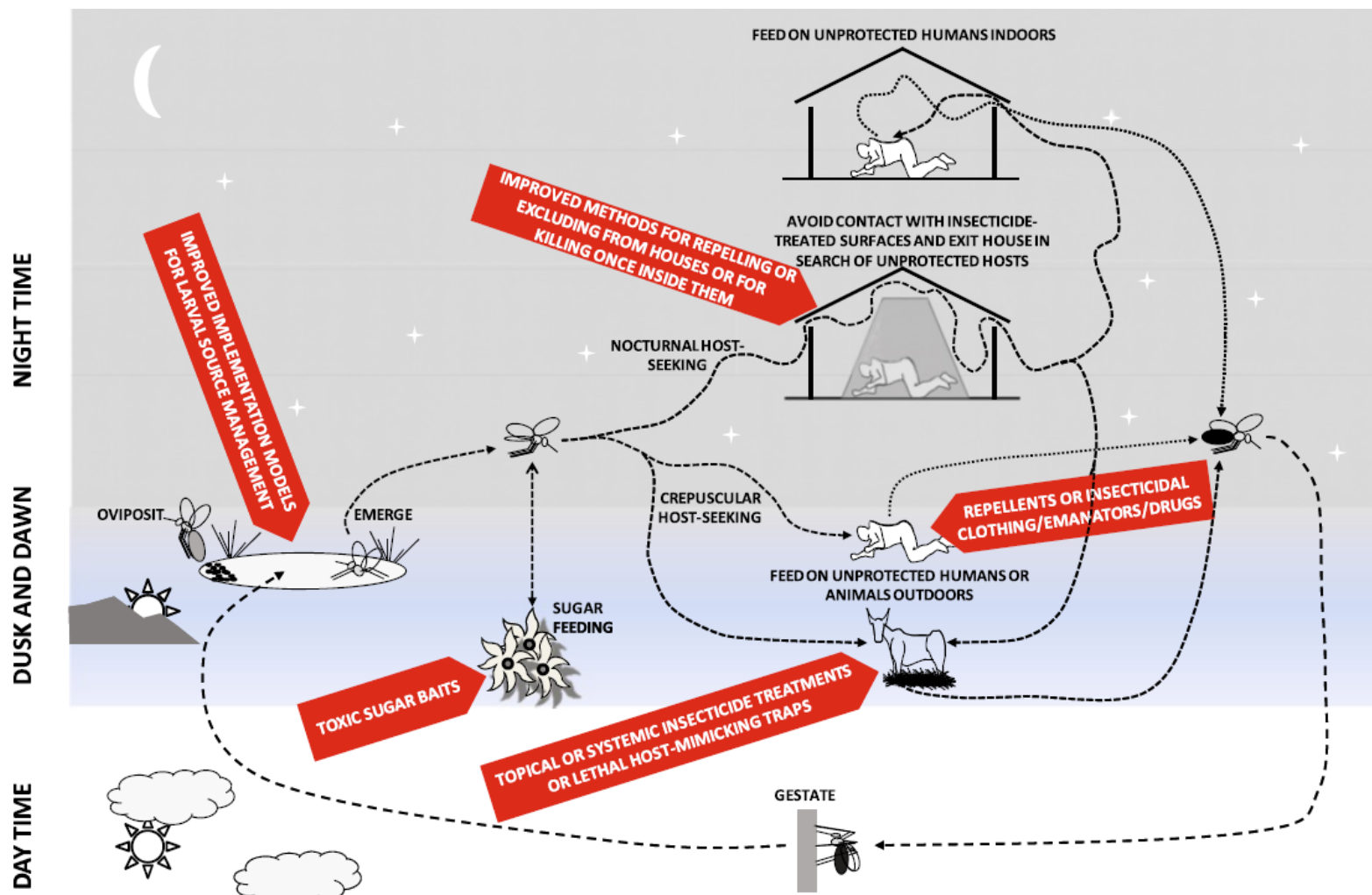


Figure 1.2 Life cycle of malaria vectors showing different behaviours that enable them to avoid conventional strategies and how these might be targeted with new interventions (Source: (Killeen 2014))

## 1.2. Semiochemicals in insects

Semiochemicals are defined as all chemicals that mediate specific responses altering the behaviour and/or physiology of recipient organism (Dethier *et al.* 1960; Nordlund *et al.* 1981; Dicke & Sabelis 1988; Prasad & Daniel 1988). These comprise toxins, all infochemicals (defined as chemicals that convey information (Dicke & Sabelis 1988)) and nutrients (Nordlund *et al.* 1981; Dicke & Sabelis 1988). Infochemicals include as pheromones, allelochemicals and apneumones (Navarro-Silva *et al.* 2009). Pheromones and allelochemicals are emitted by living organisms and mediate intra and inter specific responses respectively (Brown *et al.* 1970; Navarro-Silva *et al.* 2009). Apneumones are produced by lifeless matter such as carrion and instigate beneficial behaviour in receiving organism. Allelochemicals include kairomones that benefit only the receiver at the expense of the emitter, allomones that act vice versa, synomones that are mutualistic, and antimones that repel both emitting and receiving individual (Dicke & Sabelis 1988; Navarro-Silva *et al.* 2009). This study focuses on infochemicals.

Identified chemicals are here classified according to their effect on gravid mosquitoes. Compounds that cause active movement of gravid mosquitoes towards oviposition substrate five or more meters away (long range) without contact are termed as *attractants*. *Repellents* refers to chemicals that trigger substrate avoidance long range and without contact. In order to mediate responses from a distance attractants and repellents as described will be highly volatile (Dethier *et al.* 1960; Isoe *et al.* 1995b). In contrast with attractant and repellents, the terms *stimulants* and *deterrents* here refer to relatively less volatile compounds that mediate egg-laying only when the insect lands on substrates and makes physical contact. *Stimulants* encourage egg laying while *deterrents* inhibit the behaviour. *Attractants* and *repellents* are especially important in developing vector control strategies (Michaelakis *et al.* 2007). Attractants could be used with traps to mass collect mosquitoes. Repellents can be used to push mosquitoes away from potential substrates (Xue *et al.* 2001) thereby increasing their foraging time and reducing their success with oviposition (Gu *et al.* 2006). It is important to therefore to design experiments to distinguish these behaviours in order to identify these classes of semiochemicals (Osgood & Kempster 1971).

Insects use visual, physical and chemical cues to navigate while searching for mates, sugar meals, breeding grounds and hosts (Takken & Knols 1999). Of these cues, chemical cues are particularly important, especially in insects that have specific host



requirements and are either crepuscular or nocturnal like mosquitoes (Takken & Knols 1999; Zwiebel & Takken 2004).

Mosquitoes, like all insects have a highly developed chemosensory system. These consist of numerous sensillae primarily located on the antennae and maxillae (McIver 1982). Sensilla are porous and house many multi-branched dendrites in lymph containing odour-binding protein (OBP's). Stimuli enter through a sensillum and are conveyed through a rich array of tubules to dendrites of the olfactory receptor neurons (ORNs) by OBPs. The ORNs then transmit the signal through the central nervous system (CNS) leading to a modification in behaviour or physiology of the insect (McIver 1982; Zhou *et al.* 2004; Logan & Birkett 2007). Mosquitoes can be highly specific and sensitive to a narrow range of chemical stimuli or have a more general sensitivity to chemical compounds (Carey *et al.* 2010). Functional properties of the ORNs are conferred through a single olfactory receptor gene (*Or* Gene) (Su *et al.* 2009), 79 of which have been bioinformatically identified in *An. gambiae s.s.* (Fox *et al.* 2001; Hill *et al.* 2002).

### 1.3. Life History of the African malaria mosquitoes and the opportunity for using semiochemicals to develop new diseases control strategies

Mosquitoes go through four distinct stages in their life cycle (complete metamorphosis): Egg, larva, pupa and adult. Adult female mosquitoes lay egg batches in water or on moist surfaces likely to flood with minimal precipitation (Clements 1992). The eggs hatch into larvae within 48 -72 hours in optimal conditions of temperature but could take up to three weeks in colder climates (Bayoh & Lindsay 2004). Mosquito larvae are filter feeders that eat bacteria, algal material and other organic debris within their aquatic environment (Clements 1992). Larvae molt through four instar stages eventually metamorphosing into non-feeding pupae. Survival of the immature aquatic stages is influenced by many factors including water quality, abundance and diversity of phytoplankton as well as predation and competition by other aquatic insects (Clements 1992). The entire aquatic stage takes between 10-14 days in tropical conditions but could be longer in temperate climate (Clements 1992). After this period, the pupae split at the cephalothorax and adult mosquitoes with three main body sections emerge: A head specialised for collecting sensory information and feeding, a thorax dedicated to

locomotion and a flexible abdomen that carries sugar meals, blood meals and eggs. Adult mosquitoes unlike their larvae and pupae, are terrestrial.

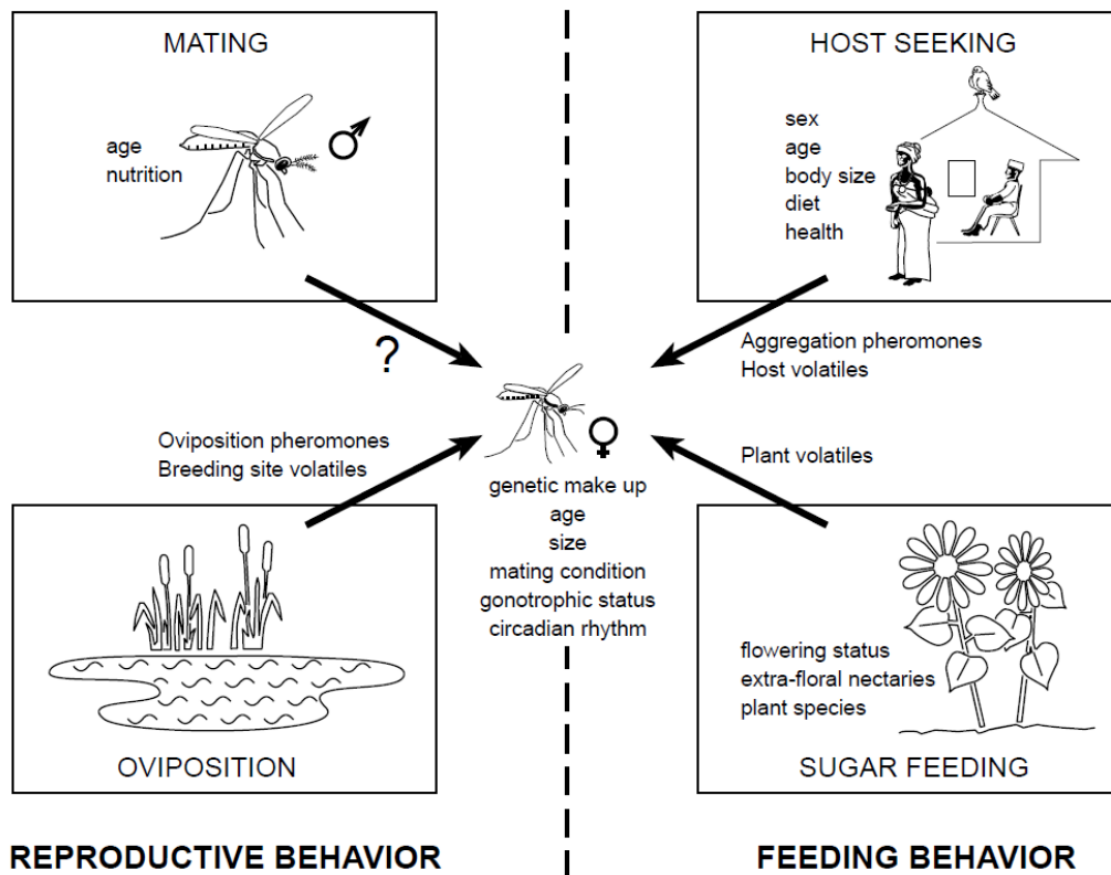
The survival and reproductive success of the adult mosquito will depend on its ability to forage for sugar and blood meals, and to mate and oviposit in appropriate breeding sites. These behaviours are believed to be genetically determined but are governed by internal and external stimuli (Takken & Knols 1999). The newly emerged mosquito begins a random flight once the threshold value of declining sugar is reached and eventually encounters external plant cues that mediate the location and selection of appropriate sugar meal plants (Nyasembe *et al.* 2014). Specificity of the choice plant sugar meal sources will depend on availability and on the internal state of the insect. For instance, a mosquito in an environment with few host plants will not be as discriminative of sugar meal sources as one in an environment with a high abundance of these (Knols & Takken 1997). Male mosquitoes do not require blood and therefore will use plant sugar as their sole source of sugar throughout their life-time.

While female mosquitoes are usually physiologically ready for insemination, male mosquitoes have to undergo physical changes in external genitalia first. There is evidence that it takes up to 48 hours for male mosquito genitalia to rotate to positions that allow mating (Provost *et al.* 1961; Jones & Gubbins 1978). Male mosquitoes use several strategies to locate conspecific females (Gjullin *et al.* 1960; Downes 1969; Nijhout & Graig 1971). Males of some species of *Mansonia* and *Aedes* have been reported to respond to host odours increasing their chances of intercepting host-seeking females (Jaenson 1985). Males of some *Culex* and *Aedes* species have also been shown to use sex pheromones to attract females (Gjullin *et al.* 1960; Nijhout & Graig 1971). In most species though including *Anopheles*, *Culex* and *Ochleroratus* however, mating is associated with swarming: an early scotophase pre-nuptial males-only dance into which female mosquitoes dive and exit in-copula with a male (Harper 1943; Downes 1969; Charlwood & Jones 1979). Female mosquitoes that fly into swarms are identified by their wing beat frequencies (Charlwood & Jones 1979; Ikeshoji *et al.* 1985; Kerdpibule *et al.* 1989). Swarms are associated with visual markers and the onset of the dark phase (Downes 1969; Marchand 1985; Yuval *et al.* 1993; Charlwood *et al.* 2002). Such swarms are often associated with visual markers such as landmarks (Marchand 1985).

It is controversial whether female mosquitoes will look for blood meal hosts only after inseminations. For *An. gambiae s.l.*, evidence suggests plasticity in this behaviour with

studies showing mosquitoes to blood feed both before (Charlwood et al 2003) and after blood meals (Lyimo and Takken 2003) probably depending on the availability of appropriate blood meal hosts. Females of the main African malaria vector species, *An. gambiae s.s.* and *An. funestus* show a high degree of anthropophagy and endophily in many areas (Service 1993). Females engage in an appetitive upwind flight behaviour caused by internal factors. Upon coming into contact with vertebrate host kairomones well before the visual range (20 – 70 meters) they begin a directional flight towards human dwelling. Within 20 meters to homesteads the concentrations of carbon dioxide above background levels become additionally important for attraction. The mosquitoes enter the houses through any available inlet and initiate a landing response on the host mediated by physical cues like heat and moisture in addition to visual and olfactory cues (Knols & Takken 1997). For mosquitoes that spread malaria, this host seeking behaviour is mainly crepuscular and nocturnal and finds human and other vertebrate hosts immobile. The mosquitoes then retreat to dark corners of the house or leave the house altogether to rest and gestate in moist environments for between 48 – 72 hours before seeking suitable oviposition sites. Importantly, blood meal and consequent physiological changes have been shown to cause changes in olfactory reception and behavioural responsiveness in female *Ae. aegypti* (Klowden et al. 1987; Siju et al. 2010) and *An. gambiae s.s.* mosquitoes (Fox et al. 2001; Takken et al. 2001; Qiu et al. 2006). Takken and others (2001) reported behavioural irresponsiveness by female *An. gambiae s.s.* mosquitoes after blood meals and showed that blood fed mosquitoes did not respond to host odours for up to 72 hours. Fox and others (2001) demonstrated the down-regulation of odourant receptors consequent to blood meals suggesting strongly that the post blood meal unresponsiveness of female mosquitoes to host odours was a result of the suppression of ORN's. With more comprehensive electro-antennography, Qiu and others (Qiu et al. 2006) later showed that while there was a down-regulation of some classes of ORN's after blood meals, there was also a marked up-regulation for the detection of some chemicals like indoles, phenols, ketones, and carboxylic acids and it was suggested that these compounds might be semiochemicals for oviposition. Taken together, these findings suggest that changes in olfactory perception and reception of *An. gambiae s.s.* may enable her locate suitable egg-laying sites and highlight the probable involvement of semiochemicals in the oviposition site selection process. These studies highlight the need to do electrophysiological studies at the time the female mosquitoes would seek an oviposition sites in nature to correctly capture olfactory responses

equivalent with that of the gravid mosquito at the time of oviposition site search and selection.



**Figure 1.3** Life history of the female mosquitoes and the probable role of semiochemicals in different behaviours

#### 1.4. Oviposition behaviour of mosquitoes

The heterogeneous distribution of mosquito immature stages in natural aquatic sites is attributable to a combination of factors including the oviposition site choices of gravid mosquitoes, survival of immature stages and the availability of suitable oviposition sites. Like many hematophagous insects, mosquitoes breed in aquatic sites often far away from their vertebrate hosts (McCall 2008). Though many studies have suggested that natural chemical cues attract gravid mosquitoes to suitable substrates in these remote sites few have systematically described these, especially for malaria mosquitoes.

Oviposition substrate (site) selection in mosquitoes is thought to be driven by visual, physical, chemical and biological characteristics of the substrate. It is thought that

physical cues such as water vapour, and visual cues act long-range to attract mosquitoes to water bodies. Near water bodies, volatile organic chemicals emanating from the potential oviposition sites influence short-range discrimination of sites (Bentley & Day 1989).

A few physical and visual cues have been shown to impact the oviposition substrate choices of mosquito species including *An. gambiae s.s.*, and *An. arabiensis*. Early laboratory studies demonstrated that *An. gambiae s.s.*, *An. atroparvus* and *An. arabiensis* all preferred to lay eggs in dark compared to pale substrates (Bates 1940; McCrae 1984). The evidence also showed that this preference was heightened when such substrates were presented against contrasting floors (McCrae 1984). Huang and colleagues later showed that visual contrast, unlike texture (Huang *et al.* 2005) or wavelength (Huang *et al.* 2007), was indeed oviposition site selection cues for *An. gambiae s.l.*. However, Balestrino and others later illustrated possible variation within the *An. gambiae* complex showing that the sympatric species *An. arabiensis* preferred humid substrate over free standing water and cups with rough walls over smooth cups (Balestrino *et al.* 2010). Beehlar and others (1992) found that *Aedes triseriatus* preferred to oviposit eggs in darker dyed oviposition site water. Collins and Blackwell (Collins & Blackwell 2000) reported that *Toxorhynchites moctezuma* and *Tx.amboinensis* preferred to oviposit on black rather than red, yellow, green or blue containers.

Studies have also indicated microbial involvement in mosquito oviposition site selection by demonstrating that microbial volatiles attract and stimulate gravid mosquitoes of (Hazard *et al.* 1967; Ikeshoji *et al.* 1975; Hasselschwert & Rockett 1988; Pavlovich & Rockett 2000; Poonam *et al.* 2002; Trexler *et al.* 2003b; Sumba *et al.* 2004a; Otienoburu *et al.* 2007; Lindh *et al.* 2008). Hazard and other (1967) found that bacterial volatiles were the primary reason hay infusions used in oviposition traps were effective baits for gravid *Cx. quinquefasciatus* and *Ae. (Stegomyia) aegypti* mosquitoes. Few studies have investigated this for anopheline mosquitoes (Sumba *et al.* 2004a; Huang *et al.* 2006a; Otienoburu *et al.* 2007; Lindh *et al.* 2008; Herrera-Varela *et al.* 2014) with contradicting results: some suggest microbial volatiles could be repellent (Huang *et al.* 2006a), others report these are attractant to gravid mosquitoes (Sumba *et al.* 2004a).

So far, direct evidence of oviposition site selection using semiochemicals is only available for a few mosquito species. Potential chemical cues for oviposition from

natural breeding sites include pheromones, metabolites of bacteria and algae and volatiles from predators and competitors (Blaustein *et al.* 2005; Ponnusamy *et al.* 2008). Depending on mosquito species, these cues may result in increased oviposition in sites ideal for larval development (McCall & Cameron 1995). Relatively few chemicals have been shown to attract or stimulate oviposition with different mosquito species (Table 1.1). In addition to these an oviposition pheromone -erythro-6-acetoxy-5-hexadecanolide (ADH)- was identified in the apical droplet of mature eggs of *Culex* (Laurence & Pickett 1982) and shown to attract *Cx. molestus*, *Cx. tarsalis*, *Cx. quinquefasciatus* synergistically with the hay infusions or the key hay infusion volatile 3-methylindole (Mboera *et al.* 1999; Mboera *et al.* 2000a; Mboera *et al.* 2000c). Another pheromone, Heneicosane, has also been identified for *Ae. aegypti* and shown to double the odds eggs being laid in water conditioned with larvae (Mendki *et al.* 2000). No pheromone has been shown yet for *An. gambiae s.s.*. In the contrary, evidence suggest that the presence of conspecifics deter oviposition in this species (McCrae 1984).

*Anopheles gambiae s.l.* generally breed in shallow, ephemeral turbid and sun-lit water bodies with algae but relatively little or no vegetation (Gimnig *et al.* 2001; Fillinger *et al.* 2009a; Mwangangi *et al.* 2010; Sinka *et al.* 2010). Unlike *An. gambiae s.s.*, *An. arabiensis* are also common in rice paddies (Mwangangi *et al.* 2010) together with *An. funestus* that breed in large semi-permanent water bodies rich in vegetation and algae (Gimnig *et al.* 2001). However, the chemoecology of oviposition in *An. gambiae s.l.* is poorly understood. Except for 2-propyl phenol and 4-methylcyclohexanol (Rinker *et al.* 2013) that were evaluated in systematic behavioural cage bioassays, many chemicals suggested to mediate the selection of oviposition substrates are yet to be confirmed as oviposition semiochemicals. Lindh and others (2008) profiled chemicals from bacteria that mediated positive oviposition responses for *An. gambiae s.s.* using solid phase micro-extraction (SPME) and gas chromatography coupled to mass spectrometry (GC-MS). Using principal component analyses and previously published electroantennography (EAG) data they suggested putative semiochemicals for oviposition in the species (Table 1.1). Many of these compounds though known to elicit electrophysiological activity in *An. gambiae s.s.* (Blackwell & Johnson 2000; Meijerink *et al.* 2000; Qiu *et al.* 2006) and *Culex* mosquitoes (Puri *et al.* 2006) have not been confirmed to affect oviposition substrate choices of the species. Some compounds

including phenol, 4-methyl phenol, 4-ethyl phenol, 4-ethyl phenol, indole, 3-methylindole, 3-methyl-1-indole, 3-carene,  $\alpha$ -terpinene,  $\alpha$ -copaene,  $\alpha$ -cedrene, d-cadinen and ethyl acetate have been presented in a recent review as oviposition attractants for *Anopheles gambiae s.l.* (Himeidan *et al.* 2013). Most of the data presented in the later paper are not supported by any behavioural evidence. This highlights the need for a very careful screening of the compounds suggested to date with accurate behavioural assays.

Table 1.1 Site-derived cues that influence oviposition with different mosquito species

Bioactive material	Description	Source	Mosquito species	Response	Authors
<b>Semiochemicals</b>					
4-methylphenol (p-cresol)	3 ppm	<i>Betula papyrifera</i> infusion	<i>Ae. triseriatus</i>	Attraction	(Bentley <i>et al.</i> 1979)
	0.01-100 µg/L	Bermuda grass infusion	<i>Cx. quinquefasciatus</i>	Synergistic Attraction	(Millar <i>et al.</i> 1992)
	0.083, 0.83, 8.3 mg/L	Synthetic	<i>Ae. albopictus</i>	Repellency	(Trexler <i>et al.</i> 2003a)
	0.01 µg/L	Bermuda grass infusion Larval rearing water Field-collected larval water	<i>Ae. albopictus</i>	Attraction	(Allan & Kline 1995)



<b>Fatty acids from C<sub>5</sub> to C<sub>13</sub></b>			<i>Cx. quinquefasciatus</i>	Repellency	(Hwang <i>et al.</i> 1982)
<b>Nonanoic acid</b>			<i>Cx. tarsalis</i> <i>Ae. aegypti</i>		
<b>Intermediate metabolites of Capric and pelargonic acids</b>		Breeding site water	<i>Cx. pipiens fatigans</i> <i>Cx. molestus</i> <i>Ae. aegypti</i>	Attraction	(Ikeshoji <i>et al.</i> 1975)
<b>Capric acid</b>		Synthetic	<i>Cx. resturans</i> <i>Cx. pipiens</i> <i>Cx. tarsalis</i> <i>Ae. aegypti</i>	Attraction	(Maw 1970)
<b>3 Methylindole (skatole)</b>	0.01-100 µg/L	Bermuda grass infusion	<i>Cx. quinquefasciatus</i>	Attraction	(Millar <i>et al.</i> 1992)
	08.3 mg/L		<i>Ae. albopictus</i>	Repellency	(Trexler <i>et al.</i> 2003a)
	0.1 µg/L	Bermuda grass infusion Larval rearing water	<i>Ae. albopictus</i>	Attraction	(Allan & Kline 1995)

		Field-collected larval water			
	0.1 µg/L	Bermuda grass infusion Larval rearing water Field-collected larval water	<i>Ae. aegypti</i>	Repellency	(Allan & Kline 1995)
<b>Indole</b>	0.01-100 µg/L	Bermuda grass infusion	<i>Cx. quinquefasciatus</i>	Synergistic Attraction	(Millar <i>et al.</i> 1992)
		Hay infusion Larval rearing water Field collected larval water	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Synergistic attraction	(Allan & Kline 1995)
<b>4-ethylphenol</b>	0.01-100 µg/L	Bermuda grass	<i>Cx. quinquefasciatus</i>	Synergistic	(Millar <i>et al.</i> 1992)

		infusion		Attraction	
		Hay infusion	<i>Ae. aegypti</i>	Synergistic	(Allan & Kline 1995)
		Larval rearing water	<i>Ae. albopictus</i>	attraction	
		Field collected larval water			
	0.1 µg/L	Hay infusion	<i>Ae. aegypti</i>	Attractant	(Allan & Kline 1995)
		Larval rearing water			
		Field-collected larval water			
<b>Phenol</b>	0.01-100 µg/L	Bermuda grass infusion	<i>Cx. quinquefasciatus</i>	Synergistic Attraction	(Millar <i>et al.</i> 1992)
	0.1 µg/L	Hay infusion	<i>Ae. aegypti</i>	Attractant	(Allan & Kline 1995)
<b>Acetic acid</b>	0.06%	Purina® Lab chow	<i>Cx. quinquefasciatus</i>	Repellency	(Hwang <i>et al.</i> 1980)
<b>Propionic acid</b>		infusion	<i>Cx. tarsalis</i>		

<b>Isobutyric acid</b>					
<b>Butyric acid</b>					
<b>Isovaleric acid</b>					
<b>Caproic acid</b>					
<b>2-propyl phenol</b>	10 <sup>-4</sup>	Synthetic	<i>An. gambiae s.s</i>	Attraction	(Rinker <i>et al.</i> 2013)
<b>4-methylcyclohexanol</b>	10 <sup>-4</sup>	Synthetic	<i>An. gambiae s.s</i>	Repellency	(Rinker <i>et al.</i> 2013)
<b>o-cresol</b>		Log pond infusion	<i>Cx. quinquefasciatus</i>	Attraction	(Gjullin <i>et al.</i> 1965)
<b><i>alpha</i>-Ethyl-p-methoxybenzyl alcohol</b>					
<b>Phenethyl methylcarbamate</b>					
<b>Ethyl methylcarbamate</b>					
<b><i>alpha</i>-conidendrol tetraacetate</b>					
<b>N-Ethyl-o-veratrylamine</b>					
<b>2,6 Dimethoxyphenol-ethylene oxide</b>					
<b>Hexadecyl pentanoate,</b>		Synthetic	<i>Ae. aegypti</i>	Repellency	(Sharma <i>et al.</i> 2009)
<b>Tetradecyl heptanoate</b>			<i>Ae. albopictus</i>		

<b>Tridecyl octanoate</b>				
<b>Octadecyl propanoate</b>	Synthetic	<i>An. stephensi</i>	Repellency	(Sharma <i>et al.</i> 2009)
<b>Heptadecyl butanoate</b>				
<b>Hexadecyl pentanoate</b>				
<b>Tetradecyl heptanoate</b>				
<b>Infusions of different plant matter</b>				
<b>Paper birch</b> ( <i>Betula papyrifera</i> )	600 grams of dried birch, 10 L water	<i>Ae. triseriatus</i>	Attraction	(Bentley <i>et al.</i> 1979)
<b>Bermuda grass</b>	450 grams grass cuttings, 5g brewer's yeast, 20g lactoalbumen hydrolysate, 75 L water	<i>Cx. quinquefasciatus</i>	Attraction	(Millar <i>et al.</i> 1992) (Allan & Kline 1995)
<b>Bermuda grass</b>		<i>Cx. tarsalis</i>		(Isoe <i>et al.</i> 1995a; Isoe & Millar 1995)
<b>Larval rearing water</b>		<i>Ae. albopictus</i>	Attraction	(Allan & Kline 1995)

		<i>Ae. aegypti</i>		
<b>Manure</b>		<i>Ae. aegypti</i> var. <i>queenslandis</i>	Attraction	(O'Gower 1963)
<b>Log pond water</b>		<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i>	Attraction	(Gjullin <i>et al.</i> 1965)
<b>Alfalfa hay</b>	10 g dried alfalfa in 200 ml distilled water	<i>Cx. pipens</i> <i>quinquefasciatus</i> <i>Ae. aegypti</i>	Attraction and stimulation	(Hazard <i>et al.</i> 1967)
<b>Purina® Laboratory chow</b>	1 gram in 100 ml, 10 days old	<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i>	Repellency	(Hwang <i>et al.</i> 1980)
<b>Breeding site water</b>		<i>Cx. pipiens fatigans</i> <i>Cx. molestus</i> <i>Ae. aegypti</i>	Attraction	(Ikeshoji <i>et al.</i> 1975)
<b><i>Panicum maximum</i></b>	40 g of grass leaves, 5 L of tap water	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Stimulation/ Attraction	(Santana <i>et al.</i> 2006)

<b>Oak leaf</b>			<i>Ae. albopictus</i>	Stimulation/	(Trexler <i>et al.</i> 1998)
			<i>Ae. triseriatus</i>	Attraction	
<b>White-oak leaf</b>			<i>Ae. aegypti</i>	Attraction	(Ponnusamy <i>et al.</i> 2008)
<b>Bamboo</b>			<i>Ae. aegypti</i>	Attraction	(Ponnusamy <i>et al.</i> 2008)
<b>Bacteria</b>					
<i>Aerobacter aerogenes</i>	10 g dry alfalfa, 200 ml water, 24 hrs incubation	Alfalfa hay infusions	<i>Cx. pipiens</i> <i>quinquefasciatus</i> <i>Ae. aegypti</i>	Attraction and stimulation	(Hazard <i>et al.</i> 1967)
<i>Pseudomonascea</i>		Unspecified hay infusion	<i>Cx. resturans</i> <i>Cx. pipiens</i> <i>Cx. tarsalis</i>	Attraction	(Maw 1970)
<i>Pseudomonas aeruginosa</i>		Breeding site water	<i>Cx. pipiens fatigans</i> <i>Cx. molestus</i> <i>Ae. aegypti</i> <i>Ae. albopictus</i>	Attraction	(Ikeshoji <i>et al.</i> 1975) Hasselschwert & Rockett 1988) (Pavlovich & Rockett 2000)

<i>Bacillus cereus</i>		Bacterial cultures	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Stimulation/ Attraction	(Hasselschwert & Rockett 1988; Poonam <i>et al.</i> 2002) (Pavlovich & Rockett 2000)
<i>Pseudomonas fluorescens</i> <i>Bacillus thuringiensis</i> <i>Bacillus sphaericus</i>	Between 100 – 2000 ppm	Bacteria filtrates	<i>Cx. quinquefasciatus</i>	Attraction	(Poonam <i>et al.</i> 2002)
<i>Psychrobacter immobilis</i> <i>Sphingobacterium multivorum</i> <i>Bacillus species</i>		Larval rearing water Soil contaminated cotton towels Oak leaf infusion	<i>Ae. albopictus</i>	Attraction	(Trexler <i>et al.</i> 2003b)
<i>Stenotrophomonas maltophilia</i>	Bacteria filtrate	Breeding site	<i>An. gambiae s.s.</i>	Repellency	(Huang <i>et al.</i> 2006a)
<b>14-18 different spp most of them Gammaproteobacteria</b>		Bamboo leaf infusion White-oak leaf infusion	<i>Ae. aegypti</i>	Attraction	(Ponnusamy <i>et al.</i> 2008)



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<i>Vibrio metschnikovii</i>	Between $1.8 \times 10^7$	Midguts and	<i>An. gambiae</i> s.s,	Attraction	(Lindh <i>et al.</i> 2008)
<i>Proteus sp.</i>	to $2.0 \times 10^8$	breeding sites			
<i>Micrococcus sp.</i>	bacterial cells /ml				
<i>Bacillus sp.</i>					
<i>Exogobacterium sp.</i>					

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## 1.5. Tools used for studying the oviposition behaviour of mosquitoes

Several bioassays have been developed and used to investigate oviposition substrate preferences and attraction in mosquitoes. Many of these bioassays were developed with *Culex* species (Isoe *et al.* 1995b) but are widely used to evaluate oviposition in other mosquito species without further modification. Some effort has been directed to customizing bioassays for *Aedes* mosquitoes (Corbet & Chadee 1993). However, to date, no behavioural bioassays have been developed strictly for use malaria mosquitoes.

Forced bioassays have been attempted to evaluate the time it takes for mosquitoes to lay eggs in test substrates (Isoe *et al.* 1995b). In such studies gravid mosquitoes were stunned by chilling before their wings were amputated to immobilize them. The immobile mosquitoes were then left on the surface of substrates and the time they took before laying eggs compared to controls. This technique was originally developed for forcefully obtaining eggs from mosquitoes in the laboratory (Ikeshoji 1966). As a bioassay technique, these have little benefit and cannot be used to investigate pre-oviposition responses or give a correct representation of the oviposition behaviour mosquitoes (Isoe *et al.* 1995b).

The vast majority of oviposition studies are done in laboratory or semi-laboratory settings within adult mosquito holding cages. Though the cages differ in shape (Kramer & Mulla 1979; Isoe *et al.* 1995b; Poonam *et al.* 2002), size (Isoe *et al.* 1995b; Poonam *et al.* 2002; Sumba *et al.* 2004a) and construction (Kramer & Mulla 1979; Huang *et al.* 2005), the principle of cage bioassays is the same: gravid mosquitoes are offered one, two or more substrates and the numbers of mosquitoes that orient to and/or lay eggs in it are estimated.

Choice egg-count bioassays are the single most commonly used oviposition tool for oviposition studies with mosquitoes. These compare the number or proportion of egg or egg raft deposited on a test substrate to that of a standard or different substrate. Egg-count bioassays have been used to describe substrate preferences by *Stegomyia*, *Culex* and *Anopheles* mosquitoes (Bentley *et al.* 1979; Hwang *et al.* 1980; Hwang *et al.* 1982; Millar *et al.* 1994; Sumba *et al.* 2004a; Sharma *et al.* 2008). However, because eggs are only the final product of oviposition choices, egg or egg raft counts provide little information on the pre-oviposition behaviour of mosquitoes. Further, egg-count bioassays allow gravid mosquitoes to come into direct physical contact with test

substrates making it difficult to distinguish between non-contact attraction and contact stimulation (Isoe *et al.* 1995b; Sumba *et al.* 2004a). The most salient flaw of these however is that egg-count bioassays assume that the number or proportion of eggs indicates egg-laying preferences. This is straight forward in for *Culex* mosquitoes that lay eggs in rafts. However for mosquitoes that lay individual eggs it should be verified (Corbet & Chadee 1993).

Alternative bioassay tools have been suggested for challenges posed by egg-count bioassays. These include detergents and sticky screens that estimate the number or proportion of mosquitoes that attempt to lay eggs in substrates. Detergent bioassays refer to behavioural assays that add minute quantities of laboratory grade surfactants to substrates to break the surface tensions and therefore drown mosquitoes attempting to land (Isoe *et al.* 1995b). Sticky screens exploit the same principle but instead of detergents use insect glue to trap gravid mosquitoes when they attempt to access substrates covered by these (Isoe *et al.* 1995b; Trexler *et al.* 2003a; Dugassa *et al.* 2012). These have been used to differentiate between volatile attractants and non-volatile stimulants for *Culex* species. However, their effectiveness is limited to mosquito species that exclusively land directly on the water surface to lay eggs. Further the olfactory and visual interferences due to the use of detergents, glues and screens could hamper accurate evaluation of oviposition.

Wind tunnels are a useful tool in evaluating short-range odour orientation in insects of agricultural and medical importance including *An. gambiae s.l.* (Kellogg & Wright 1962; Knols *et al.* 1994; Mboera *et al.* 1998; Mukabana *et al.* 2002; Olanga *et al.* 2010; Omrani *et al.* 2010). The first wind tunnel described for studying oviposition in mosquitoes was designed by Osgood and Kempster (1971). Using groups of *Culex* mosquitoes, they showed that simple air-flow wind tunnels (olfactometers) could be used to effectively identify oviposition semiochemicals and distinguish attractants from stimulants. Wind tunnels include a system that draws air through the system to initiate stimuli-laden plumes from trapping chambers. This paves the way for anemotaxis causing mosquitoes to fly into or away from compartments fitted with entry-no-return funnels or baffles and treated with test substrates. This provides for an effective method of measuring oviposition attraction. Klowden and Blackmer (1987) used the olfactometer designed by Osgood and Kempster to describe methyl propionate as an oviposition attractant for *Aedes aegypti*. Dual port wind tunnels\ olfactometers can also

been used to identify and evaluate spatial oviposition repellents (Schreck *et al.* 1970; Kline *et al.* 2003). However wind tunnels are limited in range; they cannot be used to evaluate long-range attraction over several meters such as necessary when screening putative attractants for the developments of baits for monitoring and controlling mosquitoes. For such it is necessary to design tools that evaluate mosquitoes in larger semi-field or field conditions.

Few studies have attempted to evaluate oviposition substrates in the semi-field and field condition. Mboera and others (Mboera *et al.* 2000a) used counter-flow geometry traps to evaluate the response of gravid *Culex quinquefasciatus* to the synthetic oviposition pheromone, (5R, 6S)-6-acetoxy-5-hexadecanolide. Other studies used artificial ponds to investigate the effects of the hemipteran *Anisops sardea* on habitat selection by mosquitoes (Eitam *et al.* 2002; Blaustein *et al.* 2004). No study has designed tools for screening oviposition attraction of *An. gambiae s.l.* mosquitoes under semi-field or field conditions.

## 1.6. Factors that potentially affect the responsiveness of mosquitoes prepared for oviposition studies

### 1.6.1. Blood meal

Female of mosquito species that vector diseases are haematophagous (Takken *et al.* 1998). Their blood requirement is necessitated by a physiological need to obtain serum protein needed to complete egg development (Briegel 1985; Briegel & Horler 1993). Egg development begins when the mosquito has ingested an amounts of blood sufficient to initiate follicular development (Shelton 1972; Reisen & Emory 1976; Briegel & Horler 1993). The amount and number of blood meals required depends on the mosquito species (Lounibos *et al.* 1998) and body size (Lyimo & Takken 1993; Takken *et al.* 1998). In addition to sugar, blood is a source of energy; females that are blood-fed have been shown to live longer than those fed on sugar only (Gary & Foster 2001). In nature, many vertebrates are potential host sources of blood meals for mosquitoes ranging from avians and humans to bovines and other ruminants. However, many mosquito species show host preferences (Takken & Knols 1999). The malaria mosquitoes *An. gambiae s.s.* and *An. funestus s.s.* are thought to have preference biting

human hosts (anthropophagy) while *An. arabiensis* is considered a much more general vector that bites both humans and domesticated outdoor animals. However, host preferences are mediated by several extrinsic and intrinsic factors and could vary from strain to strain (Takken & Verhulst 2013).

Captive mosquitoes intended for oviposition experiments are often blood-fed from narrow range of blood meal host sources. The choice of these is limited by the ease of handling and ethical complexities. The most common sources of blood meals in the laboratory include rabbits, guinea pigs, horses, and rats (Olayemi & Aude 2009). Blood meals are often offered by allowing sugar-starved mosquitoes timed access to immobilized hosts, a human arm or artificial membrane feeding devices (Cosgrove *et al.* 1994). The use of membrane feeding devices has been shown to negatively influence the proportions of mosquitoes that blood feed (Bunner *et al.* 1989).

Adapting specialist hematophagous insects to standard laboratory blood meal host sources is often associated with changes in their biotic potential with effects on fecundity, duration of the egg maturation period and viability of eggs (Downe & West 1963; Shelton 1972). These effects are attributable to the protein quality of blood (Jordan 1961).

### 1.6.2. Mating and insemination

In nature, mating and insemination occur early in the life cycle of most mosquito species. The age of mosquitoes has been positively correlated with mating in laboratory cages (Jones & Gubbins 1978; Verhoek & Takken 1994). Verhoek and Takken (Verhoek & Takken 1994) demonstrated that *An. gambiae s.s.* and *An. arabiensis* optimally mate when both sexes were between the ages of 5 and 7 days.

In addition to the age of the male mosquito many factors are thought to affect mating in caged mosquito colonies including body sizes of both males and females (Yuval *et al.* 1993; Charlwood *et al.* 2002; Okanda *et al.* 2002; Charlwood *et al.* 2003), sugar deprivation (Stone *et al.* 2009), sex ratios within adult mosquito holding cages (Charlwood & Jones 1979; Verhoek & Takken 1994) and lighting conditions (Charlwood & Jones 1980; Panicker & Bai 1980; Marchand 1985). No study has investigated the implication of these for oviposition studies with laboratory reared mosquitoes.

Mosquitoes are often assumed to mate before blood meals (Charlwood *et al.* 2003). However for *An. gambiae* many females have been shown to blood feed prior to mating (Lyimo & Takken 1993). The development of eggs is not dependent on the mosquitoes state of insemination, but for egg maturation and oviposition mating is mandatory (Chambers & Klowden 2001; Klowden & Russell 2004). It could be that the oviposition success rate of mosquitoes can be improved by conditioning cages where blood fed mosquitoes are held with male mosquitoes.

### 1.7. Oviposition traps for mosquito monitoring and control

The gold standard and most frequently used technique for monitoring *An. gambiae s.l.* is the human landing catches (Gama *et al.* 2013). Through human landing catches both mosquitoes that host seeking inside and outside houses can be detected and changes in their temporal and/or spatial feeding habits described (Gimnig *et al.* 2013). However, while this is the most effective method it exposes volunteers looking for doing the sampling to infective mosquito bites leading to ethical and safety concerns.

The most common alternative monitoring tools for the presence of *An. gambiae s.l.* in a target area in the recent past were collection methods and traps located inside people's houses to catch the highly endophilic and endophagic malaria vectors (Mbogo *et al.* 1993). Such tools included various light traps, pyrethroid spray catches and bed net traps (Leiser & Beier 1982; Mathenge *et al.* 2002; Sithiprasasna *et al.* 2004). Many of these tools' efficiency is density dependent and their sensitivity reduces when vector densities are extremely low (Mbogo *et al.* 1993; Hii *et al.* 2000; Overgaard *et al.* 2012). In areas with increasing coverage with insecticides inside houses mosquito densities have reduced in many areas to a very low level (Bayoh *et al.* 2010). Furthermore, vectors have adapted to this situation and avoid resting and/or feeding inside leading to a low and possible under -representative catch rate of adult vectors in the area with traditional trapping techniques (Hii *et al.* 1986; Zaim *et al.* 1986; Mbogo *et al.* 1993). It is therefore necessary to develop novel approaches to target outdoor populations of vectors in areas of high personal protection coverage (Fillinger *et al.* 2008).

Oviposition traps would provide such a tool if suitable oviposition media or semiochemicals could be identified to attract gravid *An. gambiae s.l.*. Collecting mosquitoes after blood meals provides a relatively accurate way of surveying for disease pathogens (Reisen & Meyer 1990; Meece 2002; Braks & Carde 2007; Williams & Gingrich 2007). Trapping gravid mosquitoes seeking oviposition sites provides for a

desirable method for doing so (Kline *et al.* 2006; Braks & Carde 2007). Gravid traps have been demonstrated as effective in surveillance systems for arboviral encephalitis (Reiter *et al.* 1986) and human filariases (Jayanetti *et al.* 1988). Many anopheline mosquitoes are suspected to be involved in virus transmissions and monitoring these would benefit from an oviposition trap. However, even though many ovitraps and gravid traps exist there has been none effective for the collection of *Anopheles gambiae s.l.* until recently (Dugassa *et al.* 2013).

Ovitraps were first reported by Fay and Eliason (Fay & Eliason 1966). They were essentially hay infusion baited stations that mimicked preferred oviposition sites of *Ae. aegypti*. Each station consisted of a wooden paddle wrapped up in brown blotting papers and propped into a gloss black enamel container half-filled with hay infusions. Ovitraps act as sinks for mosquito eggs and can be used for monitoring the presence of adult mosquitoes in the field (Fay & Eliason 1966). Since their introduction, ovitraps have been extensively used effectively to monitor the densities of many container breeding mosquitoes including *Ae. aegypti* (Fay & Eliason 1966; Jakob & Bevier 1969), *Ae. albopictus* (Zhang & Lei 2008), *Ae. sirriensis* (Mortenson *et al.* 1978) and the tree-hole species *Haemagogus equinis* (Tikasingh & Laurent 1981). Ordonez-Gonzales incorporated insect glue into the ovitrap design and developed a novel sticky ovitrap (Ordonez-Gonzalez *et al.* 2001). Sticky ovitraps arrest gravid mosquitoes as they land on substrate and work well for monitoring adult *Ae. aegypti* (Ritchie *et al.* 2004) and *Ae. albopictus* (Zhang & Lei 2008). The effectiveness of ovitraps depends on the oviposition substrate bait. Polson and others have shown that 10% hay infusions increase the effectiveness of ovitraps three-fold as compared to water alone for *Ae. aegypti* (Polson *et al.* 2002; Santos *et al.* 2003).

Reiter developed the first gravid traps with the intention of sampling gravid *Culex* mosquitoes and bettering surveillance systems for arboviral diseases (Reiter 1983; Reiter 1987). These traps, now available commercially as CDC gravid trap model 1712 and Box gravid trap, have two main parts: an overhead chimney-like part holding a fan that creates an upward suction to trap gravid mosquitoes and (2) a pan for dispensing oviposition media baited with attractants (Reiter 1983; Reiter 1987). Frommer introduced a trapping chamber to prevent adult mosquito specimen from being damaged resulting into a new variant of the Reiter design trap commonly known as the Frommer updraft gravid trap (CDC gravid trap model 1719). Together these three constitute the

most commonly used gravid traps (Braks & Carde 2007). These traps have low efficacy with *An. gambiae s.s.* (Dugassa *et al.* 2013). Additional variants of these trap design include the Harris county gravid trap (Dennett *et al.* 2007) and counter flow geometry (CFG) traps adapted for gravid mosquitoes (Mboera *et al.* 2000b). However, only one gravid trap – the OviART gravid mosquito trap - has been developed specifically for *An. gambiae s.s.* (Dugassa *et al.* 2013)

Trap models together with associated attributes affect the efficacy of gravid traps. Allan and Kline demonstrated that the CDC model 1712 and 1719 trapped more *Cu. quinquefasciatus* and *Cx. nigripalpus* mosquitoes compared to the box gravid trap. The study additionally showed that traps with dark and large pans were more effective in trapping these species compared to those with lighter and small pans (Allan & Kline 2004). Braks and Carde later showed that by improving the airflow within it the Box gravid trap becomes two-fold more effective (Braks & Carde 2007). Russell and Hunter (2010) replaced the collection component of the CDC 1712 design with that of a CDC light trap effectively cutting the number of specimen damaged and allowing for easier transport, freezing and removal of mosquitoes.

The sensitivity and specificity of gravid traps depends on the substrates used as a lure in the pan. Hay and grass infusions are widely used to for the surveillance of many Aedine and Culicine species including *Ae. (Ochleroratus) japonicus*, *Ae. albopictus*, *Cx. quinquefasciatus*, *Cx. nigripalpus*, *Cx. restuans*, *Cx. pipens*, *Cx. erraticus* (Dickson & Dewsnup 2005; Burkett-Cadena & Mullen 2007; McPhatter *et al.* 2009). In addition to organic infusions studies have shown that it is possible to use artificial attractants as baits for *Culex* in gravid traps (Mboera *et al.* 2000a).

*Anopheles gambiae s.l.* are not container breeders and shun the container-like design of ovitraps and gravid traps (Dugassa *et al.* 2013). While simple gravid trap that consists of acetate sheets treated with insect glue and suspended just above potential oviposition sites has been recently proposed for catching gravid anophelines when they land on a water surface to lay their eggs (Harris *et al.* 2011). However, other studies suggest that these mosquitoes can lay eggs in flight without landing (Dugassa *et al.* 2012) making sticky screens little effective. Without attractants involved to lure a large number of mosquitoes to a sticky trap only very few individuals are likely to be caught, and probably only in a high transmission setting as was the case in the test area of Harris and others (2011).



## Rationale

Following a decade-long consistent decline in malaria burden in Africa, elimination of the disease appears to be within reach for the first time in many locations. This progress is attributable to many factors but is most prominently linked to vector control through wide scale use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) for controlling peridomestic primary malaria vectors (Lengeler 2004; Pluess *et al.* 2010). These tools have together reduced malaria transmission by many orders of magnitude. However, studies indicate that even with complete coverage with LLINs and IRS, malaria elimination will remain elusive (Ferguson *et al.* 2010; Killeen 2014). Transmission of the disease will be sustained by vectors that resist insecticides and bite outdoors. Additional tools that can combine with the current frontline strategies to impact these vector populations are needed. Tools aimed at behaviours of vectors beyond blood-feeding might allow for controlling mosquitoes irrespective of their state of insecticide resistance or biting and resting inclination and are most desirable (Ferguson *et al.* 2010). For this reason, the present study seeks to provide insight for strategies targeting of gravid mosquitoes by identifying chemical factors that *An. gambiae s.s* use to locate and select oviposition substrates.

An understanding of the cues malaria mosquitoes use to locate and select oviposition substrates could instigate an exciting possibility for targeting mosquitoes that successfully bite in spite of existing control. It has been hypothesised that oviposition cues could be manipulated and combined with insecticides to attract gravid mosquitoes outdoors and kill either the gravid female or the immature mosquitoes within breeding sites (Ferguson *et al.* 2010; Killeen 2014). However, to date this has not been systematically investigated; evidence of oviposition substrate preferences is sketchy and controversial. Many volatile organic compounds have been suggested to mediate this but none supported with appropriate scientific evidence. All studies have been restricted to laboratory conditions; not one chemical has been evaluated beyond the laboratory. There is need to systematically seek and share evidence for chemicals *An. gambiae s.l.* might use to select breeding sites and test the effectiveness of this in natural conditions.

As a prerequisite to studying the response of gravid *An. gambiae s.s.* to putative semiochemicals, robust behavioural assays that accurately characterise and quantify the response of gravid malaria mosquitoes to different substrates are required. No effort has been made to empirically design or standardize such assays for this species. In spite of

at least one study with *Aedes* mosquitoes that suggested that mosquitoes that lay single eggs and skip oviposit might present unique challenges to implementing and interpreting egg-count bioassays, bioassays tools previously designed for use with other species of mosquitoes have been employed for studies with this species with no preliminary studies whatsoever to evaluate their appropriateness for this.

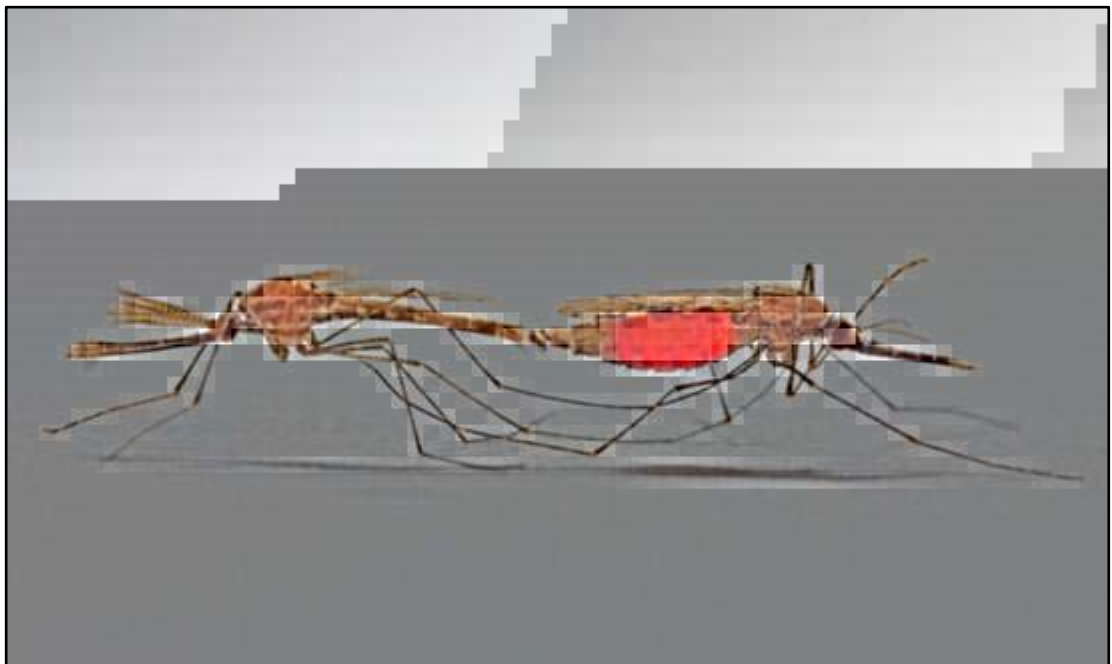
## 1.8. Overall aim and hypotheses

The aim of this thesis was to analyse the role of volatile organic compounds in oviposition substrate choices of *Anopheles gambiae* and *An. arabiensis*. Towards this elaborate studies were done to develop tools for investigating substrate preferences as well as attraction to substrates under controlled conditions and in the field.

It was hypothesized that:

- The proportion of *An. gambiae s.s.* and *An. arabiensis* that respond in controlled behavioural oviposition bioassays can be improved to over eighty percent (thereby increasing the accuracy of experiments) by standardizing operating procedures for producing gravid mosquitoes and re-aligning bioassays with the peak oviposition period of the strains.
- *Anopheles* exhibit unique egg-laying characteristics which reduces the effectiveness of choice egg-count bioassays. Such bioassays can be improved to detect relatively small differences in substrate preferences ( $\leq 20\%$ ) with sufficient power ( $\geq 80\%$ ) and confidence (95% significance) by redesigning bioassays to account for the unique features and using the right sample sizes. Novel free-flight experiments in the semi-field using mosquito traps can be used to confirm olfactory attraction to oviposition substrates.
- Gravid *An. gambiae s.s.* and *An. arabiensis* discriminate between oviposition substrates and preferentially select in which to lay eggs. This choice is partially mediated by volatile organic compounds originating from the substrates.
- Semiochemicals for oviposition substrate preferences can be manipulated to attract and trap gravid malaria mosquitoes under controlled conditions and in the field.

## Chapter 2. Optimizing the proportion of gravid *Anopheles gambiae sensu lato* that respond in oviposition bioassays



*Anopheles gambiae* mosquitoes mating (Source, Sam Cotton, University College London)

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## 2.1. Abstract

**Background:** To do effective behavioural experiments with mosquitoes it is necessary to have reproducible responses with different batches of the insects. This study aimed to improve the replicability of bioassays for oviposition by improving procedures for preparing batches of gravid *Anopheles gambiae sensu stricto (s.s.)* and *Anopheles arabiensis* so that a high (<80%) and consistent proportion of mosquitoes lay eggs in every round of experiments.

**Methods:** First the impact of insemination on oviposition was assessed for both species. The chance of egg-laying in three-day old mosquitoes blood-fed and kept in cages with an equal number of conspecific males was compared to those blood-fed and kept in cages without males. Factors that could potentially influence insemination such as the adult mosquito holding cage size and the age and size of the mosquito were investigated by monitoring the rate of inseminated mosquitoes in 30 cm<sup>3</sup> and 60 cm<sup>3</sup> cages over four days. Secondly, the impact of substitute host-sources of blood-meal on the chances *An. gambiae s.s.* maturing and laying eggs was demonstrated by comparing the proportions of mosquitoes that became gravid and oviposited when blood-fed either on a human arm or a restrained rabbit (a common alternative lab host for mass production). Thirdly, to select an ideal time-frame for experiments a time period with the most intensive oviposition was identified by comparing the proportions of mosquitoes that laid eggs between 17:00 – 21:30 h and 21:30 – 8:00 h. Multivariable analyses with generalised estimating equations were used to estimate the effect of different factors on the odds of mosquitoes being inseminated or becoming gravid and laying eggs.

**Results:** An average proportion of 84%, (95% confidence interval (CI) 77 – 89%) *An. gambiae s.s.* laid eggs when females were kept with males during and post blood-feeding. Only 25% (95% CI 15 – 41%) of *An. arabiensis* laid eggs irrespective of the presence of male mosquitoes in the cages. Larger cages did not improve the insemination rate of *An. arabiensis* (Odds ratio (OR) 0.96, 95% CI 0.89 – 1.11) and *An. gambiae s.s.* (OR 1.18, 95% CI 0.75-1.90). There was a six-fold higher probability (OR 6.68, 95% CI 2.57 – 17.4) of *An. arabiensis* being mated with every unit increment in wing size. The chances of egg-laying dropped (OR 0.30; 95% CI 0.14 – 0.66) when human arm-feeding was substituted with rabbit host. Egg numbers per mosquito were

however not affected by the host-source of blood-meal. The vast majority (96%, 95% CI 94 – 100%) of *An. gambiae s.s.* laid eggs between 17:00 h and 21:30 h.

**Conclusions:** Oviposition experiments with *An. gambiae s.s.* are best done between 17:00 h and 21:30 h with mosquitoes provided with two consecutive blood-meals 24-hour-apart from a human host, and kept in a cage with an equal number of conspecific age-mate males for at least 72 hours. More than eighty percent of mosquitoes prepared following these procedures lay eggs in experiments. Even then, mosquitoes should be individually provided with oviposition substrate in choices to eliminate the risk of including mosquitoes that do not lay eggs from the analyses and to describe skip oviposition.

## 2.2. Background

For effective electrophysiological and behavioural studies with mosquitoes and other insects it is critical to fine-tune insect handling beforehand to ensure reproducible responses from the insects during experiments (Baker & Carde 1984). Mosquito behaviour is a function of the internal physiological state and circadian rhythms (Clements 1992). Factors that influence the insects' internal state if any, should therefore be determined, regulated and standardised to improve the responsiveness during assays and make experiments repeatable. Moreover, natural behavioural changes should be followed over time to identify the peak period when experiments are best done (Baker & Carde 1984). This has never been systematically investigated for the preparation of gravid malaria mosquitoes aimed for oviposition studies.

Many egg-count bioassays done with *Anopheles gambiae s.l.* to study its oviposition behaviour report very low mean egg numbers (Sumba *et al.* 2004a; Huang *et al.* 2005; Huang *et al.* 2006a; Fritz *et al.* 2008; Balestrino *et al.* 2010). This is in spite of laboratory and field evidence that individual *Anopheles gambiae s.s.* and *Anopheles arabiensis* mosquitoes frequently lay large mean numbers of eggs (Lyimo & Takken 1993; Hogg *et al.* 1996). The low egg numbers could imply that many of the mosquitoes exposed to substrates and included in analyses failed to lay eggs during experiments. More importantly, it might suggest that many mosquitoes used for oviposition studies were not gravid and should not have been used. McCrae (1984) raised concern over the very low mean numbers of eggs commonly reported in many choice egg-count experiments with groups of *An. gambiae s.l.* mosquitoes. He noted that this implied that “the behaviour of only three or four mosquitoes was [therefore] tested”; and sample sizes for these experiments grossly overestimated. Results based on these few mosquitoes could lead to invalid conclusions. To date, no studies have sought to improve the preparation of gravid mosquitoes for experiments.

There is some evidence that whether a female mosquitoes becomes gravid or not and lays eggs or not depends on her state of insemination (Chambers & Klowden 2001; Klowden & Russell 2004), the amount and host-source of blood-meal provided to the female (Downe & West 1963; Shelton 1972; Downe & Archer 1975; Mather & DeFoliart 1983; Olayemi *et al.* 2011a), and the time period before blood-fed mosquitoes are provided with suitable oviposition media (McCrae 1983; Sumba *et al.* 2004b; Fritz *et al.* 2008; Dieter *et al.* 2012). It is beyond the scope of this study to investigate the

underlying mechanism that dictate how these factors influence the fecundity and fertility of mosquitoes. The present focus is rather to investigate the potential influence these factors have on the mean numbers of eggs laid by individual *An. gambiae s.s.* and *An. arabiensis* of the Mbita strain and the mean number of gravid mosquitoes that seek oviposition substrates during experiments. The mean number of eggs laid by mosquitoes and the mean number gravid mosquitoes that seek substrates are the primary measures in behavioural bioassays for oviposition.

“For any study of oviposition to be complete it would be valuable to know the probable time of its occurrence as a basic guide for laboratory procedures” (McCrae 1983). Putative oviposition substrates often consist of age-dependent organic infusions or concentration-sensitive chemicals which degrade in time and should be evaluated over a short period of time. Knowing the peak-time when mosquitoes oviposit would reduce challenges due to this by advising the ideal time to set up bioassays with the test species. Antennal sensitivity to semiochemicals varies with blood-meal status and the consequent physiological transition to gravidity (Fox *et al.* 2001; Qiu *et al.* 2006). Moreover, insects exhibit the lowest threshold for stimuli in the period that coincides with the natural time the specific behaviour occurs (Baker & Carde 1984). Implementing experiments and electrophysiological studies during this period of peak responses therefore leads to findings that are closer to reality and conclusions that are likely to be more true to the insects’ ecology.

Procedures for handling mosquitoes intended for studies investigating the host-seeking behaviour for *An. gambiae s.s.* are well established. As a general rule, female mosquitoes are deprived of a sugar-meals for between 4 - 8 hours before experiments (Njiru *et al.* 2006; Olanga *et al.* 2010; Smallegange *et al.* 2010). This incites an internal state of “hunger” that immediately leads to appetitive search and responsiveness when captive mosquitoes are released into the experimental arena and provided with low doses of stimuli. Moreover, host-seeking studies are best implemented at dusk representing the natural periodicity of the behaviour (Okumu *et al.* 2010b; Lorenz *et al.* 2013). Insects handled following these procedures have been used successfully in host-seeking vector studies to identify host-seeking kairomones and formulate baits to trap *An. gambiae s.s.*, (Okumu *et al.* 2010b; Mukabana *et al.* 2012). In the same way, preparation procedures for mosquitoes for gravid mosquitoes could improve the



responsiveness of insects and the integrity of conclusions made from oviposition studies.

It was hypothesized that by fine-tuning standard operating procedures for producing gravid mosquitoes and identifying the peak oviposition period the proportion of gravid mosquitoes that consistently detect and select oviposition substrates could be improved to over eighty percent. In a series of experiments carried out with caged *An. gambiae s.s.* and *An. arabiensis* of the Mbita strain local to western Kenya this study investigated (1) if keeping conspecific male mosquitoes in cages during and after blood-feeding increased the proportion of inseminated and gravid females, (2) if larger rearing cages could increase the rate of insemination in mosquitoes, and (3) if blood-feeding mosquitoes with convenient host-source of blood-meal (rabbits) affected the chance that a mosquito would lay eggs; (4) The study also sought to establish the peak oviposition time for gravid *An. gambiae s.l.* to determine the best timeframe for implementing experiments.

## 2.3. Methods

### 2.3.1. Study site

Experiments were implemented at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus at Mbita near Lake Victoria, Western Kenya (geographical coordinates 0° 26' 06.19" South; 34° 12' 53.12" East; altitude 1149 meters above sea level). Egg-count experiments were carried out in sheds, 10 m long × 5 m wide × 2.8 m high with walls constructed from dry reed mats and roofs of translucent corrugated polycarbonate sheets. Every shed contained two tables with capacity to hold 50 cages with a gap of 40 cm between each cage (Figure 2.1). Experiments were done at ambient conditions of temperature, humidity (mean daily temperature  $27 \pm 5^\circ\text{C}$ , relative humidity  $55 \pm 10\%$ ) and light.



**Figure 2.1** Experimental set-up of the two-choice egg-count bioassay (A) makeshift huts (B) cages set-up in huts at icipe-Thomas Odhiambo Campus, western Kenya (From (Herrera-Varela *et al.* 2014)).

### 2.3.2. Mosquito rearing procedures

Insectary-reared *An. gambiae s.s.* and *An. arabiensis* (Mbita strains) were used for this study. Briefly, two to three day old mosquitoes were allowed to feed on a human arm for 15 minutes on two consecutive days at 19:00 h. On the third day, blood-fed females were allowed to oviposit on wet filter papers provided overnight in the cage. An unknown number of eggs was dispensed in 20 L plastic tubs (41 cm diameter, 8 cm deep) half-filled with non-chlorinated tap water purified by filtering through a charcoal-sand filter (hereafter called tap water). Hatched larvae were fed with ground Tetramin® baby fish food (Tetra, Melle, Germany) twice daily. Pupae were collected into 10 cm diameter, 250 ml plastic cups filled with 200 ml of tap water and left overnight in 30×30×30 cm mosquito cages for adults to emerge. Adults were maintained on 6% glucose *ad libitum* using absorbent paper wicks propped in 25 ml vials filled with glucose solution.

### 2.3.3. Mosquito dissections

Female mosquitoes were immobilized by placing them in a fridge at 4°C for 15 minutes. Terminalia and the near terminal abdominal segment (segment IX) were severed in normal saline to expose spermathecae. Slide mounts of spermathecae were inspected using a microscope at 1000× magnification for the presence of motile spermatozoa - a confirmation for insemination. The abdominal segments VII and VIII were gently severed to expose ovaries. The ovaries were observed at a magnification of 200× to

evaluate stages of egg development. Mosquitoes with mature eggs, boat-shaped with fully developed floats, were categorised as gravid. To estimate the size of a female the lengths of the left wing was measured from the distal end of the alula to the wing tip (omitting the fringe setae) to the nearest 0.1 mm (Packer & Corbet 1989; Lyimo & Koella 1992).

#### 2.3.4. Cages and oviposition cups

Experiments were carried out in standard cages (30x30x30 cm) or in large cages (60x60x60 cm). The cages had a steel framework on a galvanized metallic base and covered with fine cotton mosquito netting. The cage net also had an insert sleeve for introducing and retrieving oviposition substrates and gravid mosquitoes. Oviposition substrates were offered in 7 cm diameter, 100 ml clear borosilicate crystallising glasses (Pyrex®, hereafter called oviposition cups). Prior to any experiment oviposition cups were autoclaved and kept at 200 °C for 2 hrs to reduce the possibility of bacteria and odourant contamination. Individual gravid mosquitoes (indicated by an enlarged, pale white abdomen) were introduced into the cages and provided with either one or two oviposition cups containing 100 ml of tap water.

#### 2.3.5. Experimental procedures

##### *2.3.5.1 Does holding blood-fed mosquitoes with conspecific age-mate males after feeding improve the rate of insemination and oviposition success?*

Relatively young females are often selected and blood-fed when preparing gravid mosquitoes for experiments to maximise the proportion that survive until experiments are finished two to three days after the blood-meal. The impact of male mosquitoes in put in cages with young blood-fed females on the oviposition success was explored.

To do this, two groups of 300 three-day old female *An. gambiae s.s.* mosquitoes were put in separate standard cages. These mosquitoes had spent the first three days of their adult lives in colony cages with between 1000-2000 male and female conspecific mosquitoes. In one cage 300 males of the same age were added, whilst in the other cage no males were included. Both groups were then starved of sugar solution for up to six hours. Tap water saturated cotton towels folded to a pad of 50x25 cm were placed over the cages to maintain the relative humidity (RH) between 68 to 75%. The starved mosquitoes were permitted to blood-feed from a human arm between 18:30 – 19:30 h

for 15 minutes in total darkness. Mosquitoes that were not fed after the first blood-meal were removed from the cage, killed and discarded. Sugar solution was then replaced in the preparation cage until 12:00 h the following day when the mosquitoes were starved again in preparation for a second blood-meal the same evening. The second blood-meal ensured that all mosquitoes obtain sufficient blood-meals. After blood-feeding the mosquitoes were left in the insectary at temperatures that averaged  $27 \pm 2^\circ\text{C}$  and were only retrieved 72 hrs later at the onset of experiments. The mosquitoes were seven days old when egg-count cage experiments were implemented.

Fifty females were selected from each of the two groups based on visual inspection of their abdomen and transferred individually to standard cages. Each female was offered a single oviposition cup with 100 ml of tap water. The presence and number of eggs was recorded after 16 hrs (17:00 - 08:00 h). This experiment was carried out on three occasions (rounds; 3 x 50 individual females per treatment). Identical experiments were carried out in parallel with *An. arabiensis*.

#### 2.3.5.2 Does the cage size, female age and body size affect the insemination rate of *An. gambiae s.s* and *An. arabiensis*?

The previous experiment revealed a very low (<30%) oviposition rate in *An. arabiensis* even when kept with males for seven days. Therefore, an experiment was designed to investigate the role of cage size, age and size of females on the insemination success (proportion of inseminated females) of *An. gambiae s.s.* and *An. arabiensis*.

Experiments were carried out in standard and large cages in parallel. For both species, 1400 pupae were placed in a plastic cup (10 cm diameter) filled with 200 ml of tap water and each species positioned in a standard cages for 24 hrs. Three hundred newly emerged male and 300 female mosquitoes of each species were transferred into separate cages of the two sizes. Six percent glucose solution was provided in all cages *ad libitum*. After three days, 25 females were randomly selected from each cage (standard and large for both species) by a technician unaware of the objectives of the study and dissected to evaluate insemination and to measure the wing lengths. The same number of females was dissected on days 4, 5 and 6. The experiment was implemented for three rounds with different batches of mosquitoes. Mosquitoes in this experiment were not offered blood-meals.

#### 2.3.5.3 *When is the peak oviposition time of An. gambiae s.s. Mbita strain?*

To have a consistently large proportion of females respond in oviposition experiments, it was important to establish the optimum interval between the last blood-meal and the bioassay for the local mosquito strains. Furthermore, putative test substrates for oviposition in mosquitoes (e.g. bacteria solutions, plants infusions, volatile inorganic compounds) are often unstable. It is therefore important to target the experiments during the peak in egg laying.

Cage experiments were carried out with different batches of mosquitoes 48 and 72 hrs after their second blood-meal. 'Gravid' mosquitoes for experiments were prepared following standard procedures. For each experiment, 100 *An. gambiae s.s.* mosquitoes were individually offered two oviposition cups with tap water in two-(equal) choice egg-count experiments at 17:00 h. In 50 of the 100 cages, both oviposition cups were retrieved and replaced with two new cups containing tap water at 21:30 h, the remaining 50 cages were left undisturbed through the night. The aim here was to investigate if the caged *An. gambiae s.s.* have several oviposition peaks during the night and to explore when skip oviposition (defined as the distribution of an egg batch into more than one substrate in one oviposition cycle) occurs. Half the cages remained undisturbed as a control to investigate if the exchange of cups might interfere with the oviposition response during the night. The experiment was ended at 08:00 h the following morning and the number of eggs in each cup recorded. Both experiments were carried out for three rounds.

#### 2.3.5.4 *Do non-human host sources of blood-meal have an impact on egg laying in An. gambiae s.s.?*

*Anopheles gambiae s.s.* is highly anthropophagic (Garrett-Jones *et al.* 1980) and there is evidence that different host sources of blood-meals have an impact on the oviposition rate and fecundity (Olayemi *et al.* 2011a) of this species. An experiment was designed to elucidate the impact of feeding caged *An. gambiae s.s.* on non-human hosts on the proportion of females becoming gravid and the number of eggs laid by each female.

Different groups of mosquitoes were blood-fed on either a human arm or 1 rabbit. Blood-meals on the human arm were offered as described in the previous experiment. For rabbit host blood-meals, fur was shaved on the ventral side of the rabbit in an area of 15x5 cm (approximately equal to the area exposed by an extended human arm when

the hand was covered with a latex glove). The rabbit was then held in a restrainer that limited movement and exposed the shaven underside. Mosquitoes to be fed were held in a cage positioned at the base of the restrainer allowing free access to the shaven area. In each treatment a group of 300 females were fed on two consecutive days. All mosquitoes that did not blood-feed on the first day were removed from the cages. The blood-fed mosquitoes were then held together with 300 males in standard cages for 72 hrs. A total of 100 female mosquitoes were randomly selected from each of the two cages, aspirated out by a technician unaware of the objectives of the research, and dissected to determine if they were gravid or not. Another 25 females visually appearing gravid were selected by an experienced technician from each of the two cages. These 25 females were tested individually in no-choice egg-count cage experiments to compare the proportion of females that laid eggs and the number of eggs laid per female fed on either rabbit or human blood. The experiment was done for three rounds using different batches of mosquitoes.

### 2.3.6. Statistical analyses

Multivariable analyses were implemented using generalised estimation equations (GEE) to analyse how proportions were affected by test variables. Different batches of mosquitoes from different rounds of an experiment were considered clustered (not independent) and included in the GEE model as a repeated measure. To evaluate the impact of including male mosquitoes in cages with blood-fed females on the proportions of mosquitoes that lay eggs, a GEE model was fitted with binomial distribution, logit link function and exchangeable correlation matrix. The presence of male mosquitoes (coded a 1 if a male is present, and 0 otherwise) in the holding cages was included in the model as a fixed factor. The association between the proportion of female mosquitoes inseminated and cage size (standard=0, large=1), mosquito age (a four level categorical variable coded 3, 4, 5, and 6 days) and mosquito size (measured in terms of wing length) was assessed using two separate GEE models (binomial distribution, logit link function, exchangeable correlation matrix) for the two test species. An interaction term for age and cage size was also included in the model. Similar models were fitted for the experiment on host blood-meal sources with the blood-meal source as a fixed factor; and the experiment on oviposition time with time period as a fixed factor. The mean numbers of eggs and their corresponding 95% confidence intervals (CIs) for two treatments were calculated as the exponent of the

parameter estimates based on generalised linear models with negative binomial distributions with no intercept included.

Data were analysed with IBM SPSS Statistics Version 20 (IBM-Corp 2011) and R software version 2.13.2 using various functions from the packages MASS, epicalc, lme4, effects, geepack, aod and gee (R Team 2011).

### 2.3.7. Ethical considerations

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

## 2.4. Results

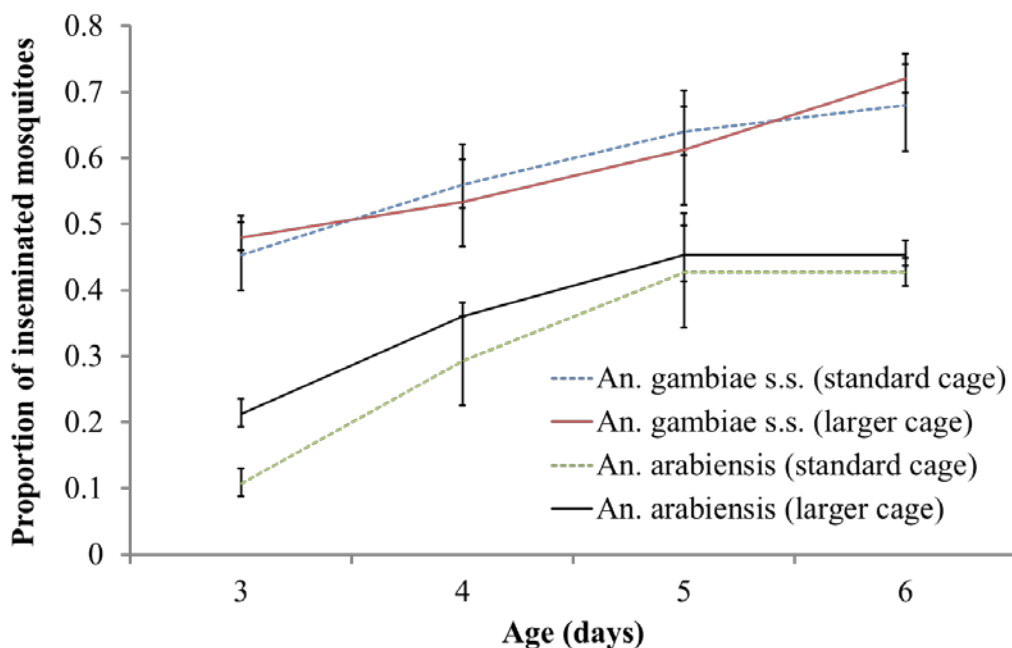
### 2.4.1. Including males in holding cages after blood-meals increases the proportion of ovipositing females in *Anopheles gambiae sensu stricto*

A female *An. gambiae s.s.* was nine times more likely to lay eggs if, after a blood-meal, she was held with males than without them (OR = 9.0, 95% CI = 7.9 – 9.5,  $p < 0.01$ ). On average 84% (95% CI 77 – 89%) of females laid eggs per experiment repeat when held with males after the blood-meal compared to 36% (95% CI 29-44%) when held without. Whilst the total number of eggs laid by females held with males (2904 eggs (95% CI 2844 – 2968)) was three times as high as the total number laid by females kept separated from males after blood-meals (994 eggs (95% CI 959 – 1030)), the mean number of eggs laid per female was similar in mixed-sex cages (66 eggs, 95% CI 44–99 eggs) and female only cages (54 eggs, 95% CI 36–82) highlighting the importance of recording individual rather than groups of mosquitoes.

Only an average of 25% of *An. arabiensis* (95% CI 15 – 41%) laid eggs. The likelihood of laying eggs was not associated with the presence or absence of males in the cages after blood-feeding (OR 1.92, 95% CI 0.62–5.98,  $p = 0.658$ ) and dissections showed that a large proportion (>50%) of females that did not lay eggs were not inseminated. The mean number of eggs laid per female that laid was 63 (95% CI 59 – 68).

#### 2.4.2. Age and body size of female mosquitoes can impact on insemination success in *An. gambiae s.s.* and *An. arabiensis* irrespective of the size of the holding cage

The proportion of inseminated mosquitoes increased with time and age for both species (Table 2.1., Figure 2.2.). However, *An. arabiensis* was 6 times less likely (OR 0.16, 95% CI 0.12-0.23,  $p < 0.01$ ) to be inseminated than *An. gambiae s.s.* The mean proportion of inseminated *An. gambiae s.s.* increased linearly to 72% (95% CI 61 – 81%) six days after emergence. The insemination rate of *An. arabiensis* peaked five days after emergence with 45% (95 CI 36 – 57%) inseminated (Figure 2.2.). Cage size did not improve insemination rate for *An. gambiae s.s.*. In *An. arabiensis*, an improved insemination rate in larger cages was only observed for three day old females but not for older females (Table 2.1.).



**Figure 2.2** Insemination rates of cages *An. gambiae s.s.* and *An. arabiensis* (Mbita strains) in standard (30×30×30 cm) and large (60×60×60 cm) cages with increasing age. (n=75).

The average length of the left wing of *An. arabiensis* was 4.20 mm (95% CI 4.16 – 4.23 mm) compared to 3.76 mm (95% CI 3.70 – 3.82 mm) for *An. gambiae s.s.* While body size did not affect *An. gambiae s.s.* insemination, *An. arabiensis* females were 6.6 times more likely to be inseminated with every unit increase in wing length (Table 2.1.).



**Table 2.1 Multivariable analysis of factors tested in association with the rate of insemination in experiments investigating the impact of cage size on the inseminations rate of *Anopheles gambiae* and *Anopheles arabiensis***

Variable	<i>Anopheles gambiae</i> s.s.		<i>Anopheles arabiensis</i>	
	OR (95% CI)	P value	OR (95% CI)	P value
<b>Age of mosquito in days</b>				
3		1		1
4	1.34 (1.30 – 1.48)	<0.001	3.40 (2.40 – 4.74)	<0.001
5	1.98 (1.62 – 2.43)	<0.001	6.23 (3.23 – 12.0)	<0.001
6	2.81 (1.75 – 4.52)	<0.001	6.20 (3.99 – 9.64)	<0.001
<b>Cage size</b>				
standard		1		1
large	1.02 (0.97 – 1.07)	0.457	2.13 (1.66 – 2.73)	<0.001
<b>Body size</b>				
Wing length	0.68 (0.33 – 1.37)	0.278	6.68 (2.57 – 17.4)	<0.001
<b>Interaction between mosquito age and cage size</b>				
3*standard		1		1
3*large		1		1
4*standard		1		1
4*large	0.81 (0.63 – 1.04)	0.099	0.61 (0.32 – 0.69)	<0.001
5*standard		1		1
5*large	0.80 (0.42 – 1.51)	0.493	0.53 (0.37 – 0.75)	<0.001
6*standard		1		1
6*large	1.06 (0.67 – 1.70)	0.799	0.47 (0.42 – 0.52)	<0.001

OR=Odds ratio; CI=Confidence interval; \*indicated interaction term

2.4.3. Blood-meals from rabbits reduce the proportion of females that become gravid and lay eggs but does not affect egg numbers laid by gravid females

Females fed on rabbit blood were less likely to become gravid compared to those fed on human blood (Table 2.2.). When selected from the cage randomly, on average 59% (95% CI 44 – 73) of those females offered blood from a rabbit were gravid and laid

eggs while 83% (95% CI 68 – 92%) of females fed on a human blood were gravid and laid eggs. Of those carefully selected as gravid based on their abdominal appearance, equal proportions of females from both treatments laid eggs when offered an oviposition medium. The mean number of eggs laid by individual gravid females also did not depend on the host source of blood-meals (Table 2.2).

**Table 2.2 Effects of host source of blood-meal on oviposition of cages *An. gambiae* s.s. (Mbita strain)**

	Mean (95% CI)	OR (95% CI)	P value
<b>Percentage of blood-fed mosquitoes gravid at dissection</b>			
<b>Human arm</b>	83 (68 – 92)		1
<b>Rabbit</b>	59 (44 – 73)	0.30 (0.14 – 0.66)	0.030
<b>Percentage of gravid mosquitoes that laid eggs in cage experiments</b>			
<b>Human arm</b>	72 (57 – 83)		1
<b>Rabbit</b>	73 (59 – 84)	1.07 (0.95 – 1.20)	0.852
<b>Number of eggs per gravid female that laid</b>			
<b>Human arm</b>	64.0 (57.1 – 71.8)		1
<b>Rabbit</b>	62.1 (52.2 – 73.9)	0.97 (0.85– 1.11)	0.661

OR=Odds ratio; CI=Confidence interval

2.4.4. Egg-count cage bioassays with *An. gambiae* s.s. Mbita strain are best implemented 72 hrs after the last blood-meal during the peak oviposition time between 17:00-21.30 h.

Due to the poor oviposition success in the colony reared *An. arabiensis* the following experiments were implemented with *An. gambiae* s.s. only.

Female *An. gambiae* s.s. were 8.7 times (95% CI 4.3 – 18.4,  $p < 0.001$ ) more likely to lay eggs when provided with substrates 72 hrs after blood-meals compared with females provided with substrate after 48 hrs. On average 81% (95% CI 71 – 93%) of females

presented with oviposition substrate 72 hrs after blood-meals laid eggs compared to only 33% (95% CI 32 –35%) 48 hrs after blood-meals (Table 2.3.).

**Table 2.3 Evaluation of egg-laying periodicity in caged *An. gambiae* s.s. (Mbita strain)**

	<b>N</b> <b>(exposed)</b>	<b>n</b> <b>(responded)</b>	<b>Percentage of</b> <b>mosquitoes that laid</b> <b>eggs (95% CI)</b>	<b>P value</b>
<b>Time since blood-meal (cups left overnight)</b>				
48 hours	200	75	33 (32 – 35)	<0.001
72 hours	150	122	81 (71 – 93)	
<b>Egg laying period (72 hours after blood-meal)</b>				
17:00 – 21:30 h		114	96 (94 – 100)	<0.001
21:30 – 08:00 h	150	3	3 (2 – 5)	
Both periods		2	2 (1 – 4)	
<b>Skip oviposition (72 hours after blood-meal)</b>				
17:00 – 21:30 h		28	90 (89 – 96)	<0.001
21:30 – 08:00 h	31	1	3 (2 – 5)	
Both periods		2	6 (5 – 7)	

Approximately 76% (95% CI 71-82) of females laid eggs whether cups were left untouched over night or changed by 21.30 h, suggesting that changing the cups did not interfere with oviposition. Ninety six percent of females that laid eggs in the experiment where cups were exchanged did so between 17:00 – 21:30 h (114/119). The tendency for individual female mosquitoes to lay eggs in both cups (also known as skip oviposition) was observed in 26% (95% CI 19– 30) of all responding females and 90% (95% CI 89 – 96%) of it took place before 21.30 h. Only two mosquitoes laid eggs before and after 21.30 h (Table 2.3.) and one only after 21.30 h.

## 2.5. Discussion

To implement empirical egg-count experiments with replicable and generalizable results it is vital to ensure a consistent and predictable oviposition rate in the test mosquitoes. This study highlighted important considerations when preparing gravid *An. gambiae s.s.* oviposition experiments: the insemination rate of the test mosquitoes, the blood-meal host source and the timing and duration of the actual experiments. It provided guidelines that lead to a high and consistent response rate in test mosquitoes (Figure 2.3).

- I. Include an equal or excess number of male mosquitoes in mosquito preparation cages:** Continued mating after blood meals causes more female mosquitoes mature eggs and lay during experiments.
- II. Provide female mosquitoes with sufficient natural host-source blood meal:** More *An. gambiae s.s.* mosquitoes become gravid after blood meals from a human host source. However, a supplemental blood meal ensures that most mosquitoes get enough blood protein to complete ovarian development.
- III. Determine the peak period for egg laying:** The shortest time window, if any, when the majority of mosquitoes lay eggs represents the ideal period for setting up choice tests. This varies and is best confirmed prior to experiments for each laboratory strain.

**Figure 2.3 Guidelines for preparing gravid *Anopheles gambiae s.s.***

Consistent with previous studies, insemination was shown to be important for egg laying by *An. gambiae s.l.* (Chambers & Klowden 2001; Klowden & Russell 2004). The proportion of test females that laid eggs in the bioassays more than doubled when they were held in cages with males after blood-feeding providing a longer period to mate. This gives further evidence that at least in laboratory settings, mating in *An. gambiae s.s.* continues after the females have taken a blood-meal. This also complements the Lyimo and Takken who showed that blood-feeding precedes mating in a large proportion of *An. gambiae s.s.* in the field (Lyimo & Takken 1993). Depending on the age of insects at blood-feeding this could be of great consequence. Cages with blood-fed females must be conditioned for insemination by including male mosquitoes especially when test females are blood-fed at a relatively young age (here two to three days). In experiments evaluating the rate of insemination with non-blood fed mosquitoes it was shown that approximately one fifth of *An. gambiae s.s.* were still virgins when six days old (the average age of test mosquitoes across studies). This might explain the similar proportion of test mosquitoes that failed to lay eggs in the bioassays even under optimized preparation procedures. Increasing the number of males in cages might improve both the rates of insemination and egg laying (Charlwood & Jones 1979). However, Verhoek and Takken (1994) have demonstrated that ratios of 3:1 male to

female do not significantly improve the rate of mating over a 1:1 ratio for *An. gambiae s.l.*.

Blood-meals taken on a rabbit resulted in a lower proportion of females that became gravid. This suggests that the common practice of substituting human hosts with rabbits, and possibly other secondary host sources of blood, (Munga *et al.* 2005; Otienoburu *et al.* 2007; Kweka *et al.* 2011) potentially reduces the proportion of gravid females and therefore increases the risk of including mosquitoes in bioassays that will not lay eggs. Excluding mosquitoes that did not lay eggs from the analysis showed that the actual mean number of eggs laid per female that became gravid after the blood-meal was the same irrespective of the source of blood. If groups would have been tested instead a false lower mean numbers of eggs with rabbit blood-meals would have been inferred. Great caution is advised in selecting gravid mosquitoes where secondary host sources of blood are used in preparing test mosquitoes. By using individuals it is possible to implement choice test even where the impact of the host-source of blood-meal is large or unknown. Mosquitoes that do not lay eggs can be removed from the final data set and reported as a separate entity of interest.

The majority of the Mbita strain of *An. gambiae s.s.* did not yet lay eggs up to 48 hours after the last blood-meal; egg laying was constrained to early evening hours of the third night ( $\approx 72$  hours) after blood-meals. This confirmed the findings of Haddow and others (Haddow & Ssenkubuge 1962). Consequently, egg-count cage bioassays with the Mbita strain were best done between 17:00 – 21:30 h on the third night after the last blood-meal. However, controversial results have been published in the past. Other studies with *An. gambiae s.s.* have shown that some strains lay eggs 48 hours after a blood-meal and it was suggested that egg-laying times depend on local conditions, blood-feeding times and temperature (McCrae 1983). Some studies also showed that *An. gambiae s.s.* can lay eggs at any time throughout the dark phase of day (McCrae 1983; Fritz *et al.* 2008). In consideration of these divergent findings, it is strongly recommend that oviposition periodicity studies precede all oviposition studies with different strains of this species. This does not only apply to behavioural bioassays but is equally important when investigating chemoreception in gravid females and changing sensilla sensitivity in response to changes to the physiological stage of a mosquito. These studies are often done 24 and 48 hours after a blood-meal (Qiu *et al.* 2006; Rinker *et al.*

2013) which might not necessarily coincide with the time a female searches for an oviposition site.

The insectary-reared Mbita strain of *An. arabiensis* showed low rates of insemination compared to *An. gambiae s.s.* from the same area. At best 45% of all female *An. arabiensis* mosquitoes were inseminated after six days when left with an equal number of males throughout the period. There is some evidence that *An. arabiensis* is more difficult to mate and colonise in the laboratory compared to *An. gambiae s.s.* (Marchand 1985), although others have shown contrasting results where the rate of insemination in *An. arabiensis* of every age between 1 – 7 days was shown to be higher than that of *An. gambiae s.s.* (Verhoek & Takken 1994). The latter findings were probably due to longer colonization of the strain which selected for this trait. Increasing the size of holding cages to increase mating activity and insemination success in *An. arabiensis* did not improve these activities. Low insemination and consequently low oviposition rates make it difficult to study the oviposition response of *An. arabiensis* to different oviposition substrates. Especially, when groups of *An. arabiensis* are used caution should be exercised in interpreting the results examining the mean egg numbers critically to ensure that the majority of the exposed females actually laid eggs. It has been shown that larger females were more likely to be inseminated compared to smaller ones. Attempting to optimize larval rearing conditions to increase adult body size and selecting for the largest females from the colony cages for experiments might thus be a reasonable approach to increasing oviposition rates in egg-count cage bioassays.

## 2.6. Conclusions

This study demonstrated that the responsiveness of gravid *An. gambiae s.s.* mosquitoes can be enhanced by optimizing procedures for handling pre-gravid mosquitoes and timing bioassays appropriately. Oviposition success with this species is affected by the female mosquito's state of insemination, the host-source of blood-meal and the timing of experiments. By ensuring that (1) female mosquitoes are inseminated, (2) provided with a sufficient amount of blood from a favourable host-source and (3) that the time when oviposition substrates are provided is aligned with the mosquito's peak oviposition period, the majority will consistently lay eggs. For the Mbita strain of *An. gambiae s.s.* used in this study, responses were optimal when mosquitoes were provided with two, 24-hour-apart blood-meals from a human host, kept in cages conditioned with

an equal number of conspecific age-mate males at and after blood-meals, and provided with substrates 72 hours after the final blood-meal between 17:00 – 21:30 h. Low oviposition rates with *An. arabiensis* were partly due to poor insemination. While this study suggests that rearing larger mosquitoes might improve this, more studies should be done with *An. arabiensis* to understand and optimize other factor that contribute to low insemination rates before laboratory strains of this species are used for oviposition studies.

## Chapter 3. Analysing the oviposition behaviour of malaria mosquitoes: design considerations for improving two-choice egg count experiments



Semi-field structures for egg-count behavioural bioassays (Source: Jenny M. Lindh)

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### 3.1. Abstract

**Background:** Choice egg-count bioassays are the most popular tool for analysing oviposition substrate preferences of gravid mosquitoes of all species. These bioassays vary widely in details but all centre on the assumption that mosquitoes lay most of their eggs or egg raft in substrates they prefer; the number of eggs or egg-rafts therefore must be the indicator for substrates preferences. This study was aimed at testing this hypothesis with *An. gambiae s.s.* and ultimately improving the design of choice egg-count experiments for measuring oviposition substrates preferences of the malaria vector *Anopheles gambiae sensu lato (s.l.)*, a mosquito that lays single eggs.

**Methods:** Simple experiments were done to measure the distribution of eggs by *An. gambiae s.s.* and test assumptions of common approaches for implementing and analysing egg-count experiments with this species. A total of 1600 gravid mosquitoes were individually provided with two oviposition cups in a cage. Both cups contained the same amount of the same oviposition substrate; 100 ml of tap water. The experiments were started at 17:00 h and the number of eggs in each cup recorded the next at morning at 08:00 h. Data from these experiments were used to describe the egg laying characteristics of this species and design a customised and improved two-choice egg-count bioassays for measuring oviposition substrate preferences in this species.

**Results:** The majority of mosquitoes provided with oviposition substrates laid eggs (1443 out of 1600). The number of eggs laid by individual mosquitoes was overdispersed (median = 52, eggs, interquartile range 1-214). Mean numbers of eggs laid per female differed widely between replicates and batches leading to a highly heterogeneous variance between groups and/or rounds of experiments. This violates a primary assumptions for parametric tests (homoscedasticity) and faults the common use of ANOVA and t-test to analyse egg-counts bioassay data for this species especially with small sample sizes. Moreover, one-third of the mosquitoes laid eggs unequally in both cups (skip-oviposited) with similar substrates giving the illusion of choice. This is masked when groups of mosquitoes are used for experiments. Sample size estimations showed that it would take 165 individual mosquitoes to power bioassays sufficiently (power=0.8,  $p=0.05$ ) to detect a 15% shift in comparative preferences of two treatments.

**Conclusion:** Two-choice egg count bioassays with *Anopheles* were shown to be best done with a two-tier design that (i) implements a parallel series of experiments with

mosquitoes given a choice of two identical substrates choices and (ii) uses a single mosquito in each test cage rather than groups of mosquitoes to assess the preference of a test or control solution. This approach, with sufficient replication, lowered the risk for detecting pseudo-preferences and drawing wrong conclusions on oviposition substrate preferences.

### 3.2. Background

*Anopheles* mosquitoes are efficient and resilient vectors of human malaria and filariases in Africa. These vectors are mainly controlled by extensive use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) of houses (Enayati & Hemingway 2010). The two interventions exploit the tendency of *Anopheles funestus* *s.l.* and *An. gambiae s.l.*, the major vectors of malaria in sub-Saharan Africa, to bite and rest indoors (Takken & Knols 1999) and have together contributed to a remarkable and consistent decline in the transmission of malaria through the last decade (Nyarango *et al.* 2006; Ceesay *et al.* 2010; O'Meara *et al.* 2010; Mharakurwa *et al.* 2013). However, like all vector control interventions, these too have limitations and when used in isolation could fall short in areas with: (1) strains selected for physiological resistance to insecticides (Chandre *et al.* 1999a; Chandre *et al.* 1999b; Ranson *et al.* 2009; Ranson *et al.* 2011) (2) secondary vector species that live and bite outdoors (Tirados *et al.* 2006; Russell *et al.* 2011), and (3) cryptic vector sub-groups that bite in the early evening and/or bite outdoors (Riehle *et al.* 2011). These and a complex of other factors including increasing drug resistance and high costs of interventions make malaria resurgence a grim reality (Cohen *et al.* 2012). New strategies with novel tools that combine with LLINs and IRS to target these elusive groups of vectors in addition to the major vectors could prevent the resurgence of disease and hasten malaria elimination.

Larval source management (LSM) can be a complementary intervention for targeting all strains of malaria vectors irrespective of their state of insecticide resistance or resting and biting tendency. However in areas with extensive oviposition sites LSM becomes challenging (Fillinger *et al.* 2008; Fillinger & Lindsay 2011). Attempts to target oviposition sites by identifying precisely the physical features of water bodies with mosquito larvae have so far been unsuccessful (Majambere *et al.* 2008; Fillinger *et al.* 2009b). Nevertheless field studies suggest that the presence of early instar larvae in water bodies is non-random, which may indicate that gravid females select particular water bodies in which to lay their eggs. These studies imply that favourable aquatic oviposition sites though highly heterogeneous in form, space and time (Fillinger *et al.* 2004; Majambere *et al.* 2008; Fillinger *et al.* 2009b; Ndenga *et al.* 2011) will display key features that act as signature cues for gravid mosquitoes seeking to lay eggs. Identifying the cues that elicit oviposition behaviour could aid the targeting of larvicides

into productive mosquito oviposition sites and allow the development of odour-baited gravid mosquito traps for *Anopheles*.

Laboratory experiments within insect cages are a simple first step in identifying cues that guide short range habitat selection in gravid mosquitoes (Isoe *et al.* 1995b). Of these experiments, choice egg-count bioassays are the most common and have been used to search for cues that are preferred or avoided by mosquitoes seeking to lay eggs (Sumba *et al.* 2004a; Bukhari & Knols 2009). Here eggs or egg rafts laid in test substrates by groups of mosquitoes are counted and compared to those laid in a reference substrate, the control. Using these choice tests chemicals that influence oviposition have been identified for *Stegomyia* (Bentley *et al.* 1979; Hwang *et al.* 1982; Sharma *et al.* 2008), *Culex* (Hwang *et al.* 1980; Hwang *et al.* 1982; Millar *et al.* 1992; Millar *et al.* 1994), and recently for *An. gambiae s.s* (Rinker *et al.* 2013). In addition egg-count bioassays have been used to investigate the response of gravid *Anopheles gambiae s.s.* to bacteria cultures (Blackwell & Johnson 2000; Huang *et al.* 2006a; Lindh *et al.* 2008) with different outcomes. Many choice egg-count experiments with *An. gambiae s.l.* have been prepared, implemented and reported in a unique way making it difficult to generalise findings.

It was hypothesized that the egg-laying behaviour of *An. gambiae s.l.* makes it necessary to re-design choice egg-count bioassays uniquely for this species (Herrera-Varela *et al.* 2014). The need to consider the design of these bioassays for species that lay single eggs and exhibit skip oviposition has been well shown by Chadee and Corbet (1993) who proposed a new study design for *Aedes*: one that entailed “recording the distribution of eggs by individual females initially provided with an array of identical sites”. However, their work has been ignored in consequent studies with this genus (Ponnusamy *et al.* 2008; Seenivasagan *et al.* 2009; Ponnusamy *et al.* 2010; Seenivasagan *et al.* 2010; Bandyopadhyay *et al.* 2011). The present study therefore aimed to present new approaches for: (1) implementing tests to compare two substrates, and (2) analysing and reporting finding of egg-count experiments.

### 3.3. Methods

#### 3.3.1. Study site

Experiments were carried out at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus at Mbita on the shores of Lake Victoria, Western Kenya (0° 26' 06.19" South; 34° 12' 53.12" East; altitude 1149 m). Choice egg-count bioassays were carried out in sheds, 10 m long × 5 m wide × 2.8 m high with walls constructed from dry reed mats and roofs of translucent corrugated polycarbonate sheets. Every shed contained two tables with capacity to hold 50 cages with a gap of 40 cm between each cage. Experiments were carried out at ambient conditions of temperature, humidity (mean daily temperature  $27 \pm 5^\circ\text{C}$ , relative humidity  $55 \pm 10\%$ ) and light.

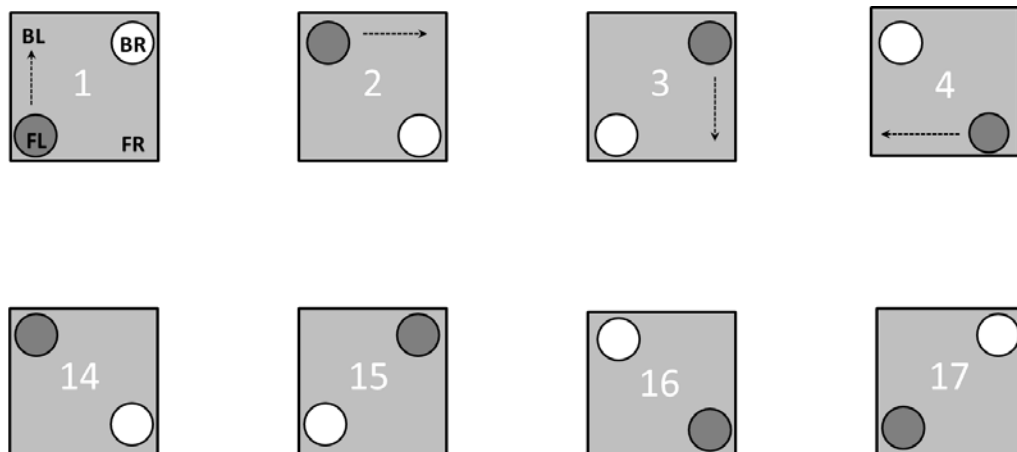
#### 3.3.2. Experimental procedures

To improve the design of two-choice egg-count bioassays and promote empirical evaluation of substrate preferences it is important to understand the natural egg-laying pattern of *An. gambiae s.s.* and take it into account when designing experiments. This experiment was aimed at identifying an appropriate layout for egg-count oviposition studies with *An. gambiae s.s.* and highlight the importance of sample size in egg-count experiments. Specifically (1) the number of eggs laid by individual *An. gambiae s.s.* mosquitoes was estimated and their statistical distributions and variances explored, (2) skip-oviposition within experimental cages was quantified and (3) the variability in egg counts and response towards two equal choices of oviposition substrate analysed between rounds.

##### *3.3.2.1. Two equal choice egg-count bioassays with individual gravid females to explore egg distribution and variability in egg-counts.*

For gravid mosquitoes 300 two to three-day old female and 300 male *An. gambiae s.s.* of the same age were kept in a standard adult mosquito holding cage. The mosquitoes were starved of sugar solution for up to six hours before they were permitted to blood-feed from a human arm at 18:30 h for 15 minutes on two consecutive evenings. Mosquitoes that were not fully engorged with blood after the first blood-meal were removed from the cage. Individual six to seven day old gravid females were presented

with two equal choices of tap water for oviposition. In order to prevent any possible bias associated with the position of the cup, the placement of cups was systematically varied between adjacent cages. The four corners of every cage were named relative to the front of the cage (inset-sleeve end) as front left (FL), back left (BL), back right (BR) and front right (FR). The first cup was placed at the FL position of the first cage and randomly referenced as ‘control’ or ‘test’. ‘Test’ cups in subsequent cages were moved one corner step in a clockwise direction (Figure 3.1). The second cups were added in the diagonally opposite corner and referenced as ‘control’. The egg-laying response of a gravid female towards these test and control cups was recorded as binary data. The numbers of eggs laid by every female in each cup was also noted. In total 41 rounds of two-choice egg-count experiments with different batches of mosquitoes were implemented. Between 20 and 50 individual female mosquitoes were exposed to the two equal choices per round but only 85-92% of all exposed females responded (laid eggs) per round (n=17-46). The response of 1443 females were analysed in total.



**Figure 3.1 Illustration of the arrangement of oviposition cups and cages in two choice egg-count bioassays.** The solid circles represent test cups which are arranged in the clockwise direction. Control cups (open) are positioned diagonally opposite. FL=front right,, BL=back left, BR=back right and FR=front right

### 3.3.3. Sample size considerations.

When implementing two choice bioassays with two different oviposition substrates the assumption is that there is no preference between the two substrates. However, it is

likely that the chances of a type 1 error (i.e. artefact preference for one substrate over then other) are increased with a small number of replicates. Therefore the data were used to estimate the sample size required for routine bioassays using power calculation for two-sample comparisons of proportions (`power.prop.test` function in R software) and for a single proportion compared to a known proportion (Brant 2015). A 50% distribution was assumed when equal choices are presented ( $p_1=0.5$ ). Power estimates were generated to predict a 15% increase or decrease ( $p_2=0.65$ ) in oviposition response to a test medium at sample sizes between 5 and 225 and generated estimates of effect sizes generated that can be detected with 80% power at a 5% significance level for the same range of sample sizes.

#### 3.3.4. Statistical analyses

Multivariable analyses were implemented using Generalised estimation equations (GEE) to analyse data from the two equal choice egg-count bioassays. One mosquito was presented with two cups with tap water for oviposition. The data derived from two cups for an individual mosquito was related and therefore counts/proportions of eggs laid in a cage by an individual mosquito were considered repeated measures in the GEE models. GEE models assuming exchangeable working correlation and with a negative binomial distribution with a log link function fitted were used to explore differences in egg counts between control and test cups and between rounds (fixed factors) whilst GEE models with a binomial distribution and logit link function fitted were used to estimate the likelihood of a female choosing the test cup over the control. All mean counts or mean proportions per treatment and their 95% confidence intervals (CIs) were calculated as the exponential of the parameter estimates for models with no intercept included.

Egg numbers laid by individual females were tested for normality using the Kolmogorov-Smirnov test. Additionally, overdispersion (i.e. meaning the variability in the data is not equal to the mean, as in the Poisson distribution) was assessed by inspecting the residual deviance which follows a Chi-squared distribution where the expected value should be close to the degrees of freedom if the data is not overdispersed. Overdispersion is a problem because it may cause standard errors of the estimates to be deflated or underestimated i.e. a variable may appear to be significant when it is in fact not. The assumption of homogeneity of variance in the correlated



count data collected from control and test cups was tested with the Pitman-Morgan test (Morgan 1939; Pitman 1939). Data were analysed with IBM SPSS Statistics Version 20 (IBM-Corp 2011) and R software version 2.13.2 using various functions from the packages MASS, epicalc, lme4, effects, geepack, aod and gee (R Team 2011).

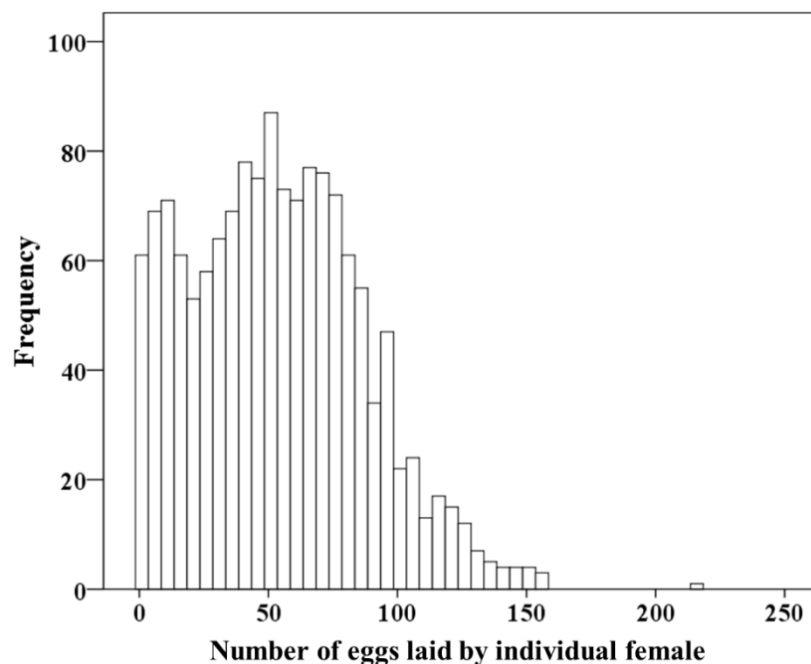
### 3.3.5. Ethical considerations

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

## 3.4. Results

### 3.4.1. Individual *Anopheles gambiae s.s.* lay a highly variable number of eggs despite standardized preparation procedures

The number of eggs laid by 1,443 individual mosquitoes provided with two cups of tap water was highly variable and ranged between 1-214 eggs (interquartile range 48), with a median of 52 eggs per female. Egg numbers were overdispersed with the variance exceeding the mean ratio indicating an overdispersed distribution (Figure 3.2) even with this large sample.



**Figure 3.2 Histogram showing the frequency distribution of egg-counts from individual *An. gambiae s.s.* (Mbita strain) (n=1443)**

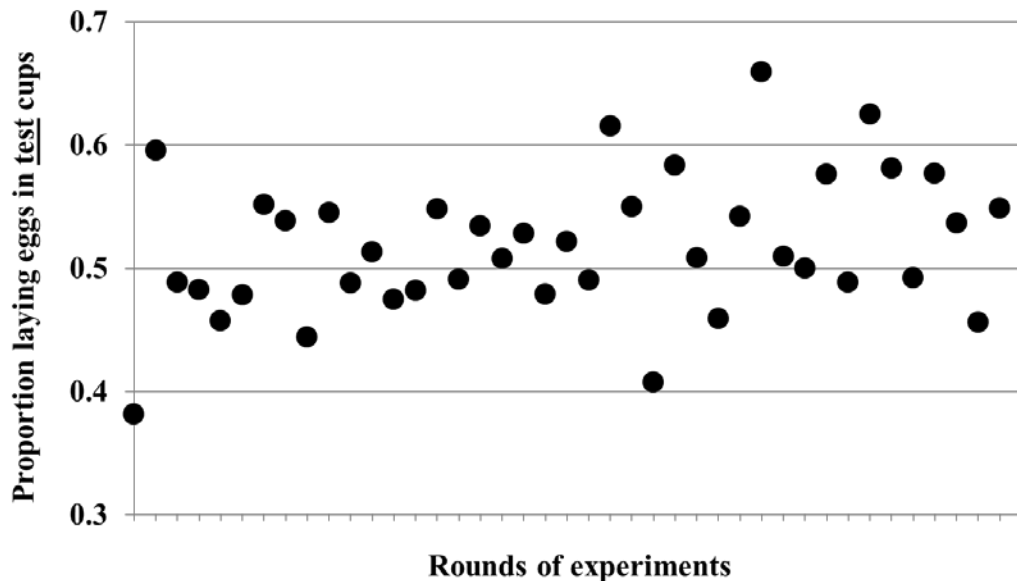
### 3.4.2. The response of gravid *An. gambiae s.s.* presented with two equal choices can be skewed when egg counts are compared

In total 77,664 eggs were laid by 1,443 females tested individually over 41 rounds; 41,113 (53%) eggs were laid in cups randomly labelled as test cups, and 36,551 (47%) in control cups. In addition to the overdispersed distribution of eggs the two correlated variances of the egg counts in control and test cups were not homogeneous ( $p < 0.01$ ). Generalised linear modelling with a negative binomial distribution fitted indicated that the differences in egg counts between control and test cups were small but statistically significant (rate ratio (RR) 1.13 (95% CI 1.01-1.25,  $p < 0.01$ ). Furthermore, counts differed significantly between rounds ( $p < 0.001$ ) with mean numbers of eggs laid per female in a cup in different rounds ranging between 17 (95% CI 13-20) and 46 (95% CI 39-55).

### 3.4.3. Comparing the proportional distribution of eggs leads to more reliable inference than using absolute egg counts

Rather than evaluating the actual egg counts, the proportion of eggs laid in test versus control cups (experiments with groups and individuals) or the proportion of mosquitoes (experiments with individuals) selecting test versus control cups for oviposition can be compared. A total of 1,902 cups (out of  $2 \times 1,443 = 2,886$  cups) received eggs; 979 test cups (51%) and 923 (49%) control cups in the 41 rounds of experiments. The distribution of individual responses towards two equal choices was therefore more balanced than the comparison of egg numbers (see above). Consequently, generalised linear modelling with a binomial distribution fitted showed that the odds of a female choosing one cup over the other when both contain the same oviposition substrate was similar ( $p = 0.08$ ) with a mean proportion of 51.4% (95% CI 49.0-53.8%) selecting the test cup for oviposition. This relatively balanced outcome is based on a very large number of samples. Looking at the individual rounds containing between 17 and 45 samples only (Figure 3.3), the proportions of females selecting the test cup is highly variable with significant between-round differences ( $p < 0.01$ ). This natural baseline variability must be measured during experiments and taken into account when

implementing choice experiments with different substrates. Otherwise, it would easily lead to false inferences especially where sample sizes are small.

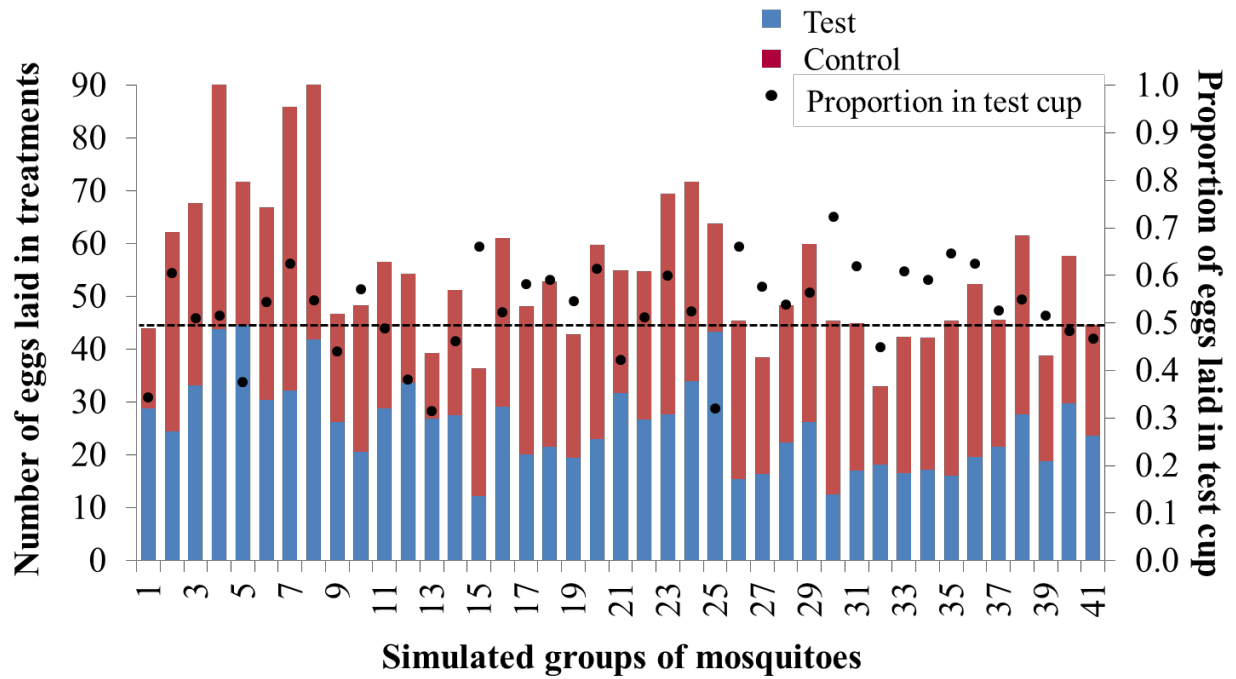


**Figure 3.3** Proportion of responses (presence of eggs) received by the test cups in two equal choice tests out of the total responses (test cups + control cups) counted per experimental round (n per round = 17-46).

3.4.4. High between-cage variability in egg counts and proportions must be expected when testing small groups of gravid mosquitoes in egg-count cage bioassays

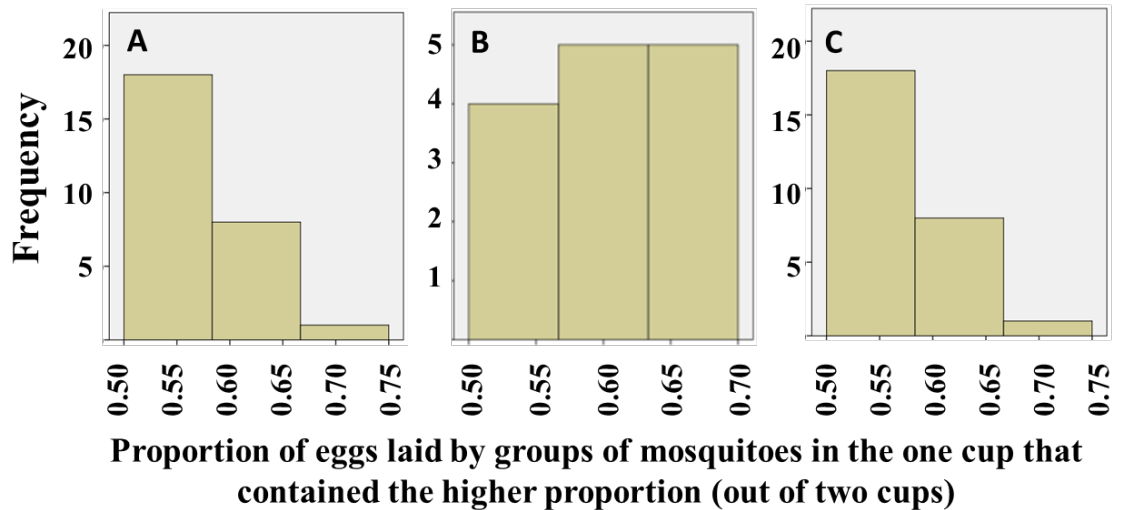
The majority of choice egg-count bioassays published for *An. gambiae s.l.* have been implemented with groups of mosquitoes (McCrae 1984; Huang *et al.* 2005; Rejmankova *et al.* 2005; Huang *et al.* 2006a; Huang *et al.* 2006b; Otienoburu *et al.* 2007). This scenario was simulated by combining the egg counts for test and control cups of all individual mosquitoes tested (responders) in a round. Therefore, the hypothesised group sizes varied from 17 to 46 mosquitoes per cage. Conventionally, the number of eggs laid per female is estimated by dividing the total number of eggs counted by the number of females introduced in the cage. Note, that in contrast to the simulation under these experimental conditions investigators cannot be sure of the actual number of females that laid and based on here presented observations it must be assumed that approximately 20% of the introduced females do not lay even when prepared under optimal procedures. The mean number of eggs per female per group was

highly variable and ranged from 33 to 92 between the assumed replicate cages (Figure 3.4).



**Figure 3.4 Mean number of eggs per female laid in test and control cup and proportion of eggs laid in test cup.** Analysis based on 41 simulated groups (replicates) of mosquitoes.

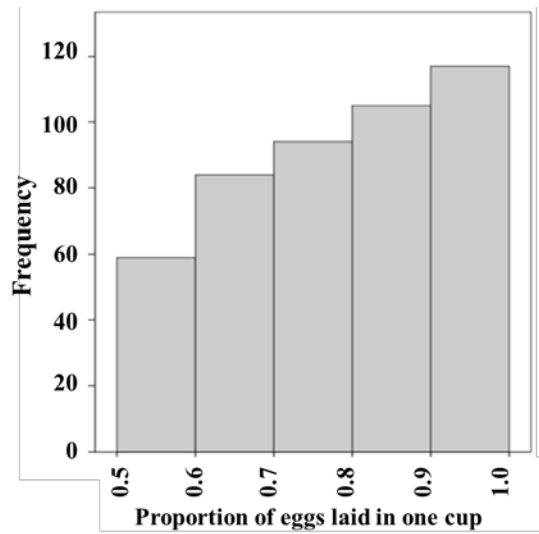
Similarly, the proportional distribution of eggs between the two cups, containing the same oviposition substrate, was in nearly half the groups unequal with one cup having > 60% of all eggs laid (Figure 3.5A). Notably, there was a negative correlation between the number of females per cage and the difference in proportion of eggs laid in test versus control cups (Spearman's  $\rho = -0.35$ ,  $p=0.03$ ). If a group consisted of less than 30 responders an unbalanced distribution of eggs (>60% of eggs in one cup) between the two equal choices occurred twice as often as a balanced one (Figure 3.5B), whilst in groups with more than 30 responders an unbalanced distribution was less frequent (Figure 3.5C).



**Figure 3.5** Frequency distribution of the proportions of eggs laid in one cup (higher proportion) over the other in two equal choice tests for simulated groups. (A) all 41 groups (B) groups with 10 individuals (C) groups with > 30 individuals.

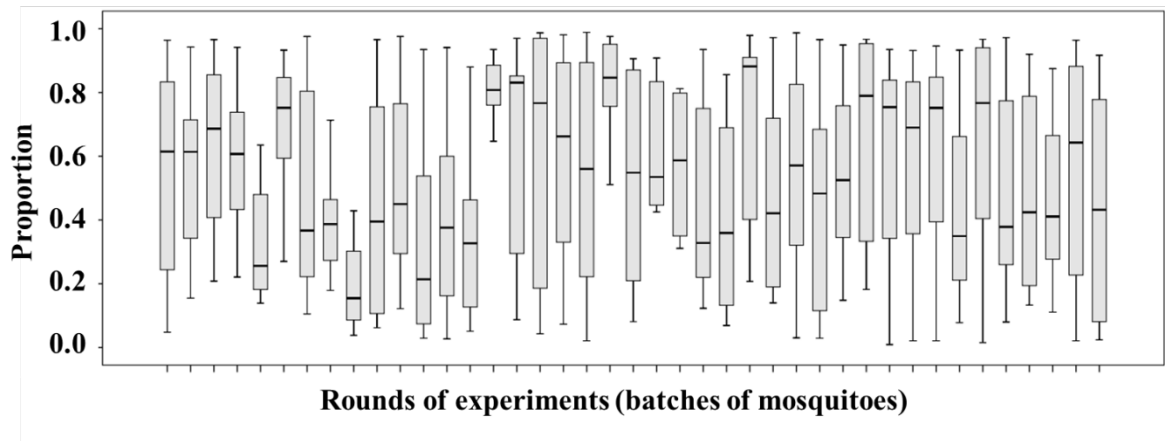
#### 3.4.5. One third of gravid *Anopheles gambiae s.s.* distribute their eggs in more than one oviposition medium ('skip-oviposition')

Individual *An. gambiae s.s.* females did not always make mutually exclusive choices of cups to lay their eggs when provided with two substrates. Of the 1,443 responders, 32% (459) laid eggs in both cups provided in the cage. Nevertheless, significant variability ( $p < 0.01$ ) was observed between batches of mosquitoes (rounds), with the proportion of skip oviposition ranging between 17% and 61% in individual experimental rounds. On average 32.4% (95% CI 29.0 – 35.8%) of the females per round laid eggs in both cups presented. Females that skip-oviposited did not lay more eggs compared to those that laid all eggs in one substrate ( $p = 0.873$ ). Importantly, most females that laid in both cups did not distribute their eggs equally in the identical substrates. The unequal distribution of eggs can therefore be wrongly interpreted as a preference for the substrate that received the higher number of eggs. In most cases a larger egg batch was laid in one cup and a smaller batch in the other cup (Figure 3.6). Three quarters of the females that skip-oviposited laid  $2/3^{\text{rd}}$  or more in one and  $1/3^{\text{rd}}$  or less of their eggs in the other cup.



**Figure 3.6** Frequency distribution of the higher proportion (>0.5) of eggs laid in one cup by skip-ovipositing mosquitoes

The unequal egg distribution might contribute to skewed egg counts, especially when the number of individuals tested in a sample and/or the number of replicate samples are low. This is illustrated by Figure 3.7 where the median proportion of eggs laid in the test cups is shown for every experimental round. Rarely were the proportions of eggs laid by skip-ovipositing females ( $n=4-20$ ) equally distributed in a single round. Nevertheless, on average for all 459 skip-ovipositing females, 54% (95% CI 45-63%) of the eggs were laid in test cups emphasizing the importance of a large enough sample size.



**Figure 3.7 Median proportion of eggs laid in the test cups by skip-ovipositing females (n=4-20) in every experimental round.**

3.4.6. To detect an increase in oviposition response of 15% as compared to the baseline proportion (80% power and 5% significance) at least 165 responders need to be tested in each treatment group.

Based on the design considerations presented above when implementing egg-count cage bioassays it is suggested to statistically compare two proportions derived from two independent (separate) random samples. The null hypothesis  $H_0$  is that the two samples' proportions are the same. The notation for the null hypothesis is  $H_0: p_1 = p_2$ , where  $p_1$  is the baseline proportion from choice experiments with two equal choices (control substrate vs control substrate), and  $p_2$  is the proportion from the experimental test comparing a putative oviposition cue against a control. The sample size will depend on the effect size one wants to detect. Here it was chosen to simulate (1) the relationship between sample size and the power of a study at 5% significance level at an effect size of 15% increase of  $p_2$  as compared to  $p_1$  and (2) the relationship between sample size and effect size ( $p_2$ ) at a fixed power of 80% at 5% significance level (Figure 3.8).

Based on sample size calculations for two independent proportions, 165 responders need to be tested in each group (165 for  $p_1$  and 165 for  $p_2$ ; total 340) to detect an increase or decrease in oviposition response of 15% ( $p_2=0.65$ ) compared to the baseline proportion ( $p_1=0.50$ ) at 80% power and 5% significance. With a smaller sample size the effect size that can be detected increases i.e. 90 replicates in each treatment arm can

detect a difference between the proportions of not less than 20% ( $p_1=0.50$  and  $p_2=0.70$ ) and 30 replicates of not less than 33% ( $p_1=0.50$  and  $p_2=0.83$ ) (Figure 3.8).

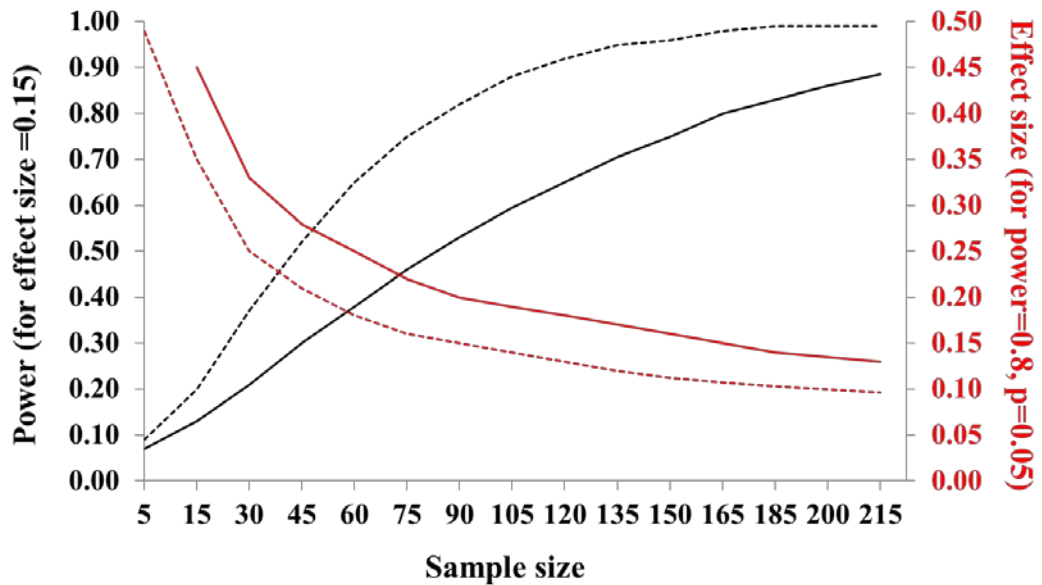


Figure 3.8 Description of the measurable powers (black) and effect sizes (red) of tests with different sample sizes (number of mosquitoes) for two proportions at the 0.05 significance level. Solid lines: Sample size considerations based on power calculation for two-sample comparisons of proportions. Dashed line: Sample size calculation for the inference for a single proportion comparing to a known proportion (0.5) suitable for testing large groups where this baseline proportion can be confirmed.

These sample size considerations apply irrespective of whether the proportions of eggs laid by groups of mosquitoes per cage or by individual mosquitoes per cage are compared since in both cases only a single data point per cage can be recorded and the proportion of non-responders in the cage is unknown. Nevertheless, if large groups (>30 responders per cage for example) are used where the baseline proportion can be predicted to be close to 50% with some certainty it might be justifiable to use the sample size calculation for the inference for a single proportion comparing to a known proportion (0.5). In this case 85 replicate cages would be required for detecting a 15% increase compared to the baseline proportion at 80% power and 5% significance (Figure 3.8). Whilst this number of replicates appears to be considerably lower it needs to be observed that over 7 times more gravid females would be required in this experimental



design ( $85 \times 30 = 2550$ ) than when using individual females and two treatment arms ( $2 \times 165 = 330$ ).

### 3.5. Discussion

Using two equal choice egg-count bioassays with individual gravid mosquitoes illustrated the importance of (1) suitable experimental design based on the behavioural ecology of *An. gambiae s.s.*, (2) estimated sample sizes and, (3) appropriate statistical analyses. This study confirmed that egg counts of individual female *An. gambiae s.s.* of the same age fed on the same source of blood and reared under standardized conditions are highly variable and overdispersed. Lyimo and Takken (1993) previously demonstrated that individual newly emerged *An. gambiae s.l.* of the Muheza strain laid between 48 – 178 (mean 111) eggs while wild field populations laid an equally variable 66 to 290 (average 150) eggs. Hogg, Thomson and Hurd (1996) later confirmed this variation showing that wild *An. gambiae s.s.* and *An. arabiensis* of Muheza laid between 20 – 180 eggs and 5 – 160 eggs, respectively. These wide disparities in egg numbers of individual females have also been shown for laboratory strains of other Anophelinae including: *An. stephensi* (Suleman 1990), *An. sergentii* (Beier *et al.* 1987), *An. multicolor* and *An. pharoensis* (Kenawy 1991). Suleman and others (Suleman 1990) noted that a small portion of *An. stephensi* females laid a very high number of eggs per batch leading to a negative binomial distribution as also demonstrated for *An. gambiae s.s.* in this study. Similar heterogeneity in egg numbers between individual females have also been shown for *Aedes aegypti* (Christophers 1960). This may be a general trait of mosquitoes that lay single eggs rendering the use of egg numbers to gauge oviposition substrate preferences inappropriate especially with small groups of mosquitoes (Corbet & Chadee 1993). It was demonstrated that the high variation in the number of eggs laid by individual females can lead to an unequal distribution of eggs in equal substrates. This disproportion persisted even with very large sample size.

Exploring the pattern of ‘skip oviposition’ in *An. gambiae s.s.* it was demonstrated that approximately one third of all gravid *An. gambiae s.s.* distribute their eggs in more than one oviposition site, a behaviour that is well known in *Aedes* mosquitoes (Chadee & Corbet 1991; Chadee & Corbet 1993), but has been poorly described in *An. gambiae s.l.* species (Ogbunugafor & Sumba 2008; Herrera-Varela *et al.* 2014) in laboratory egg-count experiments possibly because most experimenters use groups of mosquitoes,

which masks skip oviposition. There is also indirect evidence of skip oviposition from one study in the field (Chen *et al.* 2006) showing that this is not an artefact trait of colonized mosquitoes but rather an inherent trait of the species. Skip oviposition represents a response of the gravid female to the substrates and should not be excluded from analyses. Skip ovipositing females choose to use both substrates, therefore not rejecting any, an important event with reference to comparative preference of substrates. Importantly, *An. gambiae s.s.* females do not distribute their eggs in equal proportions but in most cases lay  $2/3^{\text{rd}}$  in one and  $1/3^{\text{rd}}$  in the other oviposition cup. Since observations in this study are based on equal choices, it is clear that the larger egg batch does not indicate a preference. It is important to note that individual skip ovipositing female did not lay more eggs compared to those individual females that laid in a single cup.

In experiments, where groups of females are analysed in oviposition assays, the marked heterogeneity of egg numbers laid by individual females combined with skip oviposition is likely to increase the variance in the system and this could lead to a type 1 error where an unequal distribution of eggs between the test and control solutions is wrongly considered to be true, especially if group sizes are small. Here it was illustrated that this frequently happens when group numbers per cage are below 30 responders. Considering that of those probably a fifth or more mosquitoes do not lay eggs, a skewed distribution can be expected and only a large number of cages can be able to detect true differences of substrates. Since many choice experiments with anophelines are done with groups much lower than thirty results need to be interpreted with caution (McCrae 1984; Sumba *et al.* 2004a; Huang *et al.* 2005; Rinker *et al.* 2013).

This study also demonstrated that observing individual mosquito's responses to oviposition substrates rather than groups has a number of advantages. This approach ensures that only responders are included in the data analysis. It allows the analysis of choice based on a binary outcome, the enumeration of egg numbers of individual females and the observation of skip oviposition, which has previously been shown to be influenced by the suitability of a substrate (Herrera-Varela *et al.* 2014). Last but not the least the necessary number of replications can be achieved with a smaller number of gravid females compared to when groups are used.

Sample size considerations are rarely reported for entomological studies and the number of replications hardly ever justified in publications. This study illustrates that insufficient replication might not only hamper the ability to show a significant effect due to the lack of power, but also demonstrates that a small number of replicates and small group sizes can result in significant artefact differences in oviposition responses in two choice experiments purely based on stochastic effects rather than due to a treatment effect. Misinterpretation of results can be reduced by sufficient replication and validation of the experiment by implementing a control experiment preferably in parallel (Hurlbert 1984).

The underlying hypothesis of a choice experiment is that when two (or more) equal choices are presented the response towards these choices is equally proportional with odds of success of 1:1 (baseline or control). Choices by virtue of the design of the experiment should be analysed as proportions rather than absolute counts especially when count data are highly variable. If an oviposition cue is presented that is either preferred or avoided by gravid females a significant diversion from the baseline is expected. It was shown that there is a high variability in the response towards a test and control cup containing the same substrate in individual rounds of experiments highlighting the importance of large sample sizes and the implementation of an experiment over several rounds with different batches of mosquitoes. The behaviour of mosquitoes from the same batch might be affected for example by their rearing history and/or by the climatic conditions during the experiment or other non-measurable random effects. Replicate tests with mosquitoes from the same batch implemented on the same day with the same batch of oviposition substrate should not be considered independent; it is pseudo-replication (Hurlbert 1984). In order to document the baseline including its 95% CI it is recommended that choice experiments with different test substrates in a cage must always be implemented in parallel with a control experiment with the same number of equal choices. This validates the experimental design (Corbet & Chadee 1993) and allows statistical comparison of the odds of success in the test experiment with the odds of success in the control experiment (baseline).

The classic oviposition index represents a proportional comparison of the numbers of eggs, egg rafts or females (Kramer & Mulla 1979) but is rarely used in oviposition experiments with *An. gambiae* s.s.. Frequently the mean number of eggs in test and control cups is compared using classical ANOVA and t-tests (McCrae 1984; Huang *et*

*al.* 2005; Munga *et al.* 2005; Huang *et al.* 2006a; Munga *et al.* 2006; Huang *et al.* 2007; Otienoburu *et al.* 2007; Overgaard 2007; Sumba *et al.* 2008; Balestrino *et al.* 2010; Kweka *et al.* 2011; Rinker *et al.* 2013). These assume normality of data distribution and homogeneity of variance but both assumptions are violated when looking at egg counts of *An. gambiae s.s.*. Some (log-) transform egg-counts or use non-parametric tests that do not assume a normal distribution. However, log-transforming count data for analyses has recently been challenged except when dispersion is small and means are large (O'Hara & Kotze 2010). Moreover, non-parametric tests have reportedly been invalidated even by “small differences in variance and moderate degrees of skew” (Zimmerman 1998; Zimmerman 2001; Fagerland & Sandvik 2009). When distributions are skewed (such as for negative binomial distributions) differences in means are prone to go together with differences in variance (Fagerland & Sandvik 2009). It is also imperative to appreciate the non-independent nature of the data from control and test cups in the same cage and the dependent nature of the data derived from the same rounds when analysing choice egg-count bioassays. This violation of independent observations assumption results in downwardly biased standard error estimates, overly large test statistics, and inflated type I error rates. The statistical procedure used must therefore take account of that by including repeated measure terms.

It is strongly suggested analysing choice bioassays using generalised regression models that allow for the appropriate distribution to be fit to the model rather than transforming the data (Sileshi 2006; O'Hara & Kotze 2010). Preference should be given to analysing proportions (of eggs laid or of females laying in test and control) using a binomial distribution than to analyse counts using a negative binomial or Poisson distribution. Importantly, these models allow including critical explanatory variables as well as random factors and/or repeated measures that might have affected the outcome. Based on the model, the effect size of the test can be reported using both odds ratios and predicted averages together with associated confidence intervals (Seavy *et al.* 2005).

### 3.6. Conclusion

Individual *An. gambiae s.l.* can lay a widely ranging number of eggs. A proportion of these may also skip oviposit, spreading their eggs in more than one substrate. These egg-laying patterns can lead to spurious conclusions of oviposition substrate preferences based on two choice egg-count bioassays. In order to increase the accuracy of these

bioassays designs that take into account the natural variability in the number of eggs and ensure sufficient replication are needed. In conclusion, experiments are most accurate when gravid females are prepared and selected under carefully controlled conditions and when implemented in a two tier design with 165 individual mosquitoes in each treatment arm: 165 cages each with one mosquito given a choice between a test and control solution and 165 similar cages where the mosquito has a choice between two identical control solutions. This will enable description differences in substrate preferences of as little as 15% with sufficient statistical power and significance.

Chapter 4. Water vapour is a pre-oviposition  
attractant for the malaria vector *Anopheles gambiae*  
*sensu stricto*



Air flow wind tunnels (Source: Michael Nyang'anga Okal)

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- 2.3. Stage of publication – Not yet submitted / Submitted / Undergoing revision from peer reviewers' comments / In press

**3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)**

I was involved in the conception of the idea of the study. I designed and implemented all experiments with the airflow wind olfactometer. I contributed to the implementation of experiments with WHO tubes and analysed all the data and drafted the manuscript together with my co-authors.

NAME IN FULL (Block Capitals) Michael Nyang'anga Okal

STUDENT ID NO: LSH313699

CANDIDATE'S SIGNATURE *Michael Nyang'anga Okal* Date 17th June 2015

SUPERVISOR/SENIOR AUTHOR'S SIGNATURE (3 above) *U. Stiller*

#### 4.1. Abstract

**Background:** To date no semiochemicals affecting the pre-oviposition behaviour of the malaria vector *Anopheles gambiae sensu lato* have been described. Water vapour must be the major chemical signal emanating from a potential larval habitat, and although one might expect that gravid *An. gambiae s.l.* detect and respond to water vapour in their search for an aquatic habitat, this has never been experimentally confirmed for this species. This study aimed to investigate the role of relative humidity or water vapour as a general cue for inducing gravid *An. gambiae sensu stricto* to make oriented movements towards the source.

**Methods:** Three experiments were carried out with insectary-reared *An. gambiae s.s.* during their peak oviposition time in the early evening: One with unfed females and two with gravid females. First, unfed females and gravid females were tested separately in still air where a humidity difference was established between opposite ends of a WHO bioassay tube and mosquitoes released individually in the centre of the tube. Movement of mosquitoes to either low or high humidity was recorded. Additionally, gravid mosquitoes were released into a larger air-flow olfactometer and responses measured towards collection chambers that contained cups filled with water or empty cups.

**Results:** Unfed females equally dispersed in the small bioassay tubes to areas of high and low humidity (mean 50% (95% confidence interval (CI) 38-62%). In contrast, gravid females were 2.4 times (95% CI 1.3-4.7) more likely to move towards high humidity than unfed females. The results were even more pronounced in the airflow olfactometer. Gravid females were 10.6 times (95% CI 5.4-20.8) more likely to enter the chamber with water than a dry chamber.

**Conclusions:** Water vapour is a strong pre-oviposition attractant to gravid *An. gambiae s.s.* in still and moving air and is likely to be a general cue used by mosquitoes for locating aquatic habitats.



## 4.2. Background

*Anopheles gambiae sensu stricto* and *Anopheles arabiensis* are the two major vectors of malaria in Africa. Their primary larval habitats are commonly described as small, temporary, open, sunlit pools (Muirhead-Thomson 1951; Gillies & De Meillon 1968), yet this is a gross oversimplification of the types of habitat actually colonized by these mosquitoes (Fillinger & Lindsay 2011). In reality, immature stages of both species can be found in an enormous diversity of aquatic habitats and it has been difficult to characterize these sites with precision (Fillinger *et al.* 2004; Majambere 2008; Fillinger *et al.* 2009b; Ndenga *et al.* 2011). Semi-permanent water bodies are frequently as productive or even more productive over time than the small rain-filled puddles that are often only abundant during the rainy season (Fillinger *et al.* 2004; Fillinger *et al.* 2009b; Ndenga *et al.* 2011). Nearly every type of water accumulation, apart from organically rich, may contain anopheline larvae (Chinery 1984; Fillinger *et al.* 2004; Sattler *et al.* 2005; Awolola *et al.* 2007; Majambere 2008; Machault *et al.* 2009). The presence of larvae in a water body is thought to be the result of a combination of the egg-laying choice of gravid females that deposit their eggs in water and the survival of larvae in those habitats (Muirhead-Thompson 1945), although the cues that guide the gravid female's choice are not well understood.

The attractiveness of field sites may be due to general characteristics and cues such as their relative position in relation to the resting site of gravid females, visual cues from these sites and the presence of water vapour plumes, as well as more habitat-specific chemical cues released from water bodies serving as semiochemicals which indicate the suitability of an aquatic habitat (Muirhead-Thomson 1951; Bentley & Day 1989; Clements 1999). Although some putative semiochemicals have been suggested based on coupled gas chromatography-electroantennogram detection (Blackwell & Johnson 2000; Qiu 2006; Lindh *et al.* 2008), to date, no semiochemical that affect the behaviour of gravid *An. gambiae s.l.* has been confirmed. Water vapour must be presumed to be the major chemical signal emanating from a potential larval habitat and although one might expect that gravid *An. gambiae s.l.* detect and respond to water vapour in their search for an aquatic habitat, this has never been experimentally confirmed for this species.

The present study set out to investigate the role of water vapour in the pre-ovipositional behaviour of *An. gambiae s.s.* which results in arrival at potential oviposition sites

(Bentley & Day 1989). Two separate choice tests were used: in the first test the response of unfed and gravid *An. gambiae s.s.* were compared using still air in cages connected to WHO bioassay tubes; in the second test gravid female responses were tested using moving air in a newly designed airflow olfactometer. In both systems *An. gambiae s.s.* were provided with a choice of moving towards an area of low or high humidity without visual cues or access to the water source.

### 4.3. Methods

#### 4.3.1. Study site

The study was carried out at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC), Mbita, on the shores of Lake Victoria, Kenya (0° 26' 06.19'' S, 34° 12' 53.13'' E; 1,137 m above sea level). This area is characterized by an equatorial tropical climate with an average minimum temperature of 16°C and an average maximum temperature of 28°C. The area experiences two rainy seasons: the long rainy season between March and June and the short rainy season between October and December. The average annual rainfall for 2010-2012 was 1,436 mm (icipe-TOC meteorological station).

#### 4.3.2. Mosquitoes

Insectary-reared *An. gambiae s.s.* (Mbita strain) were used for all experiments. Five-day-old females were selected 30 minutes prior to the experiment from insectary colony cages where they had been kept in groups of approximately 300 males and 300 females in 30x30x30 cm netting cages and provided with 6% glucose solution *ad libitum*. These females never had a blood-meal and are therefore referred to as unfed females. Gravid mosquitoes were prepared by transferring 150 female and 150 male mosquitoes, aged two days old, in 30x30x30 cm netting cages and provided with 6% glucose solution *ad libitum* at 25-28°C and a relative humidity between 68-75%. Saturated cotton towels, 50x25 cm in area, were folded and placed over the cages to avoid mosquito desiccation. Mosquitoes were starved of sugar for seven hours and allowed to feed on a rabbit for 15 minutes on day two and three post-emergence and rested for a further two days before use. Thus five-days-old gravid females were used for experiments.

### 4.3.3. Water

For all experiments, piped non-chlorinated water pumped from Lake Victoria was used. The water was passed slowly through a locally made sand charcoal gravel filter for purify it of debris and organic chemicals. Briefly, two 50 L buckets were placed on top of each other. The lower bucket's lid contained a hole and the upper bucket's floor was perforated with small holes for the filtered water to pass through to the lower bucket. The upper bucket contained three layers of gravel, activated charcoal and sand. Tap water was poured into the top of the upper bucket and run slowly through the layers. The aim was to remove large and small particles from the water including the majority of algae and bacteria. The purified water is referred to as 'filtered tap water'.

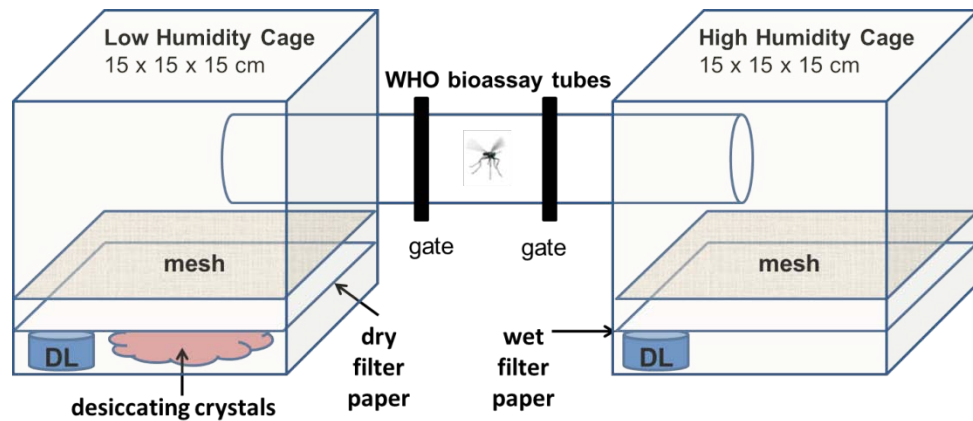
In the two bioassays described below it is hypothesized that the tap water was attractive solely because of the presence of water vapour rather than because the water contained an attractive semiochemical. This assumption is based on a preliminary experiment, that was implemented comparing the oviposition response of *An. gambiae s.s.* to filtered tap water and double-distilled water. A description of the experiment and results can be found in Appendix A. Gravid females did not have a significant preference for either filtered tap water or distilled water.

### 4.3.4. Experimental procedures

#### 4.3.4.1. WHO-tube bioassays

Choice tests were carried out in the laboratory under ambient conditions. Natural light came from a window located 2 m from the set-up. For each choice test, three WHO bioassay tubes (12mm Internal Diameter), each 12 cm long, (WHO 2006a) were connected together with open/close gates between the inner and outer tubes. The two outer tubes were inserted for approximately 6 cm into small mosquito cages measuring 15x15x15 cm. Cages were wrapped in commercially available kitchen cling-film (Figure 4.1). In one cage, 25 ml of silica gel desiccating crystals were spread evenly over the bottom of the cage, with dry filter paper covering the crystals. In the other cage, there were no desiccating crystals and the filter paper was dampened with 25 ml of filtered tap water. A 15x15 cm wire screen was fixed 5 cm above the bottom of the cages to prevent mosquitoes from making direct contact with the substrates. There were eight identical set-ups, arranged along a table 10 cm apart, with the high and low

humidity ends being alternated between each set of tubes. In the first of these eight set-ups, data loggers (Tinytag, TV4500) were placed in the two cages to record the relative humidity. A single *An. gambiae s.s.* (Mbita strain) was placed in the middle tube at 18.00 with the gates opened by 2 mm (not too wide to let the mosquito through) allowing some exchange of air within the central tube and the connected cages before the gates were completely opened at 18.30 allowing the mosquito to move freely from the tubes into the cages. This experiment was implemented with unfed and gravid females of the same age. At 19.00 the position of each mosquito either in the middle tube or in one of the two cages was recorded. The gates were closed at 21.30 and mosquitoes again counted in each cage or middle tube. The time period for observation was chosen based on preliminary experiments that have shown that out of 120 individual gravid females tested (5 round x 20 females) 95% (114/120) of the local insectary-reared *An. gambiae s.s.* (Mbita strain) laid all their eggs before 21.30, which is similar to the time reported for the same strain previously (Sumba *et al.* 2004b). Experiments were done with eight mosquitoes each evening on nine occasions with unfed females and with gravid females (total 72 per physiological stage). During the experiment with unfed females four escaped when manipulating the gates and were excluded from the analyses, similarly when implementing the experiment with gravid females six females were found dead in the middle tube and were excluded from the analysis, therefore a total of 68 unfed and 66 gravid *An. gambiae s.s.* were tested. This sample size was sufficient to detect a 33% increase in the attractiveness of humid air (i.e, 66.5% collected in the humid air cage compared with the 50% null hypothesis) at the 5% level of significance and 80% power (inference of a proportion compared to the null proportion (Brant 2013)).



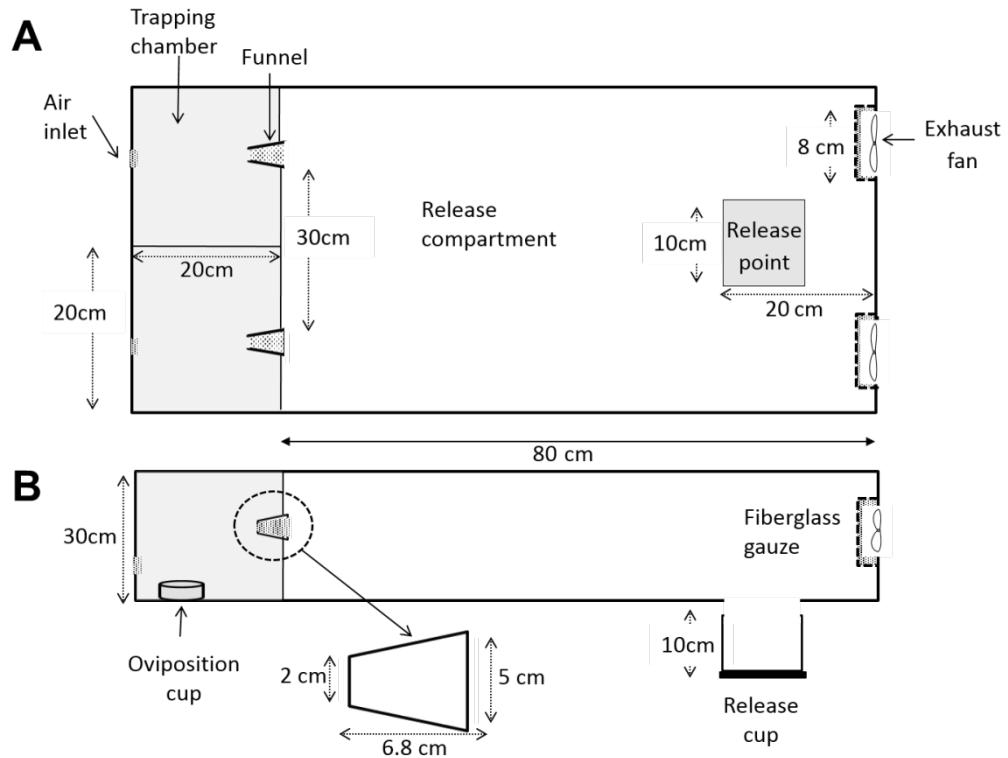
**Figure 4.1 WHO tube bioassays to observe response of individual gravid *Anopheles gambiae* s.s. towards high or low humidity. DL = data loggers**

#### 4.3.4.2. Airflow olfactometer bioassays

Three dual port airflow olfactometers were used to study the responses of gravid *An. gambiae* s.s. to filtered tap water (Figure 4.2). Each tunnel measured 40x100x30 cm and was made from polymethyl methacrylate sheets. Each tunnel was partitioned into three compartments: one large compartment for releasing the mosquitoes and two identical trapping chambers (20x20x30 cm each). Two fans (diameter 8 cm, 6V computer casing fans (Molex, China)) drew air through the trapping chambers into the release compartment at 0.48 m/s. Batches of 100 gravid *An. gambiae* s.s. females were introduced at 18.20 by inserting a 10x10x10 cm cage into the underside of the release compartment. At the same time the fans were switched on. Mosquitoes acclimated for 10 minutes and were then released by carefully opening the cage at 18.30. Mosquitoes were able to fly through a transparent polyvinyl chloride funnel into a trapping chamber. Alternative trapping chambers of each tunnel were baited with either an empty 70 mm diameter glass cup (Pyrex®) or with the same type of cup filled with 100 ml of filtered tap water. Prior to any experiment glass cups were autoclaved and heated afterwards in an oven at 200°C for at least two hours to rid them of possible odourant contamination and bacteria. Mosquitoes trapped in the chambers and those that remained in the release compartment were counted at 08.00 the following morning. Experiments were done in complete darkness, at ambient conditions (27-28°C, 60-70% relative humidity) in a room without a window.

Responses of gravid *An. gambiae s.s.* were compared for three different treatments in an olfactometer: (1) both chambers contained dry cups, (2) both chambers contained cups filled with water, and (3) one chamber contained a dry cup (control) and the other a cup with water (test). In all cases cups were randomly allocated as 'control' or 'test' (even if the same treatments were provided) to the two chambers to help facilitate the analysis.

Each treatment was replicated 24 times (the 'test' cup of each treatment was located in each of the chambers of each of the three olfactometers four times) in order to estimate the variability in responses so that sample size calculations could be done. Power calculations were based on the formula from Hayes and Bennett (Hayes & Bennett 1999) for comparing proportions of clustered data. When gravid females were provided with identical treatments in both chambers, 24 replicates resulted in a similar proportion in each chamber ( $p_1=0.5$ ). The variability of the nightly catches was used to calculate the coefficient of variation (ratio of standard deviation/mean), which was high at 0.33. Assuming that out of 100 mosquitoes released, 80 respond by entering one or the other collection chamber, 24 replicates in each arm ( $p_1$  and  $p_2$ ) can detect an increase or decrease in the catch rate of 20% ( $p_2=0.7$ ) with 90% power at a 5% significance level. Data loggers (Tinytag, TV4500) were placed in the two collection chambers and the release compartment for three nights in each of the three treatments to measure relative humidity.



**Figure 4.2 Dual port airflow olfactometer.** View from the top (A) and view from the side (B)

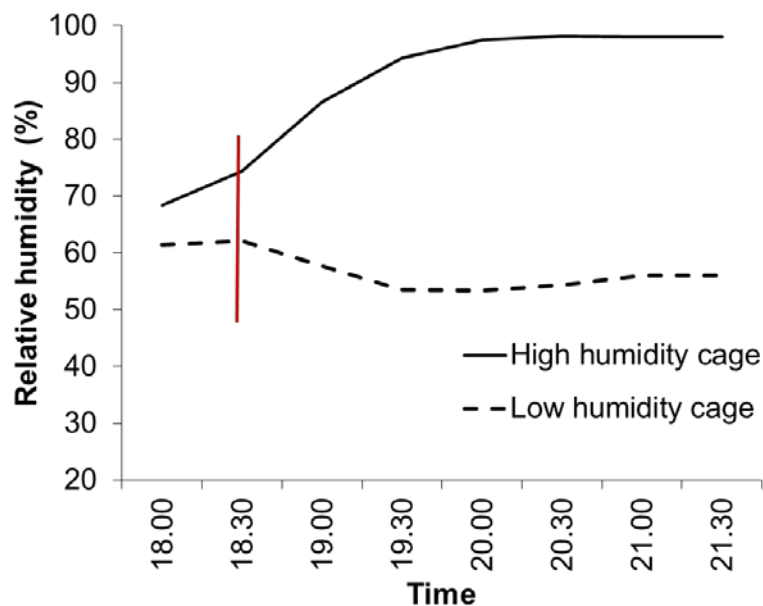
#### 4.3.5. Statistical analysis

Data were analysed using generalised linear models comparing the mean proportion of female mosquitoes responding to the test cage or the test compartment. Responses of non-fed and gravid females towards the humid cage were compared in WHO-tube bioassays. Odds ratios were calculated in reference to the response of non-gravid females. In the airflow olfactometer bioassays responses of gravid females towards the three different experimental treatments (dry-dry, water-water, dry-water) were compared. Odds ratios were calculated in reference to the wet-wet comparison (equal treatments). The experimental treatments, the olfactometer (A, B, C) and the collection chamber (left, right) were entered as fixed factors to estimate their impact on the outcome. Since the data were highly overdispersed, quasibinomial distributions were used. Mean proportions per treatment and their 95% CIs were calculated using the parameter estimates of the models by removing the intercept from the models. All analyses were done with R statistical software version 2.14.2 (R Team 2011).

## 4.4. Results

### 4.4.1. WHO-tube bioassays. *Response to water vapour in still air.*

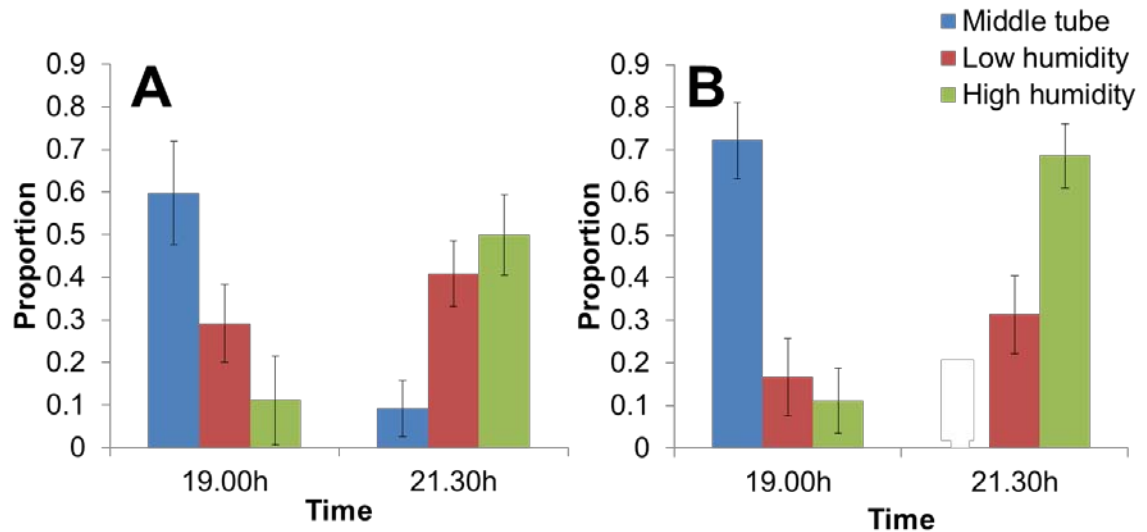
At the time when the gates of the WHO tubes were completely opened mean relative humidity differed by around 12% between high and low humidity cages. Humidity slowly decreased in the low cage and increased in the high humidity cage over the next two hours and the difference reached a maximum of approximately 44% at 20.00, with a mean relative humidity of 54% (95% CI 53-56%) in low and 97% (95% CI 95-99%) in high humidity cages (Figure 4.3). Average temperatures during the experiments ranged between 27 and 28°C. Conditions were similar in both experiments with unfed and gravid females.



**Figure 4.3 Average humidity in high and low humidity cages in tube bioassays**

At 19.00, half an hour after the gates were opened, 60% of the non-fed mosquitoes and 72% of the gravid mosquitoes remained in the middle tube; 29% of the unfed mosquitoes moved to the low and 11% to the high humidity cages. Gravid females moved in relatively small and similar proportions to the low and high humidity cages (Figure 4.4).





**Figure 4.4 Mean proportion of females (error bars = 95% confidence intervals) resting in release (middle tube), low or high humidity cage at start and end of experiment with *Anopheles gambiae* s.s. in tube bioassays. Non-fed females (A) and gravid females (B)**

Unfed females showed no preference for any of the two conditions provided (Table 4.1, Figure 4.4). When gates were closed at 21.30 half of the unfed females had moved in the high humidity cage and the other half either remained in the middle tube (9%) or moved into the low humidity cage (41%). In contrast, gravid females were 2.4 times more likely to move to the high humidity cage than unfed females (Table 4.1). All gravid females had moved out of the middle tube at 21.30 and on average 71% of them had moved into the high humidity cage.

**Table 4.1 The mean percentage of gravid *Anopheles gambiae* s.s. attracted to the test cage in the WHO-tube assays and to the test compartment in the airflow olfactometer assays**

Experimental treatment	Mean percentage (%) in test (95% CI)*	Odds Ratio (95% CI)	p-value
<b>Response towards high humidity cage (test) in WHO-tube bioassays at 21.30</b>			
Non-fed females	50 (38-62)	1	
Gravid females	71 (59-81)	2.4 (1.3-4.7)	0.018
<b>Airflow olfactometer bioassays with gravid <i>An. gambiae</i> s.s. in three experimental treatments</b>			
Wet (control) vs. wet (test)	56 (48-64)	1	
Dry (control) vs. dry (test)	50 (29-71)	0.8 (0.3-1.9)	0.598
Dry (control) vs. wet (test)	93 (88-96)	10.6 (5.4-20.8)	<0.001

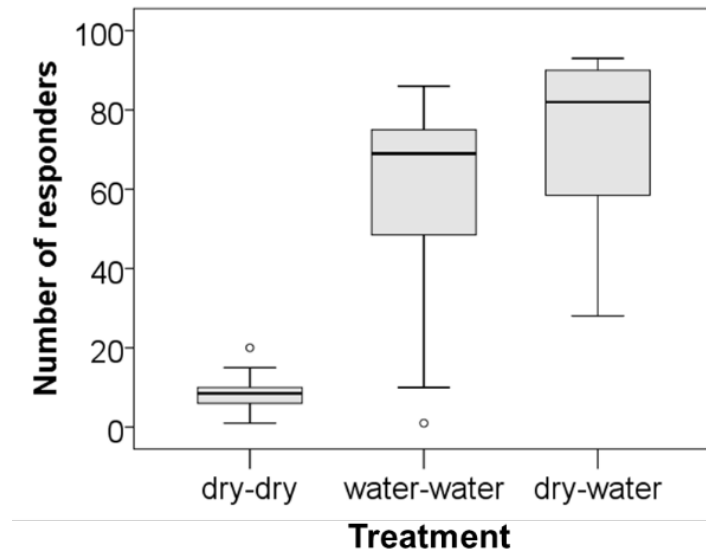
CI = confidence interval; \*based on model parameter estimates

#### 4.4.2. Airflow olfactometer bioassays. *Response to water vapour in an air stream.*

Differences in relative humidity between areas with and without water were lower in the airflow olfactometer experiments than in the cage experiments. Relative humidity was on average 20% higher in chambers that contained water than in areas that did not (collection chamber and/or release compartment). Nightly relative humidity in collection chambers containing water was 91% (95% CI 90-92%), the average relative humidity in dry release compartments or dry chambers was 71% (95% CI 69-72%). The temperature did not differ between collection chambers and release compartments irrespective of the treatments and was on average 27.7°C (95% CI 27.2-27.9°C) during the 24 nights of experiments.

High responses of gravid females were recorded in the experimental treatments that presented water in either one or both collection chambers of the olfactometer (median of 69-83%, n=100 per olfactometer/experimental unit). In contrast, when no stimulus was

provided only a median of 9% of the mosquitoes responded by flying upwind in any of the two chambers whilst the rest remained in the release compartment (Figure 4.5).



**Figure 4.5 Comparison of response rates of gravid *Anopheles gambiae s.s.* to the three experimental treatments tested in airflow olfactometers**

When presented with an identical treatment the gravid females approached both collection chambers in equal proportion (estimated ratio 1:1) whilst on average 93% of the gravid females chose the chamber with water (estimated ratio 1:11), when the other was dry (Table 4.1) irrespective of whether the test cup was presented in the left or right collection chamber and irrespective of which of the three olfactometers was used for the test (both factors were not significantly related to the outcome).

#### 4.5. Discussion

Here evidence is presented that gravid *An. gambiae s.s.* move from lower humidity towards higher humidity. This has been shown at short distances of 15-20 cm in still air and along an air stream of moving water vapour towards an area of higher humidity at longer distances of about 60 cm. Whilst one cannot be certain that gravid females are attracted to water vapour, since they could be repelled from drier areas, it is more likely that attractiveness of water vapour was responsible for the strong results observed since the relative humidity in the low humidity test areas was close to 60% and above, which is similar to the relative humidity of their resting places (Okech *et al.* 2004; Olayemi *et*

*al.* 2011b). This is supported by the results with unfed females which did not show any preference for moving into the higher humidity cage compared to the lower humidity cage. Nevertheless, it has been shown with all physiological stages that individuals can orientate to water vapour plumes or humidity differences much in the same way that a mosquito locates a host (Knols & Takken 1997). Early studies indicated that in *Aedes aegypti* humidity receptors were present on the antennae of females (Rahm 1958). In *Anopheles atroparvus* the hygrometers were located on the distal segments of the antennae bearing most of the grooved pegs (Ismail 1962). Studies with *An. gambiae s.s.* have confirmed that more than half the grooved pegs on the antennae increase their firing rate in the presence of water vapour and that some respond to low humidity, suggesting that these receptors play a role in humidity perception (van den Broek & den Otter 2000). Whilst it has been shown that humidity is important for the survival of mosquitoes (Bayoh 2001), a clear difference in the behaviour of unfed and gravid females was demonstrated in the present WHO tube experiments. It is speculated that the strong responses observed in gravid mosquitoes towards moving to areas of very high humidity is likely to increase the reproductive success of females by improving their chances of finding an aquatic habitat that might serve as a potential oviposition site. It could be an adaptive trait selected for in nature.

In the tube bioassay, only a small number of gravid mosquitoes left the central holding tube immediately after the gates were opened. This might indicate that mosquitoes remained static long enough to detect the humidity difference, especially since the difference in humidity was only around 12% at the time when the gates were opened and no airflow was created. However, at the end of the peak oviposition period 2.4 times more mosquitoes had moved into the humid cage than the drier one whilst the response of unfed females was similar towards the two treatments. The attraction of water vapour is demonstrated clearly with free-flying mosquitoes in airflow olfactometers. Here seven to eight times more gravid mosquitoes were found in the collection chambers when one or both chambers contain water than when both were dry. Furthermore, when given a choice between one chamber containing water and one that is dry, 11 times more gravid females were collected in the chamber with water. The upwind flight was probably stimulated by moist air. It is most likely that the greater attractiveness of water vapour in a wind tunnel than in the tubes was a result of moving moist air in the tunnel compared with the relatively still air in the tubes. Whilst the

evidence presented here shows the attraction of water vapour over relatively short distances, previously published work provides support that water vapour might attract females over several metres. Dugassa *et al* demonstrated that when gravid *An. gambiae* s.s. females were released into a large screened semi-field system the attractiveness of a reflecting surface was increased by 60% when presented close to water compared with when it was presented without water (Dugassa *et al.* 2012). In this case females travelled at least 5 m from the release point to the site where they were collected. *Anopheles gambiae* is highly sensitive to subtle changes in moisture as seen when selecting moist sites for ovipositing (Huang *et al.* 2005).

It cannot be totally excluded that chemicals other than water were released from the tap water in the experiments described in this paper, since water purification with charcoal-sand filters does not completely sterilize the water or remove all chemicals. Nevertheless, the observed attraction was very strong, especially in the airflow olfactometers. If this was based on semiochemicals released from the tap water, an effect should have been observed to larger degree in the preliminary experiments comparing tap water with double-distilled water. However, in these experiments only a very slight and insignificant preference for the tap water was recorded (Appendix A).

The present work supports the conclusion made by Kennedy that ‘water vapour emanating from a surface plays an important part in evoking pre-ovipository responses in mosquitoes (*An. atroparvus*, *Ae. aegypti* and *Culex molestus*)’ (Kennedy 1942). He also recognized the importance of moist air currents to activate movement and help with orientation which ‘very probably play an important part in water-finding in the field’. Such conditions existed in the olfactometer experiments. The question arises if and how gravid mosquitoes might use water vapour to navigate through the landscape. The pattern of water vapour across the savanna can be highly heterogeneous, shaped by the local climate, topography, vegetation, soil characteristics and presence and extent of water bodies (Beringer *et al.* 2011). The authors are not aware of research that has been conducted that describes the distribution, movement and concentration of water vapour at dusk in the savanna regions of tropical Africa at less than one metre above the ground; the environment encountered by gravid *An. gambiae* searching for a water body in which to lay their eggs. Such research is likely to provide further insights into the pre-oviposition behaviour of this important vector.

Water vapour is likely to be a general attractant for all mosquito species whatever their physiological status and it should not be considered the only attractive compound guiding gravid *An. gambiae s.s.* to an oviposition site. Water vapour has been shown to attract host-seeking mosquitoes (Clements 1999) and indoor-resting mosquitoes (Kirby 2005). For host-seeking mosquitoes water vapour can indicate a human host, and for resting mosquitoes it provides an environment where the insect is less likely to dehydrate and die, so increasing its chances of survival. Nevertheless, the results presented here clearly show a difference between the responses of unfed and gravid females towards water vapour suggesting that it is an important cue for a gravid mosquito locating a potential water body, though it clearly cannot be the only one. If it was the only cue mosquitoes would accumulate in large bodies of water like lakes, rivers and seas, habitats inimical to their survival. Water vapour is likely to work in a synergistic manner with visual cues possibly over a longer range (Bernath *et al.* 2012) and with semiochemicals attracting and repelling gravid *An. gambiae* mosquitoes over short distances (Bentley & Day 1989; Sumba *et al.* 2004a; Rejmankova *et al.* 2005; Lindh *et al.* 2008).

#### 4.6. Conclusion

Gravid malaria vectors need to find suitable water bodies for their aquatic life stages to develop. Water consistently evaporates from aquatic habitats making water vapour probably the major chemical signal emanating from a potential larval habitat. This study demonstrates that gravid *An. gambiae s.s.* move into areas of high humidity or along airstreams of water vapour at the time of night they are actively seeking a site to lay their eggs, implicating water vapour as an important pre-oviposition attractant. More research is needed to address: (1) how water vapour is distributed over the landscape, (2) whether it assists gravid females in locating potential aquatic habitats over longer distances, and, (3) how it interacts with other pre-oviposition cues, either visual or chemical.

## Chapter 5. Water vapour interacts with chemicals to activate and direct gravid *Anopheles gambiae sensu stricto* to oviposition sites



Semifield system at *icipe*, Kenya (Source: Michael Nyang'anga Okal)

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I contributed significantly to the conception of the idea for this research and the development of its experimental design. I developed all protocols and implemented the experiments. I analysed the data and wrote the first draft of the manuscript with the help of my supervisor.

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## 5.1. Abstract

**Background:** Cues that guide gravid *Anopheles gambiae sensu lato* to oviposition sites can potentially be manipulated to create new strategies for monitoring and controlling malaria vectors. However, progress towards identifying such cues is slow in part due to the lack of appropriate tools for investigating long-range attraction to putative oviposition substrates. This study aimed to develop a relatively easy-to-use bioassay system that can effectively test chemical attractants for gravid *Anopheles gambiae sensu stricto*.

**Methods:** BG-Sentinel™ mosquito traps that use fans to dispense odourants were modified to contain aqueous substrates. Choice tests with two identical traps set in an 80 m<sup>2</sup> screened semi-field system were used to analyse the catch efficacy of the traps and the effectiveness of the bioassay. A different batch of 200 gravid *An. gambiae* s.s. was released on every experimental night. Choices tested were (1) distilled versus distilled water (baseline) and (2) distilled water versus soil infusion. Further, comparisons were made of distilled water and soil infusions both containing 150g/l of Sodium Chloride (NaCl). Sodium Chloride is known to affect the release rate of volatiles from organic substrates.

**Results:** When both traps contained distilled water, 45% (95% confidence interval (CI) 33 – 57%) of all released mosquitoes were trapped. The proportion increased to 84% (95% CI 73 – 91%) when traps contained soil infusions. In choice tests, a gravid female was twice as likely to be trapped in the test trap with soil infusion as in the trap with distilled water (odds ratio (OR) 1.8, 95% CI 1.3 – 2.6). Furthermore, the attraction of gravid females towards the test trap with infusion more than tripled (OR 3.4, 95% CI 2.4 – 4.8) when salt was added to the substrates.

**Conclusion:** Minor modifications of the BG-Sentinel™ mosquito trap turned it into a valuable bioassay tool for evaluating the orientation of gravid mosquitoes to putative oviposition substrates using olfaction. This study describes a useful tool for investigating olfactory attraction of gravid *An. gambiae* s.s. and provides additional evidence that gravid mosquitoes of this species are attracted to and can be baited with attractive substrates such as organic infusions over a distance of several meters.

## 5.2. Background

Malaria still causes considerable human morbidity and mortality in spite of concerted control efforts that have resulted in its steady decline in the last decade (Nyarango *et al.* 2006). Effective interventions need to be scaled up (WHO 2013) and new approaches added to the armamentarium for controlling the disease and its vectors (Russell *et al.* 2011; Cotter *et al.* 2013). The two front-line interventions for controlling malaria vectors in Africa, long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS), have exploited the indoor resting and host-seeking behaviour and the susceptibility of vectors to insecticides. These tools led to a major reduction of 29% in malaria cases worldwide (WHO 2013) justifying efforts to scale up LLINs and IRS. However, because of the growing problem of insecticide resistance [5-7], increasing importance of outdoor-biting vector populations [4] as well as heritable and plastic changes in vector behaviour in response to control [4, 8-12] the effectiveness of existing approaches may be compromised and additional strategies are required.

In spite of many anticipated challenges and limitations (Okumu *et al.* 2010a), mass trapping of gravid mosquitoes using synthetic attractant baits offers an exciting possibility for an eco-friendly, sustainable complementary strategy for monitoring and controlling disease vectors. Such strategies target mosquitoes that rest and bite both indoors and outdoors irrespective of their state of insecticide resistance. Extensive behavioural and chemical ecology studies on host-seeking members of the *Anopheles gambiae* species complex (including *Anopheles gambiae sensu stricto* (s.s.) and *Anopheles arabiensis*) which are the primary vectors of malaria in sub-Saharan Africa, have led to progress towards identification of odourants from skin emanations of humans and other primary blood-meal hosts (Meijerink *et al.* 2000; Braks *et al.* 2001) and host plants (Nyasembe *et al.* 2014). These volatiles have been incorporated into baits and tested in traps (Okumu *et al.* 2010b; Nyasembe *et al.* 2014). In contrast, very little is known about the cues that gravid females of these species use to find and orientate towards an aquatic habitat to lay their eggs. Whilst a range of physical and chemical cues associated with the aquatic habitat have been suggested (Blackwell & Johnson 2000; Huang *et al.* 2006b; Huang *et al.* 2007; Otienoburu *et al.* 2007; Lindh *et al.* 2008) empirical evidence is scarce and restricted to cage and electrophysiological studies not least due to the lack of appropriate bioassay tools.

Malaria vectors bite human hosts for vertebrate blood that they require for ovarian development. The malaria parasite (*Plasmodium* sp.) inadvertently imbibed with a blood-meal will require at least eight days to complete the sexual stage within the mosquito (Florens *et al.* 2002; Barillas-Mury & Kumar 2005). In theory, this period is punctuated by two or more oviposition cycles; a period when gravid mosquitoes look for suitable breeding sites, lay eggs and recommence the search for new hosts to bite for blood (Bentley & Day 1989). Targeting gravid vectors while they forage for aquatic habitats for their offspring would thus conceivably provide an effective approach to prevent the ultimate infective bites of parous mosquitoes and reduce overall vector population densities. Relevant oviposition cues that malaria vectors use to detect, find and evaluate potential breeding sites could be identified and exploited in various attract and kill strategies by luring females either into traps or in to insecticides (Ferguson *et al.* 2010).

Laboratory evidence shows that gravid females of the *An. gambiae* complex discriminate between different oviposition substrates. They are able to detect substrates with different levels of moisture and relative humidity (Huang *et al.* 2005; Okal *et al.* 2013) and the presence or absence of bacteria (Sumba *et al.* 2004a; Huang *et al.* 2006a; Lindh *et al.* 2008). A recent study demonstrated that at short-range gravid *An. gambiae* s.s. can avoid or select substrates using olfactory cues (Herrera-Varela *et al.* 2014). In another comparable laboratory study one synthetic odourant, 2-propylphenol was shown to increase the egg-laying rate of *An. gambiae* s.s. in cage tests (Rinker *et al.* 2013). However, to the best of our knowledge no study has provided evidence that gravid females of the *An. gambiae* complex orient towards a suitable aquatic habitat over a distance of several metres using attractant chemical cues except using the bioassay here described (Lindh *et al.* 2015).

The aim of the present study was to develop a simple bioassay for measuring olfactory orientation of gravid *An. gambiae* s.s. in semi-field conditions and evaluate the response of gravid mosquitoes to soil infusions previously described (Herrera-Varela *et al.* 2014) to increase the egg-laying rate of these species in small experimental cages. Laboratory studies have shown that the addition of inorganic salts to aqueous solutions can lead to a higher release of volatile organic compounds into the headspace of the solution, an effect that is known as salting-out (Morrison 1944; Friant & Suffet 1979; Buchholz & Pawilczyn 1994; Xie *et al.* 1997; Nakamura & Daishima 2005; Gorgenyi *et*

*al.* 2006; Alonso *et al.* 2012). For instance, Mozuraitis *et al.* (2010) showed that the amount of volatiles detected in the headspace from oestrous urine of mares increased eight times when the urine sample was saturated with salt compared to samples without salt. This study used salt to alter the volatile profile of the infusions and measure the sensitivity of the newly developed tool to changes in the chemical headspaces of these infusions. As a result, an effective tool that has since been used to describe the first oviposition semiochemical for the species (Lindh *et al.* 2015) described in detail.

### 5.3. Methods

#### 5.3.1. Study site

The study was done between March 2013 and February 2014 (time of sunset between 18.30 h and 19.00 h) at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe – TOC) at Mbita on the shores of Lake Victoria in western Kenya (0° 26' 06.19" S, 34° 12' 53.13" E; altitude 1,137 m above sea level). This area is characterised by a tropical climate with temperatures ranging between a mean minimum of 16°C and a mean maximum of 28°C and two rainy seasons each year between March and June and October and December.

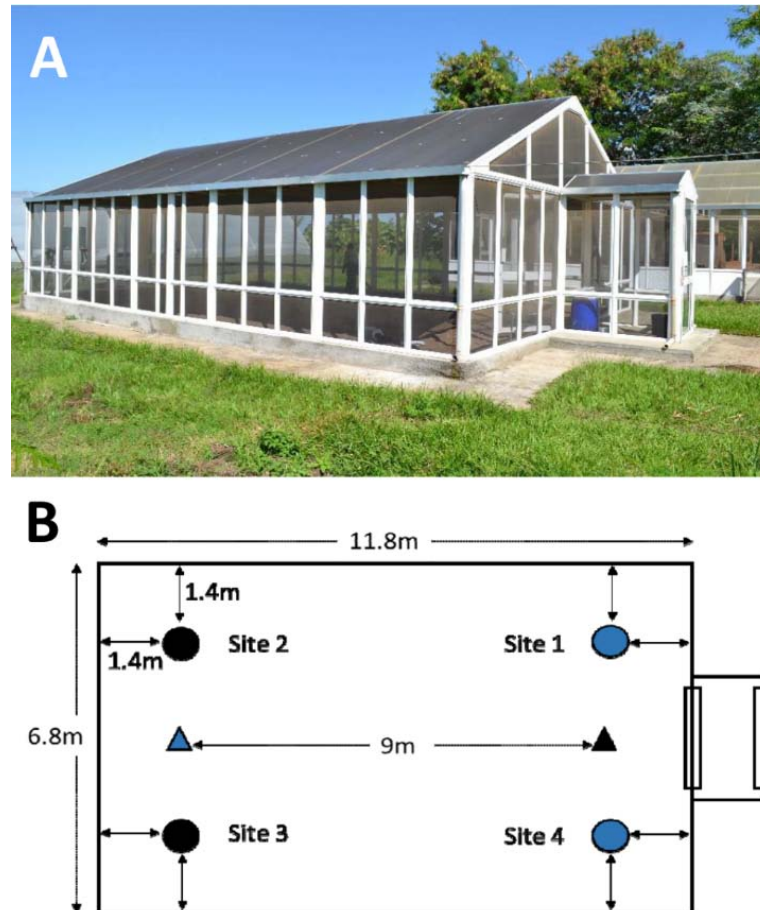
#### 5.3.2. Mosquito preparation

The Mbita strain of *An. gambiae* s.s. reared at the icipe -TOC mosquito insectaries was used for all experiments. Temperature and relative humidity in the insectary varied between 25–28°C and 68–75%. About 300 female mosquitoes held in a 30×30×30 cm netting cage with an equal number of males of a similar age were provided with two blood-meals on two consecutive nights from a human arm. Mosquitoes were starved for six hours before the blood-meal, which was offered for 15 min at 19:00 h. Mosquitoes that remained unfed after the first blood-meal were removed from the cage. A piece of cotton (50×25 cm) saturated with distilled water and positioned on top of the cage ensured that mosquitoes remained hydrated throughout oogenesis. Mosquitoes were left unattended for two days after the second blood-meal except for changing the 6% glucose solution provided as energy source and saturating the cotton on the cage with water twice a day. Gravid mosquitoes were selected by visual inspection on the third day. Females were presumed gravid when they had an opaque and pale distended abdomen.

### 5.3.3. Two-choice experiments

Two-choice experiments were implemented under semi-field conditions (i.e. ambient temperature, humidity, light conditions) in a large netting-screened (black fibreglass gauze 1.7x1.5 mm) structure; 6.8 m wide and 10.8 m long (semi-field system; Figure 5.1). A netting ceiling was stretched across the cage 2.4 m above the ground (176.3 m<sup>3</sup>). The floor was covered with sand to a depth of 50 cm. A roof made from transparent polycarbonate sheets shielded the structure from rainfall. The rectangular floor plan (two long walls, two short walls) of the semi-field system provided for four possible trap positions. Each position was arbitrarily set in each corner 1.4 m from the nearest adjoining walls (Figure 5.1). The two trap positions along the shorter walls of the semi-field system received approximately the same proportion of mosquitoes whilst there was large variability in catches when traps were set in diagonally opposite corners in preliminary tests. To reduce the statistical noise in the system the two traps constituting the dual-choice were always both placed at one of the two randomly selected short walls of the semi-field system, (site 2 + 3 and site 1 + 4; Figure 1). Gravid mosquitoes were released as far as possible from the traps near the opposite wall of the greenhouse, 9 m away from the two traps.

The location of the traps and the position of the treatments were randomly assigned for every night of an experiment. Two hundred gravid *An. gambiae* s.s. mosquitoes were released into the semi-field system at 17:30 h near the opposite shorter wall of the greenhouse, 9 m away from the two traps. Previous cage experiments (Okal *et al.* 2013) showed that the local mosquito strain has its peak oviposition time early in the evening before 21:30 h. To assess the proportion of gravid females that respond within this period the trapping chambers of the traps were changed at 21:30 h and the second pair retrieved at 08:00 h. This allowed tallying of the number of mosquitoes that were trapped with each treatment before 21:30 h and between 21:30 – 08:00 h. Each experiment was carried out on 12 nights based on previous sample size considerations (Dugassa *et al.* 2014) so that trap A and B were in each possible location three times. With this sample size an increment of 20% in the trap rate could be detected with 80% power at the 5% significance level.

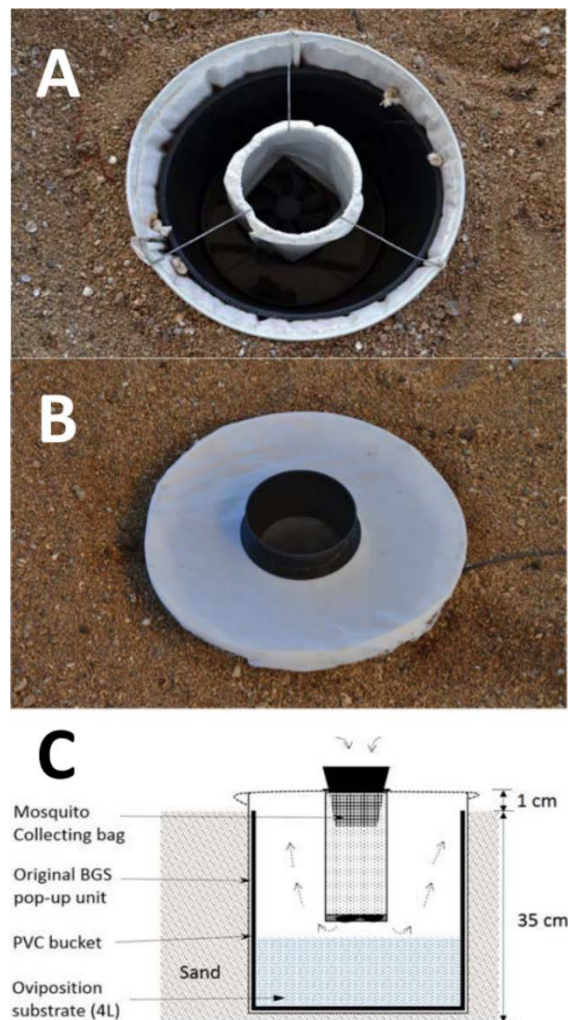


**Figure 5.1** Semi-field system (A) and schematic diagram of trap positions and release sites (B). Trap positions are shown in circles and mosquito release points in triangles. Colour codes show corresponding trap positions and mosquito release points.

#### 5.3.4. Modification of the Biogents (BG)-Sentinel™ mosquito trap into a gravid mosquito trap

Commercially available BG-Sentinel™ mosquito traps (Biogents, Regensburg, Germany) were modified and tested in this study. This is an odour-baited trap that was originally designed for mass trapping of host-seeking virus vectors like *Aedes aegypti* and *Aedes albopictus* using a chemical lure based on human body emanations (Eiras *et al.* 2004; Maciel-de-Freitas *et al.* 2006). One of the advantages of the trap is its size ‘which is large enough to incorporate additional attractants such as fragrant substances, small living animals, worn clothing, animal hairs, light and heat sources’ (Biogents 2014). The trap consists of a collapsible, white fabric container with white gauze covering its opening. The trap is 36 cm in diameter and 40 cm high. In the middle of the

gauze cover air is sucked into the trap through a black catch pipe by an electrical fan placed at its end. This draws approaching mosquitoes into a catch bag. Consequently, the air exits the trap through the gauze, generating ascending currents. The aim here was to include attractive oviposition media in the trap and to evaluate its catching efficiency under semi-field conditions. All oviposition sites of *Anopheles* mosquitoes are aquatic (or at least water saturated) and recent wind tunnel experiments suggested that water vapour is an important oviposition attractant for gravid *An. gambiae* s.s. (Okal *et al.* 2013). Consequently, the BG-Sentinel was modified to hold 4 L of aqueous test substrates by inserting a tightly-fitting black plastic bucket (Pride, Mombasa, Kenya) 34 cm high and 30 cm inner diameter into the white fabric container. Since *An. gambiae* s.s. mosquitoes rarely oviposit in container-type habitats, the entire trap was dug into the ground leaving only 1 cm of it above ground (Figure 5.2).



**Figure 5.2 Modification and set-up of BG-Sentinel trap.** (A) Interior showing bucket for holding aqueous solutions, (B) Complete trap (C) Cross-section of modified Biogents (BG)-Sentinel gravid mosquito trap.

### 5.3.5. Experimental procedures

#### 5.3.5.1. Assessing the catching efficacy of the modified BG-Sentinel gravid mosquito trap

To evaluate if and how effectively gravid *An. gambiae* s.s. are attracted to oviposition substrates contained in the traps and to generate a baseline for comparison, an experiment was carried out where mosquitoes were presented with two traps with identical substrates. Both traps (trap A and B) were filled with 4 L of fresh distilled water (Buyimpex Agencies LTD, Kenya), with the position of the traps allocated randomly.

#### 5.3.5.2. Analysing the response of gravid *Anopheles gambiae* s.s. to 6-day old soil infusions

Recently, a positive oviposition response of gravid *An. gambiae* s.s. to a six-day old soil infusion made from water mixed with soil taken from a natural breeding site located at icipe-TOC was demonstrated in cage egg-count experiments and chemical cues suggested as the reason for this response (Herrera-Varela *et al.* 2014). However, egg-count experiments do not provide information on the nature of these chemical cues, which could either be volatile and attract mosquitoes from a distance or could be less volatile and act as contact stimulants (Isoe *et al.* 1995b; Sumba *et al.* 2004a). Here, the same soil was used to prepare infusions in the same way as before (Herrera-Varela *et al.* 2014) and tested with the BG-Sentinel gravid mosquito trap. The silty clay loam top soil was dug from the same location as described by Herrera-Varela *et al.* (2014) within the icipe-TOC compound and sun-dried for 24 hours. Three litres of dry soil were thoroughly mixed with 15 L of distilled water in a 20 L plastic tub and left undisturbed at ambient conditions, but protected from rain for six days except for daily water top-up to compensate for loss through evaporation. Throughout the six days the tub was covered with mosquito netting. Just before the experiments the infusion was filtered through a cotton cloth to remove large soil particles and small debris. Exactly four litres of the soil infusion were compared to an equal volume of distilled water in choice experiments in the semi-field system. Fresh batches of infusions and distilled water were used for every experimental night.



### 5.3.5.3. Analysing the response of gravid mosquitoes to alterations in the headspace of 6-day old soil infusions

In order to be ideal for investigating attraction and formulating oviposition baits the bioassays used should be sensitive to slight changes in the release rate of odourants. An attempt was made to manipulate the release of odourants from soil infusion by adding NaCl and consequently investigate the sensitivity of the bioassay to changes in the chemical headspace of the soil infusion.

Following published data on salt concentrations (Mozuraitis *et al.* 2010), preliminary experiments were implemented where 45g of NaCl to 300 ml of soil infusion (150g/L) in a glass beaker and stirred to dissolve at room temperature. At this concentration small amounts of undissolved salt were observed to settle at the bottom of the beaker. Hence, for choice experiments, 150 g of NaCl was added per litre of the test substrates (600 g/4 L) and stirred to dissolve 10 - 20 min before the onset of experiments at 17:30 h. Two experiments were implemented. First, choice tests were done with distilled water versus soil infusion, both with NaCl. Second, the attractiveness of soil infusion without NaCl was tested against soil infusion with NaCl.

### 5.3.6. Data analysis

Data were analysed with generalised linear models with a binomial distribution and logit link function fitted to compare the probability of gravid *An. gambiae* s.s. being (1) collected in the test trap (trap B) compared with the total caught in both traps (trap A + trap B) to show substrate preference; (2) collected in both traps out of the total mosquitoes released (response rate); and (3) collected in both traps before 21.30 h out of the mosquitoes collected during the night (early responders). The underlying hypothesis of a choice bioassay is that when two equal choices are presented the response towards these choices is similar with odds of success of 1:1 (baseline or control). We expect that if an oviposition cue is presented that is either preferred or avoided by gravid females we will see a statistically significant diversion from the baseline. Consequently, the assay with two equal treatments served as reference. Initially, the trap location and the pair (wall) were included as fixed factors in the model to test for main effects and interactions. Since there were no significant associations with the outcome, these variables were excluded from the final models. The mean proportions of mosquitoes trapped in each treatment and their corresponding 95% confidence intervals (CI) were

calculated as the exponential of the parameter estimates for models with no intercept included. Data analyses were done with R statistical software version 3.00 with various functions contributed from the packages MASS, effects, epicalc, multcomp, lme4, gee, aod (R Team 2011).

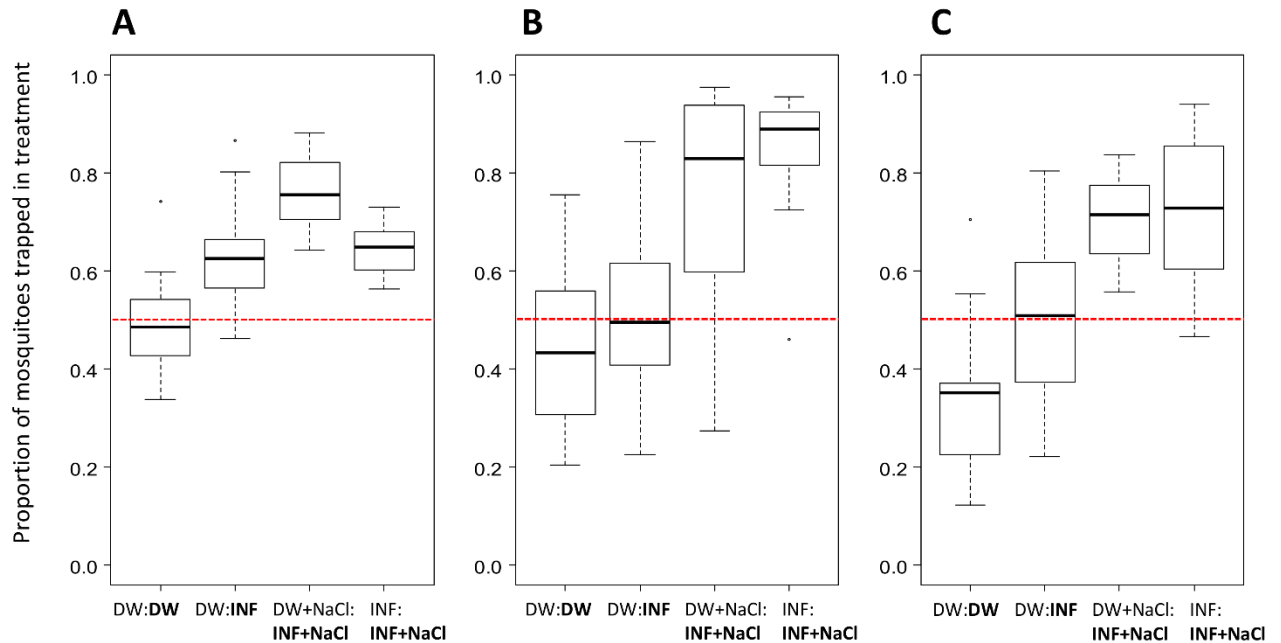
### 5.3.7. Ethics statement

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 422).

## 5.4. Results

### 5.4.1. The modified BG-Sentinel gravid mosquito trap is an effective tool for analysing oviposition attraction of malaria vectors under semi-field conditions

When two traps baited with distilled water were provided in choice tests, 45% of the released mosquitoes were recovered. Importantly, trap A and B caught equal proportions of the mosquitoes (50%, 95% CI 0.43 – 0.57%), which validates the experimental design. Only about one third of all mosquitoes (36% 95% CI 28 – 45%) were trapped before 21:30 h (Figure 5.3, Table 5.1).



**Figure 5.3 Explanatory data analyses of oviposition response of *Anopheles gambiae sensu stricto* to test substrates.** Box-and-whisker plots indicating the median value by the central horizontal line and the lower and upper quartiles by corresponding ends of the box. The whiskers show the range of the data. Dots show outlying values. (A) Proportion of females responding to the test substrate (INF =soil infusion, INFsalt=soil infusion with NaCl) compared to distilled water or infusion controls (DW=distilled water, DWsalt=distilled water with NaCl) in choice tests; (B) Response rate of the females released (N=200); (C) Response of mosquitoes before 21:30h out of the females trapped per night.

#### 5.4.2. Soil infusions contain odourants that attract gravid *Anopheles gambiae s.s.*

Gravid mosquitoes were twice as likely to be trapped in BG-sentinel gravid mosquito traps when the test trap (trap B) contained soil infusion as when the test trap contained distilled water in the bioassays with two equal choices (OR 1.8, 95% CI 1.3 – 2.6). Moreover, adding NaCl increased the attractiveness of the infusion; females were 3.4 (95% CI 2.4 – 4.8) times more likely to choose the infusion than distilled water. In direct comparisons of soil infusion with NaCl to soil infusions without NaCl, gravid females were nearly two times (OR 1.8, 95% CI 1.3 – 2.5%) more likely to be collected in the trap containing the infusion with salt (Figure 5.3, Table 5.1).

Table 5.1 Oviposition response of gravid *Anopheles gambiae sensu stricto* to substrates in two-choice tests. Generalised linear model outputs.

Oviposition substrates		Mean proportion	Odds ratio	p-value
Control (trap A)	Test (trap B)	(95% CI)	(95% CI)	
<b>Proportion of gravid females trapped in test (trap B) in two choice experiments of the females trapped</b>				
Distilled water	Distilled water	0.50 (0.43 – 0.57)	1	
Distilled water	Infusion	0.64 (0.58 – 0.70)	1.8 (1.3 – 2.6)	0.004
Distilled water + NaCl	Infusion + NaCl	0.77 (0.72 – 0.81)	3.4 (2.4 – 4.8)	<0.001
Infusion	Infusion+ NaCl	0.67 (0.60 – 0.69)	1.8 (1.3 – 2.5)	0.001
<b>Response rate of released gravid females during experiments with different substrate combinations</b>				
Distilled-Distilled		0.45 (0.33 – 0.57)	1	
Distilled-Infusion		0.51 (0.39 – 0.63)	1.3 (0.6 – 2.5)	0.438
Distilled+NaCl – Infusion+NaCl		0.74 (0.63 – 0.84)	3.7 (1.8 – 7.5)	<0.001
Infusion - Infusion+NaCl		0.84 (0.73 – 0.91)	6.8 (3.1 – 15.0)	<0.001
<b>Response of gravid females before 21:30 h of the females trapped during the night (early responders)</b>				
Distilled-Distilled		0.36 (0.28 – 0.45)	1	
Distilled-Infusion		0.56 (0.46 – 0.67)	1.59 (1.11 – 2.29)	0.003
Distilled+NaCl – Infusion+NaCl		0.66 (0.58 – 0.74)	1.92 (1.38 – 2.68)	<0.001
Infusion - Infusion+NaCl		0.64 (0.54 – 0.73)	1.76 (1.29 – 2.44)	0.001

#### 5.4.3. The presence of attractive odourants in the semi-field system increases the response rate of gravid *Anopheles gambiae* s.s.

When salt-saturated infusions were present in one of the traps it was 3.7-6.8 times more likely a mosquito would respond and be collected in either trap than when only distilled water was presented in both traps (Figure 5.3, Table 5.1).

#### 5.4.4. Odourant cues from soil infusions prompt early oviposition site seeking in *Anopheles gambiae* s.s.

The presence of soil-infusion odourants doubled (OR 1.92, 95% CI 1.38 – 2.68) the proportion of mosquitoes that responded before 21.30 h (Figure 5.3, Table 5.1).

## 5.5. Discussion

Minor modifications of the commercially available BG-Sentinel mosquito trap optimised it into a valuable bioassay tool for evaluating the orientation of gravid mosquitoes to putative oviposition substrates using olfaction. The modified traps excluded any possible contact stimuli or visual cues (e.g. light reflections from water) from the test substrates and showed a strong discrimination effect enabling the detection of small differences ( $\leq 20\%$ ) in the proportion of gravid mosquitoes attracted to one of two competing substrates (odourant blends). The BG-Sentinel mosquito trap is simple to set up and allows for rapid replacement of collection bags making it possible to evaluate the response of gravid mosquitoes at different periods during the night.

With this system we provide evidence that gravid females of the major malaria vector *An. gambiae* s.s. can detect and respond to attractive odourant cues over at least nine metres to locate and choose between potential oviposition sites. Many studies have suggested the involvement of chemical cues in the selection of breeding sites (Blackwell & Johnson 2000; Sumba *et al.* 2004a; Lindh *et al.* 2008; Herrera-Varela *et al.* 2014). However, all of these studies were egg-count bioassays done in small cages (30×30×30 cm) with gravid mosquitoes released directly over test substrates. Consequently, none of the studies were able to prove attraction or describe an attractant, defined as cues that draws insects towards substrates (Dethier *et al.* 1960; Miller *et al.* 2009). This study shows that odourants from the soil infusions reported by Herrera-Varela *et al.* (2014) attract gravid *An. gambiae* s.s.. Furthermore, our results show that oviposition attraction to odourant chemicals is affected by the strengths of the cue, as shown from the salting-out experiments. This observation is important if one wanted to use odour-baited traps for the surveillance or control of gravid mosquitoes since it indicates that olfactory cues can be manipulated to attract and mass trap gravid malaria vectors.

This study confirms earlier laboratory findings that gravid *An. gambiae* s.s. use water vapour to locate breeding sites (Chapter 5). Previous studies were done in small, closed laboratory systems, free of external odourants with standardized water vapour gradients (Huang *et al.* 2005; Okal *et al.* 2013). With the bioassay, where both traps contained distilled water only, this study provides evidence that malaria vectors use water vapour to orientate to substrates in more natural and fairly complex chemical spaces over larger

distances. It is likely that water vapour is a general selective cue, but provides no information about the quality of the habitat which might be the reason for the observed slow and low response of gravid females. In the complex chemical space of natural ecosystems it is unlikely that a species with such a highly developed olfactory apparatus should evolve to employ water vapour as the major cue for selecting favourable water bodies. Water vapour most likely indicates the presence of water bodies while chemical odourants enable mosquitoes to assess the suitability of this potential niche.

Based on the findings of this study it was hypothesised that the soil infusions tested contained at least one odourant that prompted habitat searching in gravid *An. gambiae* s.s.. The odourant bouquet of soil infusions evidently compelled passive gravid *An. gambiae* s.s. mosquitoes to fly towards the potential oviposition sites, especially when the infusions were saturated with salt. This was in contrast to the response when only distilled water was provided. A similar response has been shown for host-seeking mosquitoes when exposed to carbon dioxide which triggers long-range directed host seeking flight in otherwise inactive females of the *An. gambiae* complex (Gillies 1980). In nature such an odourant or collection of odourants would shorten the period for foraging for suitable aquatic sites by gravid mosquitoes. Gravid mosquitoes would use less energy and reduce the risk for mortality that is likely associated with prolonged habitat search and altogether improve the odds for successful breeding.

This is the first study to exploit the principle of salting-out volatile chemicals to demonstrate the potential use of NaCl in behavioural bioassays to manipulate the odour profile of organic infusions. This study shows that adding NaCl to soil infusions increased the attraction of gravid *An. gambiae* s.s. to soil infusions two-fold and the response rate three-fold. This adds proof that *An. gambiae* s.s. respond to chemical cues in soil infusion. Whilst it cannot be excluded that the addition of salt affected the microbial organisms in the soil infusion and therefore changed the chemical composition of the volatile headspace, the increase in attractiveness of the already highly attractive soil infusion suggests that it is more likely that the addition of salt led to an increased release of already present attractive odours. Numerous studies using a wide range of inorganic salts have shown that these increase the concentration of volatile organic compounds (VOC) in the headspace above the salt containing solution (Morrison 1944; Xie *et al.* 1997; Nakamura & Daishima 2005; Mozuraitis *et al.* 2010; Alonso *et al.* 2012). The presence of salt decreases the solubility of the VOCs which are

pushed into the headspace. This effect is commonly known as the salting-out effect and can be quantified by the Setschenow constant (Morrison 1944) which most frequently is positive (salting-out) but can also be negative (salting-in) (Ni & Yalkowsky 2003). In preliminary studies (Lindh JM, personal communication) aimed at optimizing the collection of volatiles in the headspace above water from mosquito breeding sites, which should be similar in chemical composition to the soil infusion studied here, addition of NaCl increased the amount detected of the majority of the compounds and pushed many previously undetected organic compounds above the detection limit. This theoretically represents an inexpensive advancement of harnessing NaCl saturated natural infusions to produce relatively inexpensive baits for gravid malaria mosquitoes for use in gravid traps. However, at very high concentrations, NaCl will corrode and quickly destroy metallic parts in the traps. More work might be useful to evaluate if smaller amounts of NaCl can still improve the odour plume and reduce the damage to the traps.

The high efficiency of BG-Sentinel gravid mosquito traps baited with NaCl saturated soil infusions in collecting gravid *An. gambiae* s.s. suggests their potential use in the field as an odour-baited gravid trap. The trap does not damage specimens, making it ideal for sampling wild mosquito populations in studies that require intact specimens or requires mosquitoes to be captured alive. The trap has been the subject of many explorative evaluations with host-seeking mosquitoes (Schmied *et al.* 2008; Gama *et al.* 2013) proving its versatility and effectiveness. This study now shows that with only small modifications it has potential for collecting gravid mosquitoes too. Its potential use in large-scale ecological studies or in vector control programmes should be evaluated in natural field conditions.

## 5.6. Conclusion

In summary this study (1) describes an efficient bioassay tool and potential new odour-baited trap for gravid females of the *An. gambiae* species complex; (2) provides evidence for the importance of olfaction in the location and selection of potential breeding sites by *An. gambiae* s.s.; and (3) describes the compulsive response of gravid females to attractive chemical cues. Research needs now to be invested in analysing the volatile chemical headspace of the attractive soil infusion to identify attractant semiochemicals for oviposition in *An. gambiae* s.s..

## Chapter 6. Insight into hay infusion avoidance by gravid malaria mosquitoes



Hay infusion in the making (Source: Jenny M. Lindh)

**This chapter is prepared for publication in the Journal of Chemical Ecology**

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2.3. Stage of publication – Not yet submitted / Submitted / Undergoing revision from peer reviewers' comments / In press


**3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)**

I contributed significantly to the conception and design of this work. I also developed all protocols for and implemented the behavioural bioassays, analysed the data and wrote the first draft of the manuscript.

NAME IN FULL (Block Capitals) Michael Nyang'anga Okal .....

STUDENT ID NO: LSH313699 .....

CANDIDATE'S SIGNATURE  Date 17th June 2015 .....

SUPERVISOR/SENIOR AUTHOR'S SIGNATURE (3 above)  .....

## 6.1. Abstract

**Background:** The responses of individual gravid *Anopheles gambiae sensu stricto (s.s.)* to hay infusions volatiles were evaluated under laboratory conditions. Such infusions have long been known to attract a few mosquito species of the genera *Aedes* and *Culex* to egg-laying sites. Consequently, these infusions and semiochemicals identified from them have been used effectively as baits for monitoring mosquitoes that vector arboviral and filarial diseases. This study investigated the response of *An. gambiae s.s.*, the chief malaria vector, to Bermuda grass hay infusions.

**Methods:** Hay infusions were formulated by adding 90 g of sun-dried Bermuda grass to 24 L of lake water and leaving the mixture in a covered bucket for three days. The proportions of eggs laid by gravid *An. gambiae s.s.* in diluted (10%) and concentrated infusions ( $\geq 25\%$ ) was compared to that laid in lake water in two-choice egg-count bioassays. Volatile compounds in the infusion's headspace were collected with Tenax TA polymer traps for 20 hours over hay infusion aliquots saturated with sodium chloride (NaCl). The polymer traps were thermally desorbed in a Gas chromatograph-Mass spectrometer (GC-MS) and compared to electronic chemical mass libraries to identify chemical constituents emitted by the infusions. Ten volatiles identified from headspace and previously indicated as putative oviposition semiochemicals for *An. gambiae s.s.* or confirmed semiochemicals for other mosquito species were tested in egg-count bioassays in concentrations of between 0.01 – 5.00 ppm chemical in lake water.

**Results:** Gravid *An. gambiae s.s.* did not discriminate between diluted hay infusion and lake water but were 10 times less likely to lay eggs in the concentrated hay infusion compared to the water (Odd ratio (OR) 0.10, 95% Confidence interval (CI) 0.03 – 0.33). Six out of ten compounds identified from the headspace of hay infusion showed behavioural activity in two-choice egg-count bioassays. Mosquitoes avoided laying eggs in nonanal, 3-methylindole, indole and 3-methyl-1-butanol at low concentrations ( $\leq 1.0$  ppm). Mosquitoes also avoided 4-ethyl phenol and phenol when the two compounds were presented at a relatively high concentration of 5.0 ppm in two choice tests with lake water.

**Conclusions:** Contrary to a number of culicine species, *An. gambiae s.s.* is not attracted to hay infusions. The compounds nonanal, 3-methylindole, indole, 3-methyl-1-butanol, 4-ethyl phenol and phenol likely contribute to the unfavourable oviposition response towards the infusions. It is speculated that these might partially explain why *An. gambiae s.s.* larvae are less common in water bodies with high organic content and why field studies with hay infusion baits in oviposition traps have never reported trapping of malaria vectors.

## 6.2. Background

Immature stages of all mosquito species (Diptera: Culicidae) are aquatic (Rozendaal & Organization 1997). Gravid adults therefore need to find suitable water bodies (or in case of many *Aedes* species suitable sites where water is likely to collect) to lay eggs in (Bentley & Day 1989). The larvae and pupae of these can be found in a large variety of heterogeneous habitats but individual species may have more defined preferences for certain habitat characteristics and water qualities (Bentley & Day 1989). For instance, *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), *Culex quinquefasciatus* (Say), *Culex tarsalis* (Coquillett) all often preferentially lay eggs in water bodies rich in organic matter (Gjullin *et al.* 1965; Kramer & Mulla 1979; Reiter 1983; Beehler *et al.* 1994; Allan & Kline 1995; Burkett-Cadena & Mullen 2007; Ponnusamy *et al.* 2010). Gravid females of these species are attracted to specific volatile chemicals that emanate from microbial breakdown of organic matter within these aquatic sites (Hazard *et al.* 1967; Rockett 1987; Benzon & Apperson 1988; Hasselschwert & Rockett 1988; Isoe & Millar 1995). For this reason, hay infusions that mimic these oviposition sites (Gjullin *et al.* 1965) have been formulated and used as lures in gravid traps and ovitraps for detection and surveillance of vectors of mosquito-borne diseases such as dengue, dengue haemorrhagic fever, West Nile virus and St. Louis encephalitis (Reisen *et al.* 2004; Lukacik *et al.* 2006; Williams & Gingrich 2007; WHO 2009; Mackay *et al.* 2013; Barrera *et al.* 2014). Recent studies in Tanzania show that semiochemicals identified from such infusions might be useful for monitoring of *Cx. quinquefasciatus* and xenodiagnosing bancroftian filariases in Africa (Irish *et al.* 2013; Irish *et al.* 2014).

*Anopheles gambiae s.l.* larvae are frequently found to share habitats with culicine mosquito species in Africa (Fillinger *et al.* 2004; Ndenga *et al.* 2011). This could

suggest that gravid mosquitoes of these diverse species can, to a certain degree, make the same choices for breeding sites. In addition, at least two volatile compounds identified from hay infusions, indole (Blackwell & Johnson 2000; Himeidan *et al.* 2013) and 4-methylphenol (Walker 2011; Himeidan *et al.* 2013) have been suggested to attract gravid *An. gambiae s.s.* in low concentrations. However, no behavioural studies have been implemented and reported investigating if hay infusions or these chemicals affect the behaviour of gravid *An. gambiae s.s.*.

Previous studies have successfully identified semiochemicals from hay infusions made from Bermuda grass that attract *Culex* mosquitoes. Millar and others (1992) characterised chemicals in Bermuda grass hay infusion through solvent extraction and guided by bioassays showed that active fractions contained phenol, 4-methylphenol, 4-ethylphenol, indole and 3-methylindole. One of the compounds, 3-methylindole, was shown to be attractive to *Cx. quinquefasciatus* in laboratory cage tests leading to further field studies (Millar *et al.* 1994; Barbosa *et al.* 2010). In a follow up study by Du and Millar (Du & Millar 1999b), using electroantennography to screen volatiles, additional odourants were detected, among them nonanal. Nonanal has also been tested as a potential replacement for 3-methylindole in commercial baits since nonanal-based lures would be less pungent and can therefore be more acceptable to users (Irish *et al.* 2013). All these compounds have been demonstrated to be physiologically active in electroantennographic studies with *An. gambiae s.s.* (James Broom, pers comm., Appendix B). In addition, many of them have been suggested as putative oviposition semiochemicals for *An. gambiae s.s.* based on presence in substrates that elicited high eggs rates in laboratory studies with these species (Blackwell & Johnson 2000; Lindh *et al.* 2008; Walker 2011; Himeidan *et al.* 2013). However, none of these have actually been confirmed with empirical behavioural studies.

This study set out to (1) evaluate the response of gravid *An. gambiae s.s.* to hay infusions made from Bermuda grass, one of the best known oviposition attractant used in traps to collect a number of mosquito species but which has never been tested for malaria vectors; (2) identify the major odourants in the headspace of the hay infusion; and (3) investigate the role of these odourants in mediating the observed oviposition responses of *An. gambiae s.s.* to the hay infusion

## 6.3. Methods

### 6.3.1. Mosquitoes

All mosquitoes used for this study were supplied by the mosquito insectaries at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC), Mbita, western Kenya (0° 26' 06.19" South; 34° 12' 53.12" East; altitude 1149 m). The mosquitoes were reared following standard operating procedures described by Herrera-Varela and others (Herrera-Varela *et al.* 2014). Approximately 300 female and 300 male mosquitoes of the *An. gambiae s.s.* Mbita strain were randomly selected from an adult mosquito holding cage with more than one thousand two to three days old mosquitoes. The selected mosquitoes were starved for about 7 hours (between 12:00 – 7:00 h) before the females were allowed to blood-feed from a human arm for 15 minutes at dusk. Cotton towels saturated with water were placed over the cage throughout to maintain the relative humidity and temperature of 68-75% and 25-28°C, respectively. Female mosquitoes that did not blood-feed, as judged by an engorged abdomen, were removed from the cage and a vial containing 6% glucose with a paper towel wick were introduced in the cage immediately after blood-feeding for ad libitum sugar supply. A second blood-meal was provided 24 hours later at 19:00 h. On the fifth days after the first blood-meal, presumed gravid mosquitoes (based on their abdominal appearance) were selected by experienced technicians from the cage at 16:30 h and used for behavioural bioassays.

### 6.3.2. Preparation of hay infusions

Hay infusions were prepared from Bermuda grass (*Cynodon dactylon* (L.)) which is widely distributed throughout the world and has frequently been used for the preparation of hay infusions for baiting gravid culicine mosquitoes (Mboera *et al.* 2000a; Mboera *et al.* 2000c; Burkett-Cadena & Mullen 2007). Here, fresh grass was harvested locally and sun-dried for 48 h to make hay. Infusions were prepared by mixing 90 g of the hay with 24 L of lake water in a plastic bucket. The mixture covered with net of mesh size 0.6 mm x 0.6 mm was left outdoors in a roofed and shaded area at ambient temperature and humidity (mean daily temperature  $27 \pm 5^\circ\text{C}$ , relative humidity  $55 \pm 10\%$ ) for three days. Thereafter, the infusion was filtered through a clean piece of cotton cloth to remove large debris. Different dilutions of the hay infusions (10%, 25%,

and 50%) were formulated for egg-count cage bioassays by diluting the infusion with lake water. A new batch of hay infusions was prepared for every round of bioassay experiments. Five, aliquots of 5L of the infusion were frozen at -70°C for chemical headspace collection.

### 6.3.3. Handling and use of glassware

All glassware were washed with a mild detergent, rinsed with water and acetone and then placed in a 200°C oven over night. Volatiles were sampled from Erlenmeyer flasks (E-flasks) fitted with gas wash bottle heads (QuickFit joined ware, Staffordshire, United Kingdom). Oviposition substrates were offered in 7 cm diameter, 100 ml clear borosilicate crystallising glasses (Pyrex®, hereafter called oviposition cups). Prior to any experiment oviposition cups were autoclaved and kept at 200 °C for two hours.

### 6.3.4. Behavioural bioassays with hay infusion.

To evaluate the response of gravid *An. gambiae s.s.* to hay infusions, two-choice egg-count bioassays were implemented following a recently described method (Okal et al, submitted). In this approach individual gravid females are exposed to two putative oviposition substrates in a cage. Two glass cups (Pyrex®, 100 ml, 70 mm diameter), one test cup, and one control cup, were set in diagonal corners of each 30x30x30 cm cage. In the two choice experiments, the control cup was filled with 100 ml of lake water and the test cup with an equal amount of the infusion. Bias that could stem from the position of oviposition cups within the cages was minimized by systematically altering the position of the cups in each cage relative to the preceding cage. The first test cup was randomly set in one of the four possible corners in the first cage. Subsequent test cups were rotated in the next possible corner in a clockwise direction relative to the position of the test cup in the preceding cage. One cup containing lake water was added in each cage diagonal to the test cup to complete a two-choice set-up. An equally replicated set of cages with lake water in both cups (two equal choices) was implemented and used as a baseline with which to compare responses in the choice tests with two different substrates.

One gravid mosquito was introduced in each cage at 18:00 h. The presence and number of eggs was scored for every cup the next morning at 08:00 h. Mosquitoes that did not lay eggs were excluded from the analysis. Six round were implemented with between

ten to fifty replicate cages per round for every treatment (Table 6.1). All experiments were done in make-shift sheds at icipe-TOC at ambient conditions of temperature, humidity and light but protected from rain.

### 6.3.5. Dynamic headspace sampling of volatile compounds released from hay infusion.

Adsorbent traps were made by adding 25 mg of Tenax TA of mesh size 60/80 (Supelco, Sigma-Aldrich Sweden AB, Stockholm, Sweden) in a GERSTEL-Twister Desorption glass liner (GERSTEL, Muelheim an der Ruhr, Germany) and held in place with glass wool (Supelco, Sigma-Aldrich Sweden AB, Stockholm, Sweden). The traps were washed 10 times with 2 ml of methyl-tert butyl ether (MTBE, Supelco, Sigma-Aldrich Sweden AB, Stockholm, Sweden) and then placed in a 50°C oven with both ends covered with PTFE tape for at least six hours before use.

Previously frozen infusion was naturally thawed at room temperature (25 – 28°C) on the day of headspace sampling. Headspace samples were collected over 300 ml aliquots of defrosted and undiluted soil infusion in a 500 ml E-flasks. Exactly 150 g/L of sodium chloride (NaCl, reagent grade Scharlau, Sentmenat, Barcelona, Spain) was added to every aliquot. Charcoal filtered air was pumped through the E-flasks at 0.5 L/min and drawn out through Tenax TA adsorption traps. All connections were made airtight using glass and polytetrafluoroethylene (PTFE) tubing and sealed with PTFE tape. Volatiles were sampled for twenty hours. Empty E-flasks served as baselines for analyses. This was repeated over five rounds. After headspace collection the polymer traps were sealed with PTFE tape and stored at -70 °C.

### 6.3.6. Chemical analyses of hay infusion headspace samples.

Samples were analysed with an Agilent 7890A gas chromatograph (GC) connected to an Agilent 5975C inert MSD mass spectrometer (MS) (Santa Clara CA, United States). The GC system was fitted with GERSTEL Multi-Purpose Sampler (MPS: Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) and an Agilent HP-5MS (5% phenyl and 95% dimethyl polysiloxane) capillary column (30 m, 250 µm internal diameter and 0.25 µm film thickness).

Tenax traps were thermally desorbed in splitless mode in a GERSTEL thermal desorption unit (TDU) at initial temperature 40°C, then increased by 120°C / min to 270°C which was held for 5 min. One microliter heptyl-acetate (3.16 ng / µl) was added to the Tenax trap in the TDU unit prior to analysis. The desorbed volatiles were focused in a GERSTEL CIS inlet at 10°C. The CIS inlet operated in splitless mode was then heated at a rate of 12°C/s to 280°C. The GC oven was programmed to start at a temperature of 40 °C for 1 minute; then increased by 4°C / min to 280°C. The final temperature was held for 3 minutes. Helium at a pressure of 34 psi was used as the carrier gas. The MS was set to full scan and identified mass ranges from 30-400 m/z with electron ionization at 70 eV and ion source temperature at 230°C.

GC-MS data was captured and processed with the enhanced ChemStation software version E.02.01.1177 (Agilent Technologies, Santa Clara, CA, USA). All peaks that had unique retention times and/or mass spectra were manually integrated. The empty bottle baselines/ controls were used to adjust for background volatiles. Peaks present in both the empty bottle and sample collections were only retained if they were at least two times as large in the sample. The areas of such peaks were adjusted by subtracting that of the matching peak in the empty bottle. An internal standard was used as a marker to match peaks between different samples. Mass spectra of all peaks were compared to those of the NIST 2008 library for tentative identifications.

The identity of compounds of interest were confirmed with authentic standards; (3-methyl-1-butanol, 3-methylindole, phenylmethanol and indole (Sigma-Aldrich Sweden AB, Stockholm, Sweden); phenol, 2-phenylethanol and 1-octen-3-ol (Lancaster, Chemtronica, Stockholm, Sweden), 4-methylphenol and 4-hepten-1-ol (Alfa Aesar, Chemtronica, Stockholm, Sweden), 4-ethylphenol (TCI Europe NV, Chemtronica, Stockholm, Sweden). All standards tested had a purity of at least 95%. For each compound, one microliter of a 10<sup>-4</sup> M dilution in methyl-tert-butylether was injected into thermal desorption unit using the same GC-MS settings described above.

### 6.3.7. Behavioural bioassays with chemicals identified from hay infusions

Ten compounds (purchased from Sigma Aldrich, St. Louis, USA and >99% pure, unless otherwise stated) were analysed in two choice bioassays following the same procedures as described for the bioassays with infusion. A chemical identified from the chromatograms was considered for further analyses if it was: (1) a dominant constituent



of the hay infusion headspace: 4-hepten-1-ol (97%, Alfa Aesar, Chemtronica, Stockholm, Sweden), 4-methyl-phenol, 4-ethyl-phenol, 3-methylindole (98%, Acros organics, New Jersey, USA); (2) detected in the headspace of the hay infusion and previously suggested to influence the oviposition behaviour of *Anopheles*: 3-methyl-1-butanol, phenylmethanol, 2-phenylethanol (>99%, Fisher scientific, Loughborough, UK) and indole (>99%, Acros organics, New Jersey, USA); (3) extensively referenced in other oviposition studies with mosquitoes and identified in the headspace of hay infusions: phenol and nonanal (both 95%, Sigma Aldrich, St. Louis, USA)). The compounds were tested at various concentrations of between 0.01 – 5.00 parts per million (ppm) in lake water. Details available in Table 6.1

### 6.3.8. Statistical analyses

Generalised linear models with quasibinomial distributions were used to analyse behavioural data from two-choice egg-count bioassays with hay infusions and putative semiochemicals. The proportion of eggs laid by gravid female mosquitoes in the test cup in the experiments with two different choices was compared to that in the test cup in cages with two equal choices. It was hypothesised that gravid females presented with equal choices respond to both in an approximately equal proportion ( $p=0.5$ ). The statistical analyses were aimed at revealing if the treatment of interest (e.g. different concentrations of grass infusion or chemicals) elicited an increase or decrease in the proportion of eggs laid as compared to the distribution in the experiments with equal choices. The test treatment (lake water, infusions or chemicals) and the round of experiment were included in the model as fixed factors. Mean proportions and associated 95% confidence intervals were predicted from the fitted model. Data were analysed in R (R Team 2011).

### 6.3.9. Ethical considerations

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no.422).

**Table 6.1 Summary of two-choice egg-count bioassays to evaluate the egg laying preferences of gravid *Anopheles gambiae* s.s. to hay infusion volatiles compared to lake water**

Test substrate	Concentration of test substrate <sup>a</sup>	No. of rounds	No of mosquitoes	
			Tested	Laid eggs
Hay infusion	0	8	375	075 <sup>b</sup>
	10%	3	150	126
	25%	3	030	022
	50%	3	030	025
	100%	3	030	028
3-methyl-1-butanol	0	5	250	209
	0.010ppm	3	151	133
	0.100ppm	5	250	197
	1.000ppm	4	300	209
4-hepten-1-ol	0	9	265	185
	0.100ppm	6	148	110
	0.500ppm	4	119	091
	1.000ppm	4	116	088
	5.000ppm	5	149	094
phenol	0	9	265	185
	0.100ppm	5	107	107
	0.500ppm	3	148	110
	1.000ppm	3	090	071
	5.000ppm	5	147	102
phenylmethanol	0	6	210	175
	0.500ppm	5	175	144
	1.000ppm	5	175	138
	2.500ppm	5	210	149
	5.000ppm	6	230	169
4-methylphenol	0	11	417	319
	0.100ppm	5	208	130
	0.500ppm	4	119	087
	1.000ppm	5	238	171
	5.000ppm	5	146	109
2-phenylethanol	0	7	200	165
	0.100ppm	4	200	162
	0.500ppm	5	155	125
	1.000ppm	5	155	131
	2.500ppm	4	180	144
	5.000ppm	6	195	168
nonanal	0	4	400	360
	0.050ppm	4	150	116
	0.100ppm	3	250	190

	0.500ppm	4	060	032
	1.000ppm	4	060	043
4-ethylphenol	0	6	220	169
	0.100ppm	5	148	110
	0.500ppm	3	080	060
	1.000ppm	3	087	060
	5.000ppm	3	132	109
indole	0	5	430	203
	0.100ppm	4	060	037
	0.500ppm	4	060	040
	1.000ppm	5	225	160
	5.000ppm	5	205	158
3-methylindole	0	8	290	195
	0.010ppm	8	130	102
	0.100ppm	8	130	098
	0.500ppm	8	160	110
	1.000ppm	8	250	170

**a** “0” indicate pure lake water i.e. two equal choices used as control (baseline); **b** Data from seventy five control-control cages was randomly selected from a total of 375 cages from eight different rounds in previous experiments using an excel add-in random sorter.

## 6.4. Results

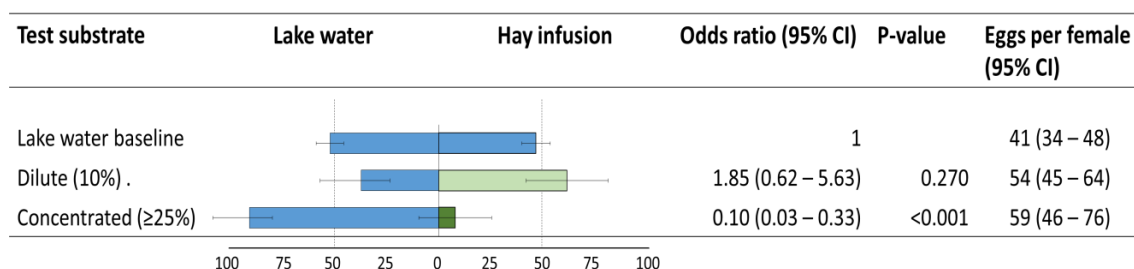
### 6.4.1. Oviposition response of *Anopheles gambiae s.s.* to hay infusions.

When two equal lake water choices were presented, females laid a similar proportion of eggs in the test and control cup (0.47, 95% CI 0.32 – 0.63) validating the experimental design and serving as baseline for comparison. The distribution of eggs in the two choice test with a dilute 10% hay infusion did not significantly differ from the baseline (Figure 6.1). However, gravid *An. gambiae s.s.* laid a lower proportion of eggs in 25% infusions (0.11, 95% CI 0.03 – 0.33), 50% infusions (0.07, 95% CI 0.02 – 0.26) and 100% infusions (0.06, 95% CI 0.02 – 0.22) when given a choice to lay in lake water instead. The differences in the response to infusions with concentrations of 25% and above were not significant ( $P=0.69$ ) and the data was pooled for final analysis. Eggs were 10 times less likely to be laid in the test cup when the choice was a concentrated hay infusion  $\geq 25\%$ ; test) versus lake water (control) than when the choice was lake water (test) versus lake water (Figure 6.1).

#### 6.4.2. Chemical constituents of the hay infusion headspace

The most dominant compounds in the headspace of hay infusions were 4-heptan-1-ol, 4-methylphenol, 3-methylindole and 4-ethylphenol. Other key compounds included 3-methyl-1butanol, phenylmethanol, 2-phenylethanol, indole and phenol which have all been suggested to attract gravid *An. gambiae sensu stricto* (Blackwell & Johnson 2000; Lindh *et al.* 2008; Himeidan *et al.* 2013).

**Figure 6.1 Egg-laying responses of individual gravid *Anopheles gambiae sensu stricto* to hay infusion compared to lake water**



Nonanal was not detected from the infusion headspace using our chemical analysis method but was tested because it has been detected previously in hay infusions (Leal *et al.* 2008) and has been used in attractant blends for other mosquito species (Irish *et al.* 2014). All these compounds except for 4-heptan-1-ol have been previously shown to elicit electrophysiological responses in *An. gambiae s.s.* (Blackwell & Johnson 2000; Carey *et al.* 2010). (Table 6.1)

**Table 6.2 Frequency and abundance of key volatile organic compounds detected in the headspace of hay infusions and evaluated in egg-count bioassays**

Putative semiochemical (in order of abundance)	No. of samples (rounds) with compound	Average amount detected (ng/μL)
4-hepten-1-ol	5	11.261
4-methylphenol	5	9.625
3-methylindole	4	5.336
4-ethylphenol	5	6.300
indole	4	2.176

phenyl methanol	1	0.822
2-phenylethanol	4	0.656
phenol	4	0.777
3-methyl-1-butanol	5	0.051
Nonanal	0	0.000

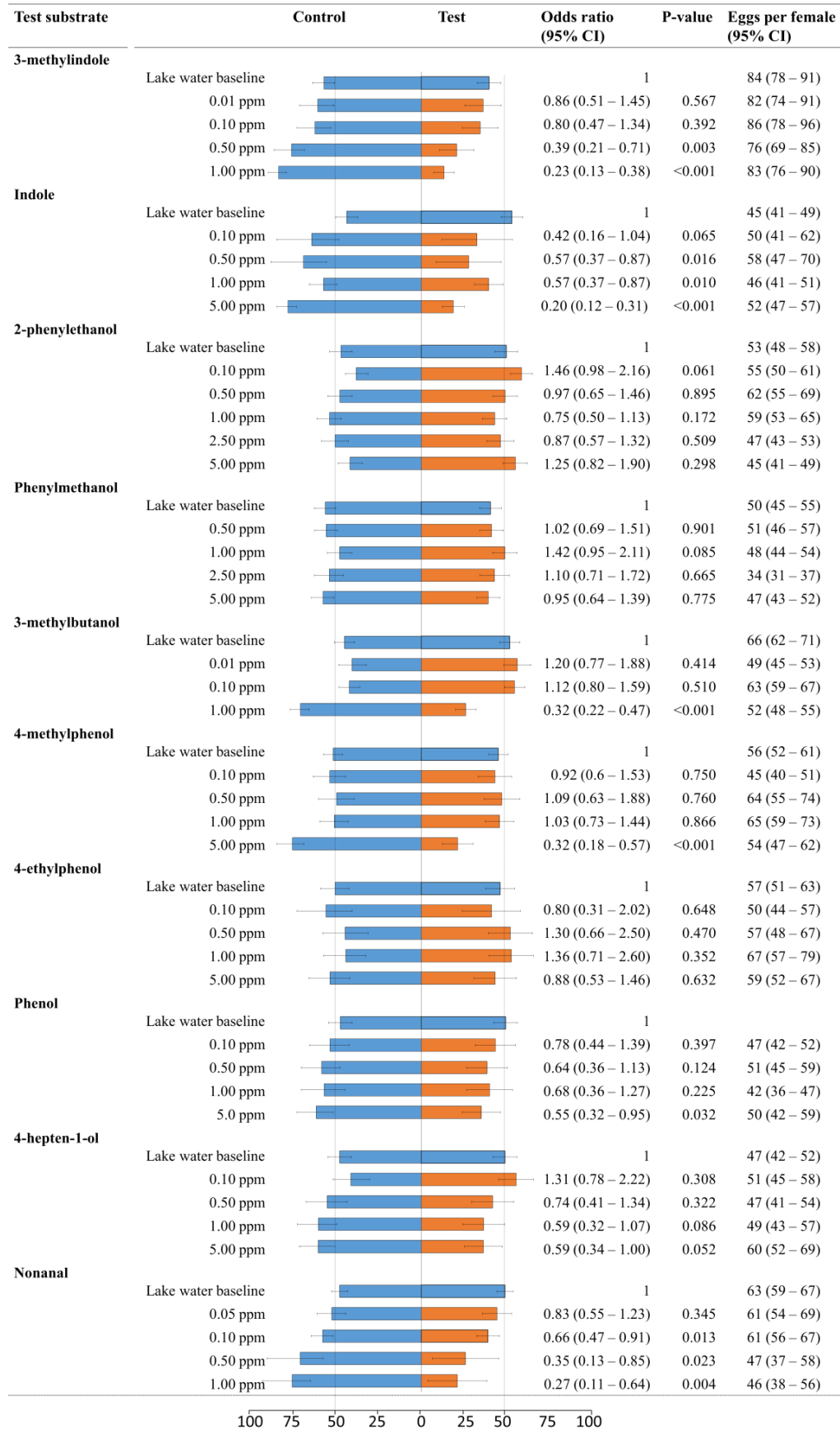
#### 6.4.3. Oviposition response of *Anopheles gambiae sensu stricto* to key hay infusion volatiles

Six out of ten test compounds affected the egg-laying choices of *Anopheles gambiae s.s.* (Figure 6.2). The mosquitoes laid a lower proportion of eggs in very low doses of the alkyl aldehyde nonanal ( $\geq 0.1$  ppm) compared to lake water. The proportion of eggs laid in 3-methylindole and indole only dropped significantly when the test doses were raised to 0.5 ppm. This same effect was recorded for 3-methyl-1-butanol at a ten-fold higher concentration of 1.0 ppm. The chemicals 4-ethyl phenol and phenol were only avoided for egg-laying when presented at a high concentration of 5.0 ppm; 2-phenylethanol, phenylmethanol, 4-ethylphenol and 4-hepten-1-ol did not affect the oviposition choices of *An. gambiae s.s.* at any of the tested concentrations (0.01 – 5.00 ppm). None of the chemicals increased the oviposition response of *An. gambiae s.s.*

### 6.5. Discussion

This study demonstrated that hay infusions are an unfavourable oviposition substrate for *An. gambiae s.s.* except at very low dilutions. At a low dose of 10% mosquitoes laid the same proportion of eggs in the hay infusion and lake water. However, there was only a one-in-ten chance of finding an *An. gambiae s.s.* egg in more concentrated hay infusions (25-100%) compared to lake water. This is in sharp contrast to *Cx. quinquefasciatus*, *Cx. cinereus*, *Cx. tigripes*, and *Ae. albopictus* which are all attracted to such infusions and readily deposit eggs and egg rafts in it (Allan & Kline 1995; Mboera *et al.* 1999) and might be the primary reason why field studies with hay infusion-baited traps rarely report trapped gravid *An. gambiae s.s.* even when implemented in areas with high densities of these species. A notable example is the studies by Mboera and others (2000a) in Muheza, Tanzania who evaluated traps baited with hay infusions at a time when the densities of *An. gambiae s.s.* in the area were markedly high (Mboera *et al.*

2000b) but failed to trap any. One might argue that the commercially available Box gravid traps models (BioQuip, Rancho Dominguez, CA) used in the majority of these studies were not designed for *An. gambiae* s.s. and that might be a reason for the lack of efficacy in field trials. However, a recent study showed that these traps capture a considerable number of this species in the semi-field when baited with just lake water (Dugassa *et al.* 2013).



**Figure 6.2** Egg-laying responses of individual *Anopheles gambiae sensu stricto* to key organic volatiles from hay infusions

Furthermore, another study by Mboera and other (2000a) used counter-flow geometry traps (CFG) - a popular choice for odour baiting of *An. gambiae s.s.* (Njiru *et al.* 2006; Qiu *et al.* 2007; Schmied *et al.* 2008; Smallegange *et al.* 2010; Nyasembe *et al.* 2014) with similar negative results. Herrera-Varela and others (2014) recently demonstrated high mortality of *Anopheles* larvae in infusions with high organic content and suggested that the avoidance of such infusions might be an evolutionary trait that female mosquitoes exhibit to enhance the survival of offspring.

Gravid *An. gambiae s.s.* also avoided or disregarded synthetic equivalents of volatile organic compounds identified from the organic infusions. All these compounds, except 4-heptan-1-ol have been shown to elicit electrophysiological responses in *An. gambiae s.s.* (Blackwell & Johnson 2000; Meijerink *et al.* 2000; Qiu *et al.* 2006; Carey *et al.* 2010). The latter chemical did not instigate or suppress egg-laying suggesting that it is not an important semiochemical in the context of oviposition with this species even though it was the most dominant chemical released from the infusion. Similarly 2-phenylethanol and phenylmethanol, though strongly suggested to be oviposition attractant for gravid *An. gambiae s.s.* previously (Lindh *et al.* 2008; Himeidan *et al.* 2013), did not attract more eggs compared to lake water. Another compound, 4-ethylphenol, though detected frequently and in relatively high amounts had also no effect on oviposition choice in spite of the evidence that female *An. gambiae s.s.* mosquitoes are sensitive to it post blood-meal (Qiu *et al.* 2006). This apparent disregard for compounds in spite of these being in high abundance and showing electrophysiological activity proves that while these indices could be predictors of behavioural importance, only behavioural bioassays can confirm the relevance of any compound as an oviposition semiochemical.

*Anopheles gambiae s.s.* laid few eggs in low concentrations of 3-methylindole and indole. These compounds are well-known attractants of gravid *Cx quinquefasciatus* both in the laboratory (Millar *et al.* 1992) and in the field (Mboera *et al.* 2000c) at a low average concentrations of 0.8 ppm. Trexler and others (2003a) have also found that the compound repelled *Aedes albopictus*, but at a relatively high concentration of 8.3 ppm. In the same study however, *Aedes albopictus* did not show antennal detection of the compound (Trexler *et al.* 2003a). It appears therefore that 3-methylindole may have acted as a physical irritant at very high concentration to deter egg-laying as opposed to being an olfactory repellent. Concentrations tested in this study were constrained to



between 0.1 – 5.0 ppm, which was considered to be a good range in which the potential olfactory response threshold would probably be included. Nonanal, although not readily identified in this study is a known constituent of Bermuda grass hay infusions (Du & Millar 1999a). It elicited a reduced egg-laying at a very low concentration. This compound has also been identified in rabbit food pellet infusions (Leal *et al.* 2008) that has been recently shown to repel gravid *An. gambiae s.s.* (Herrera-Varela *et al.* 2014). It might be an important cue for the avoidance of such organic infusions. Since the compound repelled mosquitoes at the lowest concentration it might be speculated that it could be an early warning avoidance cue for gravid females to avoid environments that would be highly stressful to their larvae. All these compounds, especially, indole and 3-methylindole are common in nature and a common constituent of stagnant water bodies (Kaushik *et al.* 2013) and might to some degree explain habitat partitioning between the different species of mosquitoes in the wild.

One of the tested compounds, 3-methyl-1-butanol, has been shown to be a synergistic attract for host-seeking *An. gambiae s.l.* and constituted into novel baits used for monitoring and controlling the host-seeking vectors (Mukabana *et al.* 2012). Gravid *An. gambiae s.s.* however preferred to lay eggs in lake water compared to water treated with this chemical. Notably, it had been suggested to be an oviposition attractant for malaria vectors after it was detected in the headspace of bacterial isolates from field collected water samples shown to be colonized by *An. gambiae s.l.* (Sumba *et al.* 2004a; Lindh *et al.* 2008).

Two compounds, 4-methylphenol and phenol only affected egg-laying at the highest concentrations tested. At this high concentrations these compounds likely cause physical irritation to the advancing gravid adult as opposed to providing an olfactory signal for avoidance. The commonly found compound 4-methylphenol has been suggested to attract egg-laying *An. gambiae s.s.* at low doses (Walker 2011; Himeidan *et al.* 2013). All doses evaluated here however did not attract more egg-laying than did lake water alone. Results from the volatile analyses showed that this compound was one of the most abundant in the hay infusion. It is therefore possible that it exists in the infusions in sufficiently high amounts to elicit an irritation or repellence: The hay infusion might have contained enough 4-methylphenol to inhibit egg-laying by *An. gambiae s.s.*

This study also rekindles the question: *What is the role of bacteria and other microbes in the oviposition substrate choices of An. gambiae s.l.?* Whilst most agree that *Anopheles gambiae s.s.* are sensitive to bacteria derived odours (Sumba *et al.* 2004a; Huang *et al.* 2006a; Lindh *et al.* 2008), the exact role of these volatiles remain unknown. There is contradicting information on the perceived role of these; a few studies appeared to have provided some evidence that some microbes and their volatiles could attract gravid *An. gambiae s.s.* to oviposition sites (Sumba *et al.* 2004a; Sumba *et al.* 2008) while others only record a repellent effect (Huang *et al.* 2006a). All the compounds identified and evaluated in this study are associated with microbial activity and metabolism. Some of these have been previously identified from bacteria (Lindh *et al.* 2008). This study indirectly adds evidence that *An. gambiae s.s.* avoids substrates rich in bacteria produced metabolites and volatiles. If there are any bacteria derived volatiles that increase oviposition responses remains elusive and warrant further investigation.

This study also highlighted three novel chemical constituents of Bermuda grass hay infusions: 3-methyl-1butanol, 2-phenylethanol, phenylmethanol and 4-heptan-1-ol. This is probably due to the use of the porous adsorbent polymer Tenax TA that is particularly effective for collecting volatiles over samples with high moisture content and the highly sensitive thermal desorption alternative to the common liquid desorption. However, our approach failed to detect nonanal, a compound which based on the bioassay results is important for oviposition choices in *An. gambiae s.s.*. While this could stem from non-standardised infusions profiled in various studies, it likely confirms that different methods for volatile sampling and analyses lead to different number and quantity of volatiles detected (Agelopoulos & Pickett 1998). One method is not sufficient for exhaustively profiling the headspace of organic samples; multiple approaches provide a better chance of discovering behaviourally active compounds.

## 6.6. Conclusion

*Anopheles gambiae sensu stricto* avoid laying eggs in organic matter rich hay infusions. This unfavourable oviposition response is probably mediated by bacterial volatiles including 3-methylindole, indole, nonanal and 3-methyl-1-butanol, compounds that have been previously shown to be highly attractive to a number of culicine disease vectors. This study also highlighted the need for behavioural assays. It demonstrated that electrophysiological activity alone cannot be used to confirm semiochemicals. With

behavioural bioassays it is was shown that many compounds suggested to be oviposition attractants based on electrophysiology repelled mosquitoes or failed to elicit behavioural activity.

## Chapter 7. Discovery of an oviposition attractant for gravid malaria vectors of the *Anopheles gambiae* species complex



**OviART field team collecting samples in Rusinga Island (Source: Maria Ulbrich)**

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## 7.1. Abstract

**Background:** New strategies are needed to manage malaria vector populations that resist insecticides and bite outdoors. This study describes a breakthrough in developing ‘attract and kill’ strategies targeting gravid females by identifying and evaluating an oviposition attractant for *Anopheles gambiae s.l.*.

**Methods:** Previously, the authors found that gravid *An. gambiae s.s.* females were two times more likely to lay eggs in lake water infused for six days with soil from a natural oviposition site in western Kenya compared to lake water alone or to the same but autoclaved infusion. Here, the volatile chemicals released from these substrates were analysed with a gas-chromatograph coupled to a mass-spectrometer (GC-MS). Furthermore, the behavioural responses of gravid females to one of the compounds identified were evaluated in dual choice egg-count bioassays, in dual-choice semi-field experiments with odour-baited traps and in field bioassays.

**Results:** One of the soil infusion volatiles was readily identified as the sesquiterpene alcohol cedrol. Its widespread presence in natural aquatic habitats in the study area was confirmed by analysing the chemical headspace of 116 water samples collected from different aquatic sites in the field and was therefore selected for evaluation in oviposition bioassays. Twice as many gravid females were attracted to cedrol-treated water than to water alone in two choice cage bioassays (odds ratio (OR) 1.84; 95% confidence interval (CI) 1.16-2.91) and in experiments conducted in large-screened cages with free-flying mosquitoes (OR 1.92; 95% CI 1.63-2.27). When tested in the field, wild malaria vector females were three times more likely to be collected in the traps baited with cedrol than in the traps containing water alone (OR 3.3; 95% CI 1.4-7.9).

**Conclusion:** Cedrol is the first compound confirmed as an oviposition attractant for gravid *An. gambiae s.l.*. This finding paves the way for developing new ‘attract and kill strategies’ for malaria vector control.

## 7.2. Background

Mosquitoes of the *Anopheles gambiae* species complex (*An. gambiae sensu lato (s.l.)*) including *An. gambiae sensu stricto (s.s.)* and *Anopheles arabiensis* are among the most efficient vectors of malaria on the planet and are responsible for most deaths from this disease in sub-Saharan Africa (WHO 2013). The most effective way to prevent malaria to date is vector control. The interventions used to reduce vector numbers primarily target host-seeking mosquitoes indoors (Lengeler 2004; Pluess *et al.* 2010). While these interventions are effective, increasing evidence suggests that malaria elimination is not achievable by these methods alone since residual malaria transmission is maintained by vectors that feed and rest outdoors or feed on animal hosts (Killeen 2014). The development of an efficient attract-and-kill strategy for oviposition site-seeking females could be one of the novel vector control tools that is urgently called for (Ferguson *et al.* 2010; Govella & Ferguson 2012).

To date, there has been relatively little research investigating how *An. gambiae s.l.* females find and choose oviposition sites. It is known that water vapour helps to guide them (Huang *et al.* 2005; Okal *et al.* 2013), however, in nature many aquatic sites remain uncolonized suggesting that some are more attractive to gravid females than others (Fillinger *et al.* 2009b; Ndenga *et al.* 2011; Gouagna *et al.* 2012). Recently, the authors found that mosquitoes were two times more likely to lay eggs in lake water infused for six days with soil from a natural oviposition site in western Kenya compared to lake water alone in two-choice egg-count cage bioassays. This preference was lost when the infusion was autoclaved (Herrera-Varela *et al.* 2014) suggesting that volatile chemicals, rather than visual cues attracted the mosquitoes. Although a number of chemicals have previously been proposed as oviposition semiochemicals for *An. gambiae s.s.* (Blackwell & Johnson 2000; Lindh *et al.* 2008; Rinker *et al.* 2013), none of these have been shown to attract gravid females over a larger distance (more than a few cm) in laboratory, semi-field or field settings.

Volatiles released from autoclaved and unmodified soil infusions, and the lake water used as control in the study by Herrera-Varela and others (2014) were analysed. One of the compounds was selected for evaluation in: i) two choice egg-count cage bioassays to test for preferential egg-laying; ii) large semi-field systems with free-flying females to test for attraction over larger distances; and, iii) under natural field conditions. Through

these experiments the first confirmed oviposition attractant for gravid *An. gambiae s.l* is described.

### 7.3. Methods

#### 7.3.1. Volatile collections from soil infusions

All glassware used was first washed with an odourless detergent (Teepol, general purpose detergent, Teepol Industries, Nairobi, Kenya) rinsed in water and acetone and then placed in an oven at 200°C for at least two hours before use. Volatiles released from lake water, autoclaved and unmodified six-day old soil infusions were collected in parallel with behavioural cage bioassays previously published (Herrera-Varela *et al.* 2014). All the unmodified infusions elicited higher oviposition responses than the lake water or the autoclaved infusion in these bioassays (Herrera-Varela *et al.* 2014). Infusions were prepared by mixing 15 L of lake water with 2 kg of soil sourced from a natural *Anopheles* breeding site, located within the compound of the International Centre of Insect Physiology and Ecology-Thomas Odhiambo Campus (icipe-TOC) at Mbita, western Kenya (0°26'06.19" South; 34°12'53.12" East; altitude 1,149 m). The soil was collected and sun-dried for one day prior to preparation of the infusion. On the day of the experiment the infusions were sieved through clean pieces of cotton cloth to remove large debris from the soil. One half of the infusion was autoclaved at 120°C for 20 minutes and left to cool to ambient temperatures. Volatiles were collected on Tenax traps made from GERSTEL-Twister Desorption glass liners (GERSTEL, Muelheim an der Ruhr, Germany), glass wool (Supelco, Bellefonte, PA, USA) and 25 mg of Tenax® TA polymer (60-80 mesh, Supelco, Bellefonte, PA, USA). The traps were washed with 3 ml of methyl-tert butyl ether (MTBE, Sigma-Aldrich, Steinheim, Germany) the openings covered with polytetrafluorethylene (PTFE) tape and kept in an oven at 50°C for at least two hours before use. Dynamic headspace collections were performed from 300-ml aliquots of the three sample types in 500-ml conical borosilicate glass Erlenmeyer flasks with 24/29 sockets (Quickfit® glassware). Forty-five grams of sodium chloride (NaCl, ≥99.8%, Sigma -Aldrich, Steinheim, Germany) were dissolved in all aqueous samples before volatile collections to improve the release of volatile chemicals (Morrison 1944; Mozuraitis *et al.* 2010). E-flasks were fitted with gas wash bottle heads and charcoal-filtered air was pumped at 100 ml/minute through the inlet and drawn out at the same speed through the Tenax trap over 20 hours after which the traps were stored at -70°C. Empty bottles sampled the same way served as control for



background compounds. Volatiles were collected in parallel from empty bottles, lake water and duplicates of soil infusions (autoclaved and non-autoclaved). This was repeated over seven rounds.

### 7.3.2. Analysis of soil infusion volatiles

The gas-chromatograph-mass spectrometer (GC-MS) system consisted of a 7890A GC (Agilent Technologies, Santa Clara, CA, USA) fitted with a 30-m long HP-5MS column (Agilent Technologies) with an inner diameter of 0.25 mm and 0.25  $\mu\text{m}$  film thickness coupled to a 5975C MS (Agilent Technologies) with electronic ionization set at 70 eV, the ion source at 230°C and the quadrupole at 150°C. Tenax traps were thermally desorbed in a GERSTEL thermal desorption unit (TDU, GERSTEL, Muelheim an der Ruhr, Germany) initially held at 20°C and then increased at 120°C/minute to 250°C, the end temperature was held for five minutes. The volatile chemicals were transferred in splitless mode to a cooled injection system (CIS) injector fitted with a Tenax liner (GERSTEL). The CIS injector was held at 10°C during the TDU programme and was then heated at a rate of 12°C/second to 260°C during which the volatiles were transferred to the column in a splitless mode. Helium was used as carrier gas at a pressure of 34 psi. The temperature of the GC oven was held at 40°C for one minute and then increased by 4°C/minute to 260°C and kept there for three minutes.

Heptyl acetate (35 ng, SAFC, Sigma-Aldrich, Steinheim, Germany) in Methyl tert-butyl ether (MTBE) was injected as external standard with each sample. A hydrocarbon standard with the C8-C20 compounds (10 ng of each in cyclohexane) was run and used to calculate Kovats retention indices (RI). GC-MS data from the lake water and soil infusion samples were compared to those of the empty bottle controls for each round. All peaks that were present in the samples (both duplicates for the soil samples) and had a different retention time and/or mass spectra compared to the empty bottle control were manually integrated. Volatiles with a peak-area at least twice as big in the sample compared to the control were also included. The peak-area of the control was subtracted from the peak-area of the sample when a volatile was present in both chromatograms. The area of each integrated peak was normalized against the area of the external standard heptyl acetate injected with each sample and Kovats retention indices (RI) calculated (Appendix B). Peaks with similar RI and mass spectra were given the same

compound identification number (ID). Mass spectral data were compared using to the electronic mass spectral library, NIST 2008 for a tentative identification.

### 7.3.3. Identification of cedrol in the soil infusion samples with authentic standard

The identity of ID 276 was confirmed using an authentic standard: (+)-cedrol,  $\geq 99.0\%$  sum of enantiomers, GC, optical activity  $\alpha_D^{20} +10.5 \pm 1^\circ$  (Sigma-Aldrich, Steinheim, Germany). The compound was diluted in MTBE to 0.8 mg/ml and 1  $\mu$ l was injected in a CIS-injector, set to a splitless mode, held at 40°C for 0.5 minutes and then heated at a rate of 12°C/second to 260°C. All other GC-MS parameters were as for the soil infusion samples above.

### 7.3.4. Standard curve for cedrol

Eight different amounts (0.008, 0.016, 0.032, 0.08, 0.1, 0.2, 0.4, 0.8  $\mu$ g) of cedrol  $\geq 99.0\%$  (sum of enantiomers, GC, Sigma-Aldrich, Steinheim, Germany) dissolved in MTBE were injected in preconditioned Tenax traps in the TDU unit on the GC-MS system (described above). All settings and temperature programmes were as described above for the soil infusion samples. The area of the peaks was utilised to create a standard curve, which was used to calculate the amount of cedrol collected in the soil infusion samples.

### 7.3.5. Screening of volatile collection samples from field sites

Water samples were collected from 116 natural water bodies (puddles, pools, ponds, drains, swamps, and pits) on Rusinga Island, western Kenya (0°24'33.08" South; 34°10'14.84" East; altitude 1,377 m), during the long rainy season in 2012. Water samples were filtered into 250-ml wide-neck polypropylene bottles (Thermo Scientific, UK) through a clean piece of cotton cloth to remove large debris and transported in a cool box to the laboratory. The samples were transferred into 500-ml E-flasks. Volatiles in the headspace above the water samples were collected on polydimethylsiloxane/divinylbenzene (PDMS/DVB) solid-phase microextraction (SPME) fibres (65  $\mu$ m Stable Flex™, Supelco, Bellefonte, PA, USA) for 20 hours. A bottle containing distilled water, stored, transported and sampled the same way as the field samples, served as control for background compounds. SPME fibres were analysed immediately after

volatile collection on a GC-MS system with the same instruments, GC-column and settings as described above. The GC injector was kept at 250°C in a splitless mode; helium with a flow of 1.2 ml/minute was used as carrier gas. The oven temperature programme started at 40°C for three minutes followed by an increase of 5°C per minute to 260°C which was held for three minutes.

The GC-MS files were screened for the main ions of the four compounds closely associated with the unmodified soil infusion samples in the principal component analysis (PCA) (compound IDs 51, 263, 276 (cedrol) and 286). Only cedrol was found. The amount of cedrol in the field samples was often close to the detection limit of the volatile collection method. Hence, all samples with a peak containing two of the main mass spectra ions of cedrol (95 and the compound specific 150) at the retention time that matched cedrol were scored as positive for the compound.

#### 7.3.6. Mosquito preparation

Laboratory and semi-field experiments were carried out with insectary-reared *An. gambiae s.s.* (Mbita strain) supplied by the mosquito insectaries at icipe-TOC, Mbita, and reared following standard operating procedures. Gravid mosquitoes were prepared by selecting 300 female and 300 male mosquitoes, two to three days old, from their rearing cages at 12.00 hours and keeping them in 30x30x30-cm netting cages at 25-28°C and 68-75% relative humidity. To avoid mosquito desiccation, folded cotton towels, saturated with tap water were placed over the cages. Mosquitoes were starved of sugar for seven hours before blood-feeding and allowed to feed on a human arm for 15 minutes at 19.00 hours. Afterwards unfed female mosquitoes were removed from the cages. Mosquitoes were then provided with 6% glucose solution *ad libitum*. This procedure was repeated the following day. Fed female mosquitoes were kept together with males for two days after the second blood-meal and used on the third day for experiments (i.e., four to five days after first blood-meal). At 16.30 on the day of an experiment visually presumed gravid females (enlarged, pale white abdomen) were selected from the holding cage (Herrera-Varela *et al.* 2014).

### 7.3.7. Experimental procedures

#### 7.3.7.1. Preparation of cedrol solutions for bioassays and field experiments

Stock solutions of 10,000 ppm cedrol in ethanol were prepared by adding 150 mg of (+)-cedrol ( $\geq 99.0\%$ , sum of enantiomers, Sigma-Aldrich) to 15 ml of absolute ethanol (puriss. pa, absolute,  $\geq 99.8\%$  (GC), Sigma-Aldrich). Dilutions were made by adding the appropriate amount of stock solution to lake water. For example, to make a 5-ppm solution of cedrol in water, 3.5 mL of the stock solution was added to 7 L of lake water; for each round 2.5 L of this was used for cage bioassays and 4.5 L for semi-field experiments with free-flying mosquitoes. The same formulation procedures were used to create 5-ppm cedrol preparations for all traps in the field.

#### 7.3.7.2. Dual-choice cage bioassays to study substrate preferences

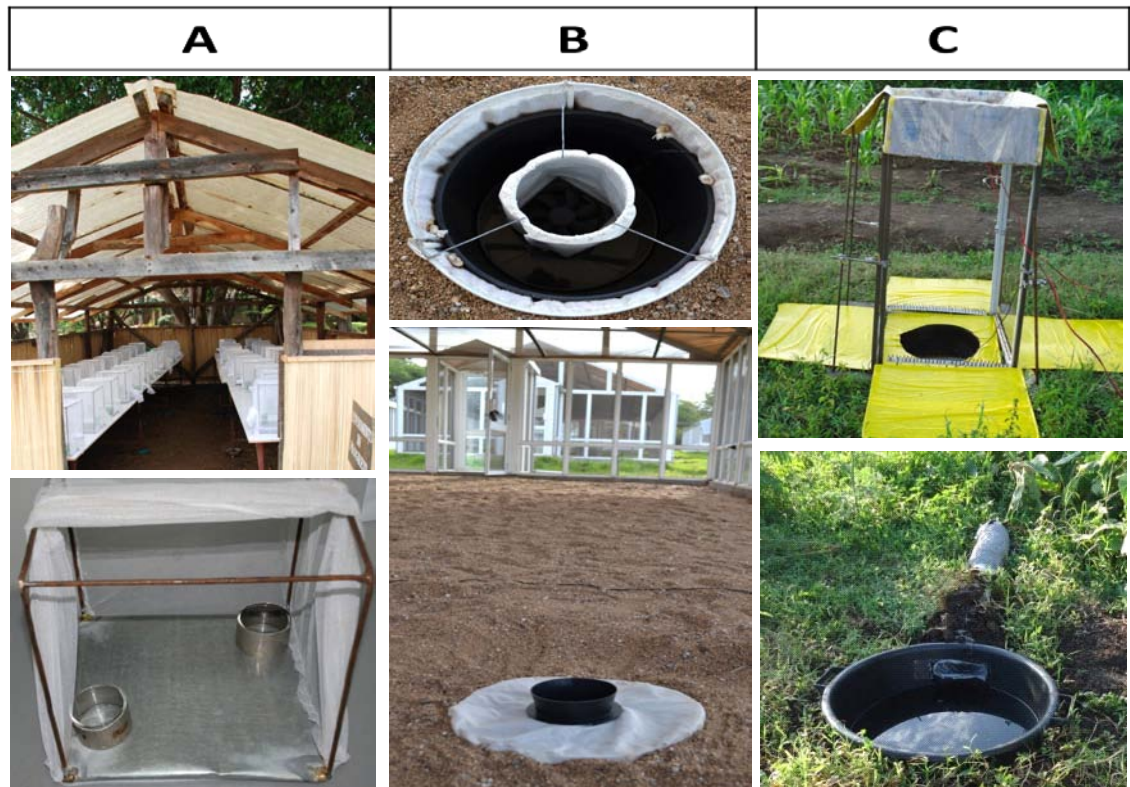
Experiments were done in previously described (Herrera-Varela *et al.* 2014) make-shift sheds at icipe-TOC (Figure 7.1A). All experiments were carried out at ambient conditions of temperature, humidity (mean daily temperature  $27 \pm 5^\circ\text{C}$ , relative humidity  $55 \pm 10\%$ ) and light. Each cage (30x30x30 cm) had two glass cups (Pyrex®, 100 ml, 70 mm diameter) covered with a metal ring and filled with 100 ml of either the control or test water. The control water was lake water pumped from Lake Victoria, stored in a settlement tank and drawn from a tap. The test water was the same water treated with the respective concentration of cedrol. The position of the test cups were randomly allocated to one of the four corners of a cage and alternated between adjacent cages to control for possible position effect. One control cup was added in each cage diagonal to the test cup to complete the two choice set-up. Five treatments were tested in parallel: 1) two untreated cups of lake water in a cage which served as the reference group; 2) lake water (control) *versus* lake water treated with 2.5 parts per million (ppm) cedrol (test); 3) control *versus* 5 ppm test; 4) control *versus* 10 ppm test; and, 5) control *versus* 20 ppm test. Cage experiments were implemented over 15 rounds with fresh cedrol stock solution and different batches of mosquitoes for every round. Fifteen to 25 replicate cages per treatment were set up per round. Cages were set at a minimum distance of 30 cm. A single gravid female was introduced per cage at 18:00. The next morning between 08.00 and 09.00 the absence or presence, and the number of eggs was

recorded for the control and test cup in each cage. Non-responders (mosquitoes that did not lay eggs) were removed from the analysis.

#### *7.3.7.3. Semi-field experiments with free-flying gravid mosquitoes to study attraction and odour discrimination*

Experiments designed to evaluate attraction (defined as the oriented movement of an insect to the source of a chemical cue from a distance of several metres (Dethier *et al.* 1960)) of free-flying gravid female *An. gambiae s.s.* were done in a screened semi-field structure (10.8 m long × 6.7 m wide × 2.4 m high) at icipe-TOC, using modified BG-Sentinel mosquito traps (Biogents AG, Regensburg, Germany). The BG-Sentinel mosquito traps were sunk into the sand and a plastic container inserted to hold 4.5 L of aqueous solutions (Figure 7.1B). Two traps were set 1.5 m away from the shorter wall of the semi-field system so that they were 4.5 m apart and equidistant to the mosquito release point, 9.5 m away towards the opposite short wall. Treatments were randomly allocated to four possible corners in a randomized complete block design. Two-hundred gravid mosquitoes were released per round over 12 rounds. Mosquitoes were introduced at 18:00, about five minutes after the BG-Sentinel traps were started. Gravid mosquitoes that oriented towards either trap were sucked into a catch bag in the trap.

The peak oviposition time of the caged *An. gambiae s.s.* is between 19:00 and 21:30 (Okal *et al.* 2013). To be able to compare the oviposition response within this time period to the remainder of the night the catch bags were changed at 21.30 and then collected the next day between 08:00 and 09:00. Two treatments were tested: 1) two traps with 4.5 L lake water, this served as the reference group; 2) 4.5 L lake water (control) *versus* 4.5 L lake water with 5 ppm cedrol (test).



**Figure 7.1 Experimental set-up** (A) Cage bioassays with individual gravid females under ambient conditions in makeshift huts; (B) Modified BG sentinel traps in a semi-field system; (C) Field set-up of square of electrocuting nets (up) and OviART gravid trap (down).

#### 7.3.7.4. Estimation of release rates of cedrol from bioassay cups and BG-Sentinel mosquito traps

Cage bioassays and BG-Sentinel mosquito traps were set up in the same way as described for experiments (section 7.3.7.2). Tenax traps prepared and cleaned described in section 7.3.1 were used to collect volatiles above the oviposition cups and gravid traps. A pump was used to draw air through the Tenax traps at a speed of 100 ml/minute. Collections were made 3 cm above the water surface of untreated lake water and lake water containing 5 or 10 ppm cedrol in the bioassay cups between 17:30 and 08:30. BG-Sentinel traps were set up in the semi-field system and collections made 5 cm above the netting covering the trap where the air current leaves the trap. The BG-Sentinel traps were baited with untreated lake water or lake water containing 5 ppm cedrol. Tenax traps were changed hourly for 12 hours. Two rounds of samples in duplicates were taken for cage tests and three for semi-field tests. Tenax traps were

eluted with 200  $\mu$ L of MTBE containing 20 ng of  $\beta$ -caryophyllene ( $\geq 98.5$  sum of enantiomers. Sigma-Aldrich, Steinheim, Germany) as an internal standard. The samples were analysed using the same GC-MS instrumentation, settings and programme as described for SPME fibres above. The amount of cedrol in the samples was determined by comparing peak areas to that of the internal standard and converted to per minute release rates by dividing with the collection time.

#### 7.3.7.5. *Field assessment of trapping efficiency of wild mosquitoes with odour-baited gravid traps*

Fieldwork was implemented during the end of the long rainy season in June 2014 approximately 7 km south of icipe-TOC in Kaugege location. Collecting gravid malaria vectors has never been done routinely and gravid traps have only been developed recently (Harris *et al.* 2011; Dugassa *et al.* 2013). Whilst the modified BG-Sentinel mosquito traps worked well as gravid traps in the semi-field system (Okal *et al.* 2015) and were therefore an obvious choice to be taken to the field for comparison with the semi-field data, they had never been tested under field conditions prior to this work. Two other novel gravid traps, a square of electrocuting nets (E-nets) (Dugassa *et al.* 2012) and the OviART gravid traps (Dugassa *et al.* 2013), had previously been developed and preliminary field tests had shown that they performed well in the study area (S Dugassa, pers comm). Therefore, E-nets and OviART (Figure 7.1C) gravid traps were run in parallel to BG-Sentinel traps in the field to evaluate the effect of cedrol treatment and trap type on the collection of gravid mosquitoes. The operating procedures for these devices have been published in detail elsewhere (Dugassa *et al.* 2012; Dugassa *et al.* 2013).

Three study sites in close vicinity to residential houses and within 200 m of the lake shores were selected. The sites were separated by between 70 and 500 m. In each site four trap locations were chosen 10-20 m apart from each other and 5 m from the nearest house. One out of the three sites was randomly selected to receive two squares of E-nets and two OviART gravid traps whilst the other two sites received BG-Sentinel traps in all four locations. The different trap types were not set simultaneously at the same site to avoid a competition between visual and chemical cues. The OviART gravid trap and the square of E-nets provide a visual stimulus with their open water surface whilst the BG-Sentinel trap relies exclusively on chemical cues released from the trap with its

convection currents. However, the trap types were rotated randomly through all three sites so that the OviART gravid trap and square of E-nets were tested in all three locations. All trapping devices provided artificial oviposition sites filled with lake water; the BG-Sentinel trap contained 4.5 L whilst the OviART gravid trap and the square of E-nets contained 8 L each. At each study site half of the traps (per type) were treated with 5 ppm of cedrol whilst the other half remained untreated. Treatment location per site was allocated randomly in such a way that each location had received the test treatment twice during the test round (eight days). Cedrol treatment was done just before the traps were switched on at 17.00. Mosquitoes were collected from the traps in the morning at 06.00. All traps were freshly set up in the afternoon in the same position for eight days, then the location of the OviART gravid traps and E-nets were relocated randomly to another study site. This was repeated twice to ensure that the alternative traps (OviART and E-nets) were in each site once (three rounds of eight days).

In order to have an estimate of the mosquito population density in the area, more established host-seeking vector collections were implemented weekly in parallel to the gravid collections from 12 households a minimum of 100 m apart from each other and within 1 to 2 km from the locations of the gravid traps. Collections were made indoors in inhabited houses with CDC light traps (Service 1993) and outdoors with cattle baited traps (CBT) (Tirados *et al.* 2006). The two different collection methods were chosen to gain a better estimate of potential malaria vectors with varying feeding and resting behaviour. Mosquitoes were morphologically identified to genus level and *Anopheles* mosquitoes to species level (Gillies & De Meillon 1968; Gillies & Coetzee 1987). Molecular tools were used to identify members of the sibling species of the *An. gambiae* complex and the *Anopheles funestus* complex following published procedures (Scott *et al.* 1993; Koekemoer *et al.* 2002).

### 7.3.8. Statistical analyses

GC-MS data were explored using PCA with supplementary variables. Only volatiles present in at least four out of the seven rounds for at least one of the sample types were included in the analysis. The data was centred and standardized by volatiles prior to analysis with Canoco 5 (Ter Braak & Smilauer 2012).



Dual choice cage bioassays and semi-field experiments were analysed using generalised linear models with a quasibinomial distribution fitted to account for overdispersion in R statistical software version 2.13 (R Core Team 2014). The proportion of responses (eggs laid or females trapped) received by the test cups in cage bioassays or test traps in the semi-field systems of the experiments with two different choices were compared with the responses received by the test cups/traps in cages/semi-field systems with two equal choices (lake water in both cups/traps). It was hypothesized that gravid females presented with identical treatments respond to both cups/traps in an approximately equal proportion ( $p=0.5$ ). The statistical analysis aimed to reveal if the test treatment of interest (e.g., increasing concentration of cedrol) received an increased or decreased proportion of responses as compared to the lake water only treatment. The experiment (two-choice, equal choice) and the round of experiment were included as fixed factors to analyse their impact on the outcome. Rounds were not significantly associated with the outcome in any of the experiments and therefore removed from the final models.

Field data were analysed using generalised estimating equations (GEE) in IBM SPSS version 20. Prior to the final analysis the data was tested for significant between-group variations in trap location and study area. Only study area varied significantly and was included in the final analysis as repeated measure with an exchangeable correlation matrix. The data fitted a negative binomial distribution. Treatment and trap type were included in the model as fixed factors. Interactions were tested but no significant associations found. All reported means and their 95% confidence intervals (CIs) were estimated as the exponentials of the parameter estimates for models with no intercept included.

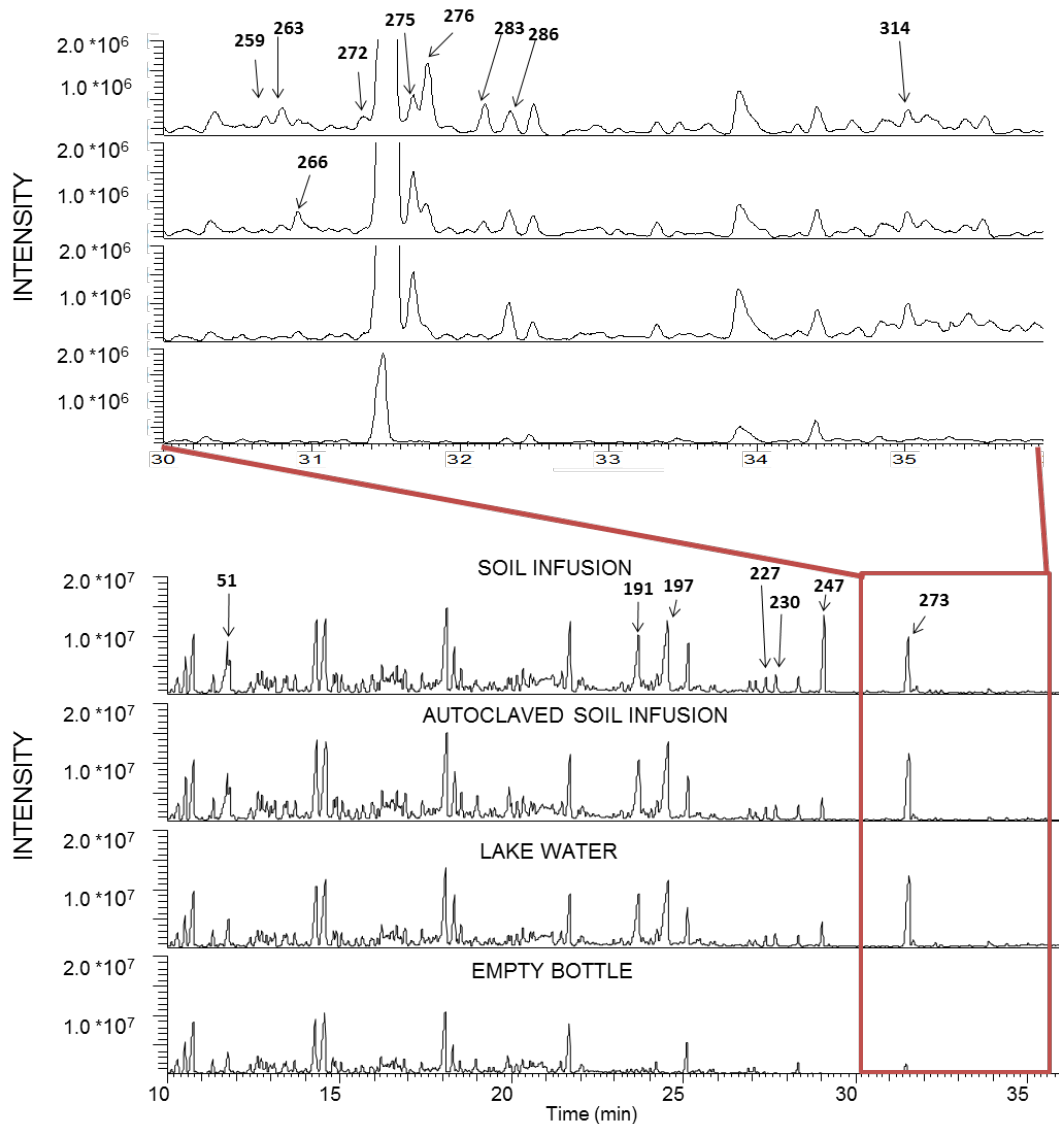
### 7.3.9. Ethical considerations

Ethical approval for this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 363 and protocol no.422).

## 7.4. Results

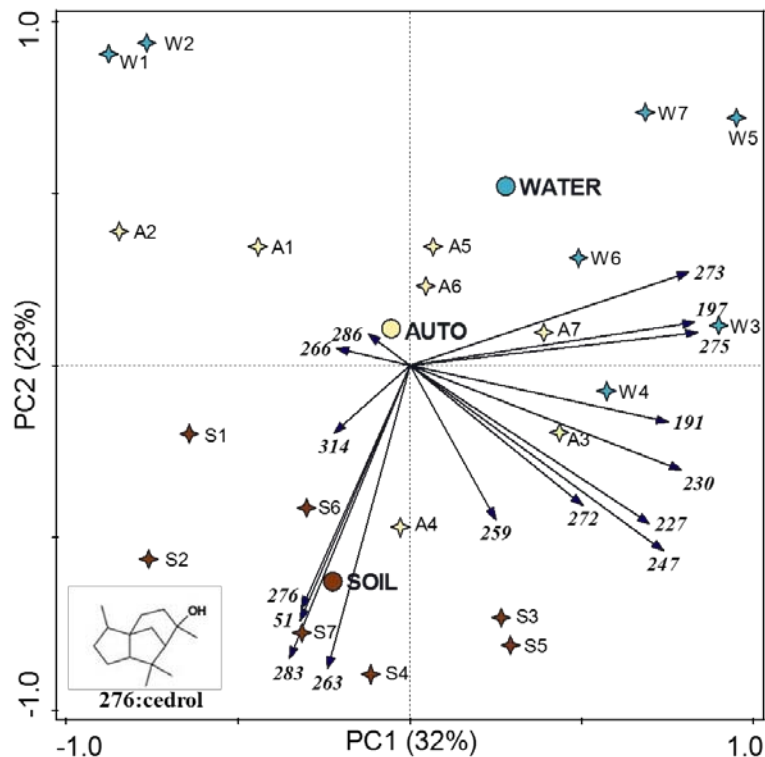
### 7.4.1. Identification of putative oviposition semiochemicals

Volatile chemicals emitted from autoclaved and unmodified soil infusions as well as the lake water were sampled in parallel to behavioural assays and analysed by GC-MS (Figure 7.2).



**Figure 7.2** Example chromatograms from round five of volatile collections. One chromatogram of each sample type (unmodified soil infusion, autoclaved soil infusion and lake water) and empty bottle control. All compounds included in the multivariate analysis are marked by the corresponding ID number. Kovats retention index (RI) and mass spectral data for each compound can be found in Appendix A.

Exploration of the GC-MS data using PCA indicated similarities in volatile profiles within the replicates of the same sample type but different chemical profiles between the treatments (Figure 7.3). Four compounds (IDs 51, 263, 276, 283) grouped closely with the unmodified soil samples. GC-MS data with volatiles emitted from water samples from natural aquatic habitats situated along the shores of Lake Victoria in western Kenya were screened for these four compounds. ID 276 was above the detection threshold in 62 of the 116 samples whereas none of the other three compounds was detected. ID 276 was identified as the sesquiterpene alcohol cedrol by comparison of mass spectral data to the NIST08 library and an authentic standard. Based on its presence in natural *Anopheles* oviposition sites and the ease of its identification, cedrol was selected for further evaluation.



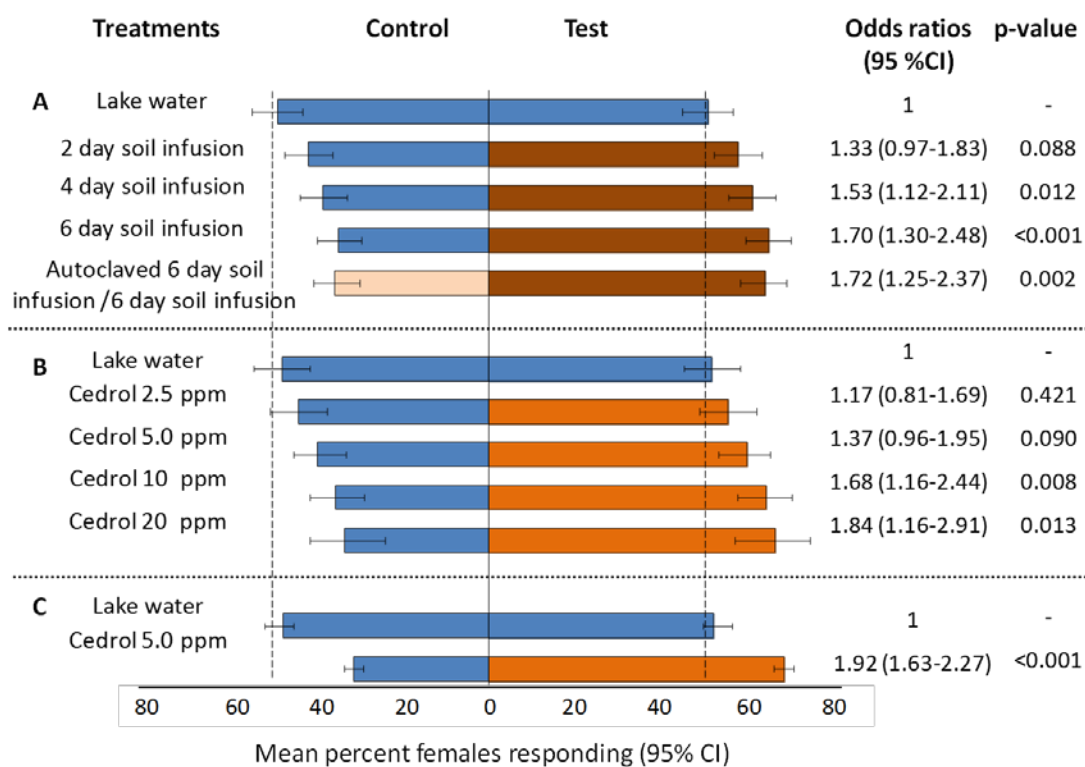
**Figure 7.3 Biplot of the GC-MS data from lake water, unmodified and autoclaved soil infusions.** The three sample types form distinct groups, mainly separated by the second principal component. Four compound IDs (51, 263, 276 and 283) group closely with the unmodified soil samples. Data from seven rounds of each sample type were centred and standardized by the volatile compounds before being subjected to principal component analysis with supplementary variables. The supplementary variables were the three sample types indicated with WATER (lake water), AUTO (autoclaved soil infusion) and SOIL (unmodified soil infusions). Each sample is indicated with a letter;

W, A or S for lake water, autoclaved soil infusion and unmodified soil infusion respectively. The number following the letter indicates the round; volatiles were collected in parallel from samples with the same number.

Cedrol was present in all the soil infusion samples investigated (n=14 for unmodified and autoclaved samples combined) and the amount was three times as high in the unmodified soil infusion (mean 15.8 ng, 95% CI 9.36-22.2), which was preferred for egg-laying in the previous study, compared to the non-preferred autoclaved infusion (mean 5.7 ng, 95% CI 4.6-6.7). In contrast, it was only detected in two out of seven lake water samples (mean of those two samples: 4.2 ng, 95% CI 3.8-4.5).

#### 7.4.2. Cedrol attracts laboratory-reared gravid *Anopheles gambiae s.s.* females

A series of experiments was carried out in the laboratory and semi-field with insectary-reared *An. gambiae s.s.* to determine whether gravid females respond to cedrol (Figures 7.3 and 7.4). The cage bioassays demonstrated a dose-dependent response of gravid females with increasing concentrations of cedrol increasing the probability of a female laying her eggs in the test solution. Interestingly, the dose-response matched the previously observed (Figures 7.4A and 7.4B) results for the soil infusions of increasing incubation time when compared to lake water.



**Figure 7.4 Mean per cent of gravid *Anopheles gambiae* responding to control and test treatments in choice experiments** (A) Cage bioassays with soil infusions of increasing incubation time and comparison of autoclaved versus unmodified infusion. The data from Herrera-Varela and others (Herrera-Varela *et al.* 2014) have been re-analysed for this figure to show the per cent of females responding. These data present the background for the current study. Headspace collections for identification of volatile chemicals were implemented for autoclaved and unmodified six-day old soil infusions in parallel to these behavioural assays. (B) Cage bioassays with cedrol-treated lake water in increasing concentrations. (C) Semi-field evaluation of response of free-flying gravid females to cedrol-baited traps.

Since these egg-count cage bioassays cannot distinguish between contact stimulants and long-range attractants (Isoe *et al.* 1995b) experiments were implemented in a large (174 cu m) semi-field system using modified BG-Sentinel traps (Figure 7.1B). These odour-baited traps enabled the researchers to assess the relative attractiveness of volatiles released from a trap, without the influence of visual cues or contact stimulants since the mosquitoes are prevented from seeing or accessing the test substrate. The experiments confirmed that cedrol was attractive with 69% (95% CI 66-71%) of released females collected in the treated trap (Figure 7.4C). The response towards the cedrol-baited trap was consistent and high from night-to-night with very little variation. Furthermore, on

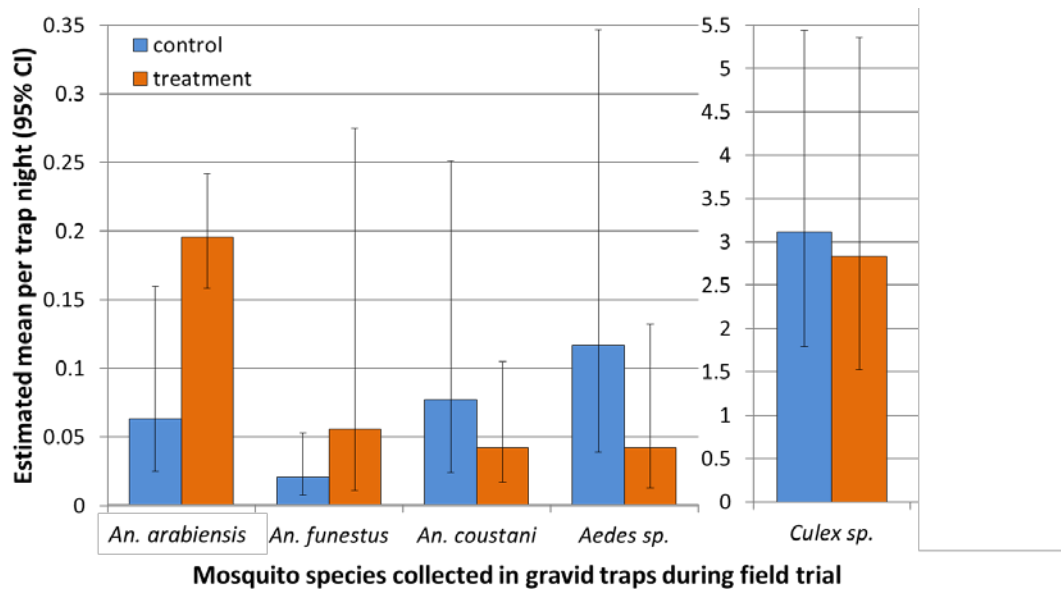
average 89% (95% CI 84-92%) of released gravid mosquitoes were recaptured during the choice experiment when a cedrol-baited trap was present. This was in sharp contrast ( $p < 0.001$ ) to the experiment where both traps contained only lake water in which only 34% (95% CI 29-38%) of the released females were recaptured.

The peak oviposition time of the caged *An. gambiae* used in this study has previously been determined to be between 19:00 and 21:30 (Okal *et al.* 2013). In the semi-field experiment 68% (95% CI 57-78%) of the females were collected during this period, with 74% (95% CI 70-76%) choosing the cedrol-treated trap over the trap with lake water only. However, the response after 21.30 was nearly balanced, with only a slightly higher proportion of females collected in the 5 ppm test trap (58%, CI 53-62%).

Volatile headspace collections from both bioassay systems confirmed that cedrol was released from the test substrates but not from the controls. Besides the cedrol peak, no consistent difference was observed in the chromatograms from test and control treatments hence, no breakdown products of cedrol were detected. Oviposition cups treated with 5 ppm cedrol released 8.7 ng/minute (95% CI 5.9-12.7 ng/minute) and those treated with 10 ppm released 22.8 ng/minute (95% CI 18.0-29.0 ng/minute) during the 12 hours of experiment. The release rate from the BG-Sentinel traps treated with 5 ppm cedrol was on average 8.0 ng/minute (95% CI 5.4-12.0 ng/minute). Cedrol was released at consistent rates over the 12-hour experimental period with no significant difference ( $p=0.293$ ) between the peak oviposition time (19:00-21:30) and the rest of the night.

#### 7.4.3. Cedrol attracts wild malaria vectors

Under natural field conditions a total of 933 female mosquitoes were collected in 288 gravid trap nights (12 traps per night for 24 nights); 91% were *Culex* species. Of the *An. gambiae* species complex, only *An. arabiensis* were collected in the field sites, representing 4% of the total catch. In addition, a small number (1%) of the malaria vector *An. funestus* s.s. were collected. Trap catches also included 2% of the secondary malaria vector *Anopheles coustani* and 2% *Aedes* species. Traps baited with cedrol were 3.3 times (95% CIs 1.4-7.9) more likely to trap a female *An. arabiensis* than traps containing lake water only, irrespective of the trap type (Table 7.1, Figure 7.5).



**Figure 7.5** Estimated mean number of female mosquitoes per trap night (all trap types pooled) collected during the field trial Error bars represent 95% confidence intervals.

However, the three trap types performed differently under field conditions with more *An. arabiensis* females caught in devices that included visual water cues like the squares of electrocuting nets and the OviART gravid trap irrespective of site and location (Table 7.1). Collections of host-seeking females indoors with CDC light traps and outdoors with CBTs at the same time confirmed that the overall population density of vectors in the study area was low during the study period. In CDC traps a mean of 0.73 (95% CI 0.28-1.90) and in CBTs a mean of 2.1 (95% CI 1.1-4.0) females of the *An. gambiae* complex were collected per trap night; 96% of which were *An. arabiensis*, confirming the predominance of this sibling species in the field setting.

**Table 7.1 Probability of a mosquito female being trapped in field tests**

	<b>Rate ratio (95% confidence interval)</b>				
	<i>Anopheles arabiensis</i>	<i>Anopheles funestus s.s.</i>	<i>Anopheles coustani</i>	<i>Aedes sp.</i>	<i>Culex sp.</i>
<b>Treatment</b>					
Control	1	1	1	1	1
Test	3.3 (1.4-7.9)	2.6 (0.97-6.96)	0.5 (0.3-0.8)	0.4 (0.3-0.6)	0.8 (0.7-0.9)
<b>Trap</b>					
BG	1	1	1	1	1
OviART	5.2 (0.9-30.9)	6.3 (1.6-25.4)	- <sup>a</sup>	- <sup>a</sup>	1.1 (0.5-2.3)
E-nets	10.0 (5.6-18.0)	12.4 (2.9-52.5)	12.9 (5.0-32.6)	3.5 (1.3-9.1)	8.7 (5.0-15.1)

Interestingly, the data indicate that *An. funestus* might show a preference for cedrol-treated oviposition sites, however due to the small sample size this result is only of borderline significance ( $p=0.057$ , Table 7.1). On the contrary, *An. coustani*, *Aedes* species and the abundant *Culex* species preferred the untreated traps (Table 7.1).

## 7.5. Discussion

This study describes the identification of the first oviposition attractant for malaria vectors of the *An. gambiae* species complex. Caged gravid females selected lake water treated with cedrol over lake water without cedrol for laying their eggs. Furthermore, the odourant attracted colonized free-flying gravid mosquitoes in large semi-field structures and increased the trap catches of wild gravid mosquitoes in the field. The attractiveness of cedrol was established in comparison to natural water from Lake Victoria which constitutes the majority of the natural, highly productive anopheline habitats in the study area (Fillinger *et al.* 2004) and which previously was found “to be the most stimulatory water treatment [for *An. gambiae*] uncovered to date” in egg-count cage bioassays (Otienoburu *et al.* 2007). This comparison is considered more realistic than one using distilled water as a comparator, since it is an artificial water source that wild mosquitoes are unlikely to encounter. It can though not be excluded that volatile compounds released from the lake water contributed to the attractiveness of cedrol.



However, preliminary cage bioassays (unpublished) implemented with distilled water gave similar results as those with lake water.

The recently developed systems of analysing oviposition responses in comparison to a baseline that provides two equal, untreated choices (Herrera-Varela *et al.* 2014), and of measuring attraction of gravid mosquitoes to oviposition substrates with modified BG-Sentinel mosquito traps (Okal *et al.* 2013) allowed a more detailed description of the behaviour of gravid *Anopheles* in response to odourants, since the response of individual females could be studied and stochastic effects affecting the distribution could be estimated and included in the analyses. It was shown here that cedrol not only increased the proportion of gravid females that were caught in the test trap out of the total number caught, but it also increased the proportion that responded out of the mosquitoes released. Furthermore, the presence of cedrol in the system induced a fast response, with two thirds of gravid mosquitoes trapped by 21:30.

With the ethanol-based cedrol formulation utilized here, cedrol was released in consistent rates over the entire 12 hours trapping period each night and therefore does not explain the nearly balanced response of gravid females to the traps in the semi-field experiment after 21.30. Less than one third of the collected mosquitoes were trapped after 21.30. It might be that these specimens were not fully gravid and therefore responded to high humidity to locate a resting place rather than to locate an oviposition site. For future studies, there may be value to work out better ways to formulate and dispense cedrol. The fact that it is a stable compound of relatively low volatility means that it should be well suited for development of long-lasting attractive baits.

The field study was implemented in an area of relatively low vector density as confirmed by collections of host-seeking mosquitoes. Considering that only a proportion of mosquitoes that host seek obtain sufficient amount of blood and survive long enough to become gravid, it was not unexpected that collections in gravid traps were an order of magnitude lower than catches in host-seeking traps. Despite low densities, it was three times more likely to trap *An. arabiensis* (the predominant species of the *An. gambiae* species complex in the study area) when the trap was cedrol-baited than when it only contained lake water. Previous reports from the study area show that the two sibling species *An. arabiensis* and *An. gambiae s.s.* share the same aquatic habitats (Chen *et al.* 2008; Ndenga *et al.* 2011; Minakawa *et al.* 2012) and therefore it is

not surprising that they appear to use the same odourants for orientation and selection of oviposition sites. The collections from the gravid traps also suggested that it is worth testing the attraction of the malaria vector *An. funestus* to cedrol since a slight preference for cedrol-treated traps was recorded. Finding a semiochemical or blend that could attract gravid females of the three most important vectors of human malaria in Africa, *An. gambiae*, *An. arabiensis* and *An. funestus*, would represent a tremendous breakthrough for the development of novel interventions. The fact that *Anopheles* were caught in an area with very low densities and that cedrol attracted *An. arabiensis*, a vector that is becoming increasingly important in areas where indoor interventions have impacted mosquito densities, indicates a promising future for the development of an odour-baited surveillance tool (Tirados *et al.* 2006; Bayoh *et al.* 2010).

The results presented here confirm that the modified BG-Sentinel mosquito traps work extremely well under semi-field conditions but were less effective in the field. It is hypothesized that visual cues interact with olfactory signals (Bernath *et al.* 2012), explaining the better performance of traps with open water surfaces in the study. Further understanding of the interaction between visual and chemical cues which may result in more effective traps will increase the possibility to efficiently lure vectors into oviposition traps when competing with natural oviposition habitats.

Cedrol-treated lake water, attracted similar proportions of gravid females in the semi-field experiments as the soil infusions from which it was identified (Herrera-Varela *et al.* 2014). To achieve this, a release rate of cedrol, which was much higher than from the natural source, was required. A lower concentration of cedrol might be enough to attract gravid malaria vectors if released in combination with other attractants. For instance, blends of synergistic attractants have been shown to be essential for effective trapping of host-seeking *Anopheles* mosquitoes (Braks *et al.* 2001; Okumu *et al.* 2010b; Mukabana *et al.* 2012). The analysis of the GC-MS data suggests another four putative semiochemicals, yet to be identified, that may play a role in the attractiveness of the six-day old soil infusion to gravid mosquitoes however, in contrast to cedrol none of these could be detected in the samples from natural oviposition sites in Kenya.

Cedrol is a sesquiterpene alcohol best known for its presence in the essential oil of conifers, especially in the genera *Cupressus* and *Juniperus*. However, it has been found in a large variety of plants including *Sorghum* (Khwatenge 1999), *Artemisia* (Mercke *et*

*al.* 1999) and swamp grasses of the genus *Cyperus* (Olawore *et al.* 2006), which are all common in the study area. Sesquiterpenes are also known metabolites of fungi and to some extent bacteria (Agger *et al.* 2008; Nakano *et al.* 2011; Kramer & Abraham 2012). It was shown here that the amount of cedrol released from a soil infusion was higher than from the same infusion that had been autoclaved and previously that the oviposition preference increased with increasing incubation time of the infusion (Herrera-Varela *et al.* 2014). This suggests that the release of cedrol is associated with microbial activity, possibly by metabolism of plant products. Finding the source of cedrol might further elucidate why *An. gambiae* s.s. and *An. arabiensis* prefer to lay eggs in habitats containing this compound and might help predict habitat selection and guide malaria vector control interventions.

## 7.6. Conclusions

This study provides evidence that gravid females of the *An. gambiae* complex can use attractive chemical cues when orienting towards potential oviposition sites. The findings demonstrate that these chemical cues can be exploited for trapping female malaria vectors. The discovery of an oviposition attractant provides prospects for novel ecological studies and progress in developing ‘attract and kill’ strategies against gravid malaria vectors. This could provide a novel tool in targeting residual malaria transmission in areas where current gold-standard indoor vector control interventions are applied at full coverage but are not enough to eliminate malaria (Govella & Ferguson 2012; Killeen 2014).

## Chapter 8. Synthesis

The tremendous success in controlling malaria transmission in the last decade should restore confidence in vector control as an effective frontline strategy for curbing the spread of malaria and other mosquito-borne diseases. Peridomestic malaria vector control using LLINs and IRS has lowered the burden of malaria and renewed hopes for eliminating the disease from sub-Saharan Africa (WHO 2013). However transmission of the disease is sustained by vectors that defy interventions by biting and/or resting outdoor or by surviving insecticides. These challenges make malaria elimination elusive in the region and instigate a growing need for supplementary vector monitoring and control methods (WHO 2013). To break the transmission cycle of the diseases strategies that take into account the ecology beyond blood-feeding processes are more desirable (Ferguson *et al.* 2010).

All major malaria vectors in Africa (*An. gambiae s.l.* and *An. funestus s.l.*) lay eggs in outdoor water bodies away from their human hosts dwelling. If identified, volatile organic chemicals that guide oviposition substrate location and discrimination in these species could be manipulated to attract and trap or kill gravid females foraging for oviposition substrates or combined with insecticides to kill their offspring. This study paved way for the development of such approaches by (1) originating and calibrating effective tools for analysing putative semiochemicals for oviposition in *An. gambiae s.s.*, (2) identifying and characterizing the role of multiple volatile organic chemicals for oviposition and (3) providing proof that odour baited traps can be used for targeting gravid *An. gambiae s.l.* and *An. funestus s.l.*.

### 8.1.Key findings

8.1.1. Improved responsiveness of gravid *An. gambiae s.s.* with standardized production of mosquitoes and re-alignment of oviposition bioassays with the peak oviposition period.

The study recorded the highest responsiveness and egg-rates of gravid *An. gambiae s.s.* in oviposition studies with the species. More than 80% of mosquitoes either laid eggs or flew to oviposition substrates in different experiments. This improvement was the

consequence of systematic standardization of procedures for preparing mosquitoes and careful considerations to re-align the periods for implementing bioassay with the natural peak oviposition period of this species. Firstly, it was shown that conditioning mosquito preparation cages containing young, 2-3 day old, blood-fed females with conspecific age-mate male mosquitoes, can enhance post-blood meal mating and improve the average proportion of mosquitoes that laid eggs to a consistent eighty percent. Secondly, preliminary evaluations demonstrated that secondary host-sources of blood meal such as rabbits could hamper the responsiveness of mosquitoes. The majority of mosquitoes blood-fed on rabbits failed to lay eggs during experiments. Consequently, human host-source of blood meals were recommended for the production of *An. gambiae s.s.* mosquitoes aimed for oviposition studies. Thirdly, scheduling oviposition experiments 72 hours after blood meal at dusk (which coincided with the peak oviposition time for the Mbita strain of mosquitoes) ensured prompt oviposition constrained to the early evening. This fine-tuning increasing the odds of mosquitoes remaining alive throughout the oviposition studies whilst also indicating an ideal time-frame for evaluating unstable substrates that degrade with time.

However, these guidelines did not improve the responsiveness of the *An. arabiensis* (a sympatric species of the *An. gambiae s.s.* complex) highlighting the need for customised production procedures with every species before oviposition studies. This study indicated that this is a secondary impact of low insemination rates especially in relatively smaller female mosquitoes.

### 8.1.2. Unique egg-laying characteristics of the malaria *Anopheles gambiae s.s.* and *Anopheles arabiensis* reduces the effectiveness of egg-count bioassays.

Unique egg-laying characteristics of *An. gambiae s.s.* and *An. arabiensis* highlighted the detrimental effects of these on egg-count choice tests. Studying the egg-laying in a large number of mosquitoes individually allowed to measure and describe a number of important characteristics. Firstly, colonised *An. arabiensis* were shown to be little responsive to oviposition substrates. An average proportion of just 25% of mosquitoes laid eggs in spite of careful preparation guidelines. The markedly low egg-rate of

colonised *An. arabiensis* had been reported in relatively old studies (Marchand 1985) but its potential consequence ignored by recent oviposition studies with this species. This study warns that substrate preferences reported in such oviposition studies might be based on the response of only a few mosquitoes that laid eggs; the conclusions might be misleading. Secondly, *An. gambiae s.s.* females were shown to exhibit unique egg-laying patterns that necessitated the development of different approaches to implementing choice egg-count bioassays. Egg numbers recorded for individual mosquitoes were overdispersed. In addition to this, the mean numbers of eggs laid by individual female mosquitoes were found to be highly variable between different replicates of experiments leading to a highly heterogeneous variance (heteroscedasticity). These characteristics violate primary assumptions of parametric tests widely used for analysing eggs numbers including t-tests and ANOVA. Recent statistical advances show that heteroscedasticity will also weaken non-parametric tests. Importantly, one-third of mosquitoes laid eggs unequally in identical substrates when presented in a two-choice test (skip-oviposited) giving an illusion of choice. Skip-oviposition makes it difficult to interpret unbalanced egg numbers in two cups in a choice test; showing oviposition substrate preferences especially with the low numbers (sample size) of mosquitoes. Similar challenges had formerly been recorded for *Ae. aegypti* another species known to lay single eggs and skip-oviposit, and important considerations for bioassaying mosquitoes that lay individual eggs suggested (Corbet & Chadee 1993). Their suggestions were almost universally ignored.

### 8.1.3. Improved and new tools and approaches for detecting egg-laying substrate preferences and measuring the attraction of gravid *Anopheles gambiae s.s.* to putative oviposition cues.

Effective existing or new tools and methods for behaviourally evaluating the chemoecology of oviposition with *An. gambiae s.s.* were evaluated in this study. To investigate relative preferences for egg-laying substrates a new approach for using egg-count bioassays was developed. Two-choice egg count bioassays were shown to be best done in two tier designs that (i) implement a parallel series of experiments with mosquitoes given a choice of two identical substrate choices and (ii) uses a single mosquito in each test cage rather than groups of mosquitoes. This approach with

sufficient replication, lowered the risk of detecting pseudo-preferences and drawing wrong conclusion oviposition substrate preferences. Sample size estimations and power tests for these bioassays were done. This marks the first reporting on these in oviposition studies with *An. gambiae s.l.*. It was demonstrated that with 165 individual in each treatment arm: 165 cages each with one mosquito given choice between a test and a control substrate and 165 similar cages where the mosquito has a choice between two identical control solutions. This enabled the description of differences in substrate preferences of as little as 15% with sufficient statistical power (80%) and significance (95%). Importantly, the appropriateness of (1) using generalised linear models with untransformed data instead of parametric methods and (2) analysing egg proportions instead of numbers were both demonstrated. A wide range of substrates were analysed using these.

In the laboratory, a WHO tube bioassay method was developed for investigating the response of gravid mosquitoes to humidity in the short range. Consisting only of two small cages, silica gel and two, fifteen centimetre pipes, this simple bioassay provided a good model of a robust but simple bioassay for individual mosquitoes. A larger air-flow wind tunnel built from polymethyl methacrylate (Perspex) sheets provided a dependable tool for investigating the ovipositional response flight of *An. gambiae s.s.* to putative cues in the short range. These two tools used together effectively described the role of water vapour in the oviposition choices of *An. gambiae s.s.*.

Finally, with little modification the BG-Sentinel mosquito traps were turned into effective tools for investigating the oriented flight of gravid mosquitoes to water vapour and volatile chemical substrates in the semifield and under field conditions. This tool provides a way measure pre-oviposition flight over a distance of several meters.

#### 8.1.4. Volatile organic chemicals interact with water vapour to attract gravid *Anopheles gambiae s.s.* to oviposition substrates. Such chemicals can be identified and used in the field to attract and trap gravid African malaria mosquitoes

The role of water vapour in the pre-oviposition behaviour of gravid *An. gambiae s.s.* was described. Using standardized laboratory bioassays water vapour was shown to be a

major pre-oviposition cue that mosquitoes use to locate potential oviposition substrates. It attracted gravid *An. gambiae s.s.* in still and moving air in the shorter range and under semifield conditions when water was provided in BG sentinel traps. However, the response to water vapour was found to be non-specific; the cue is little indicative of the quality of the oviposition site and probably provides the mosquito with little information with which to select suitable breeding sites, this was nicely demonstrated when presented in modified BG sentinel traps in two equal choice tests in the semifield. When only water was provided less than half the released mosquitoes responded slowly to the cue and were trapped. This was in sharp contrast to the experiments with an attractive organic chemical. Volatile organic chemicals were shown to interact with water vapour to mediate oviposition substrate choices of *An. gambiae s.s.* that either led to the selection/preference of an oviposition substrate or the avoidance of it.

*Anopheles gambiae s.s.*, unlike many culicine species (Allan & Kline 1995; Mboera *et al.* 2000a), consistently avoided laying eggs in  $\geq 25\%$  hay infusions and laid eggs in lake water instead. Many of the chemicals identified from the hay infusion elicited a similar negative response when presented singly in water at low doses. Many of these chemicals found in infusions such as 3-methylindole and *p*-cresol are known attractants for *Culex* species (Bentley *et al.* 1979). On the contrary, an infusion made from soil collected from a vibrant larval habitat of *An. gambiae s.l.* attracted gravid females to modified BG sentinel traps from a distance under semifield conditions. The first reported oviposition attractant for *An. gambiae s.s.* was identified from this soil infusion, the sesquiterpene alcohol cedrol. When added to water, cedrol treated substrates consistently attracted more eggs compared to lake water in egg count bioassays and attracted twice as many gravid females to BG sentinel traps in the semifield. The addition of the chemical also caused a stronger overall response of the majority of gravid females locating the substrates before 21:30 h. In addition to this, it attracted *An. arabiensis* under field conditions and led to an increased but not significantly higher number of *An. funestus* which warrants further testing with the latter species. The compound was found to be widespread in natural aquatic habitats in the study area. This study provided proof of principle that it is possible to attract and trap gravid African malaria vectors in the field using semiochemicals for oviposition. This opens up ways for the development of new strategies targeting gravid mosquitoes for monitoring and controlling malaria.



This study screened a large number of compounds that have previously been suggested to attract gravid *An. gambiae s.s.*. Many of these suggestions are based on evidence from electrophysiological studies that this species could detect these compounds. However, this study demonstrated that not all compounds that elicit electrophysiological activity are attractive to mosquitoes or important for oviposition site selection. Here, ten compounds many of which were suggested oviposition attractants only repelled gravid mosquitoes.

## 8.2. Limitations of the study

### 8.2.1. The role of semiochemicals and water vapour in the oviposition substrate choices of *Anopheles gambiae s.s.* in the wild is still unknown

While this study clearly demonstrated that water vapour and volatile organic chemicals contribute to oviposition substrate choices of *An. gambiae s.s.* it did not evaluate this in their natural oviposition environment. The entire study, except for the small scale field test at the end was done in standardized laboratory and semi-field systems where the potential role of additional cues such as vision was limited. Furthermore, the semiochemicals that were described were derived from studies of arbitrarily formulated infusions (hay and soil) and not natural breeding sites. Therefore, until large scale field ecological studies are done to confirm the presence and role of these compounds in the natural ecosystem, these should by no means be regarded as the determinants of oviposition site selection with the species.

### 8.2.2. Geographically limited study and findings

This is the first study to confirm the existence of olfactory oviposition attractants for *An. gambiae s.s.* and report an empirically tested semiochemical attractant. Cedrol was shown to attract gravid mosquitoes in the laboratory, semi-field and field. Its occurrence was discovered and demonstrated to be wide within the study area. Nonetheless, the whole study was done in one geographical area and it is possible that the compound may only be a regional specific cue as has been suggested for the species (Ogbunugafor & Sumba 2008). The compound should be evaluated in other regions with different local mosquito strains. There is also evidence that mosquitoes like many insects exhibit spatial memory and could improve their ability to find resources, oviposition sites and mating sites by learning the location of these (McCall *et al.* 2002). While the olfactory

memory (Kaur *et al.* 2003) in gravid *An. gambiae s.s.* is yet to be demonstrated and remains unlikely (Herrera-Varela *et al.* 2014), this study cannot rule out or confirmed its contribution to the attractiveness of cedrol.

### 8.2.3. Unknown sources of semiochemical evaluated in the study

The sources of the compounds described and tested in this study remain unknown. While the hay infusion volatiles tested have been clearly attributed to bacteria in various studies (Allan *et al.* 2005; Lindh *et al.* 2008; Allan *et al.* 2010), the exact metabolic pathway that leads to their formation and the bacteria species that instigate these processes in the substrates tested were not investigated. The cedrol detected in oviposition sites across the study area could have been from a wide range of sources including microbes or plants (Khwatenge 1999; Mercke *et al.* 1999; Olawore *et al.* 2006; Agger *et al.* 2008; Nakano *et al.* 2011; Kramer & Abraham 2012).

## 8.3. Future work

A number of new questions arose from the here presented studies that are worth pursuing in the future. Providing answers to the questions might be invaluable to the development of attract and kill strategies for malaria mosquitoes across Africa.

### 8.3.1. Identification of unknown putative oviposition attractants

In the presented experiments a release rate of cedrol which was much higher than from the natural source was required to observe a preferential oviposition response. This might be because a single compound was used rather than a blend of odourants which would present the more natural situation for a mosquito. This has been extensively shown for host-seeking mosquitoes, where blends are required for efficient attraction (Braks *et al.* 2001; Okumu *et al.* 2010b; Mukabana *et al.* 2012). A lower concentration of cedrol might be enough to attract gravid malaria vectors if released in combination with other attractants. This study strongly associated at least six compounds with the oviposition attraction of gravid *An. gambiae s.s.*. GC-MS evaluation and PCA analyses linked these to the soil infusion that strongly attracted the species. However, only one of these compounds was readily identified as cedrol. This is because the remaining compounds were only detectable in trace amounts insufficient for further analyses

through advanced techniques such as nuclear magnetic resonance. Even though these other unknown compounds were not as widespread in the study area as cedrol, evidence of their contribution to the oviposition substrates preferences should be investigated further. Their identification might lead to the formation of blends of semiochemicals that are stronger attractants of *An. gambiae s.s.*. In addition more natural habitat sources need to be investigated to identify a range of attractive (and repellent) chemicals involved in oviposition.

### 8.3.2. Investigating the origin of cedrol for oviposition in *Anopheles gambiae s.s.*

The attractant cedrol is best known for its occurrence in the woody plants *Cypressus* and *Juniperus*. However, it has also been associated with common African plants such as *Sorghum*, *Artemisia*, and the swamp grass *Cyperus* (Khwatenge 1999; Mercke *et al.* 1999; Olawore *et al.* 2006). Certain grass species have been implicated to play a role in habitat selection in the South American malaria vectors *An. albimanus* by releasing attractive semiochemicals (Torres-Estrada *et al.* 2005). Cedrol also can be metabolite of fungi (Nakano *et al.* 2011; Kramer & Abraham 2012), a group of microbes that has not been extensively investigated for its role in mosquito oviposition site selection. Finding the source(s) of cedrol might further elucidate why *An. gambiae s.l.* prefers to lay eggs in habitats containing it and might help predict habitat selection and guide malaria vector control interventions. The sources may provide for the isolation of a marker for high potential oviposition sites. Such markers could benefit mosquito control through target larviciding thus tremendously improving the efficiency and cost-effectiveness of such interventions. Furthermore, more attractants than those identified here might be produced from these sources.

### 8.3.3. Development of optimal formulated chemical baits and repellents

Work on formulation and presentation of cedrol (and other semiochemicals) will be a critical next step in the development of an attract and kill (or push and pull) strategy. Cedrol is crystalline at ambient temperatures (melting point 82-86°C) and not water

soluble, making it difficult to standardise the presentation in bioassays and traps. Here cedrol was mixed with ethanol to dissolve the crystals in a stock solution. However, the high variability in response rates observed in the cage bioassays indicates that our final test solutions might have contained different amounts of the active ingredient between test nights. Studies need to be done to improve the dose, release rates and delivery of cedrol and other semiochemicals identified in this study.

#### 8.3.4. Improvement of gravid traps

It was shown that the modified BG-Sentinel gravid trap worked extremely well under semi-field conditions and is an important new tool in evaluating putative oviposition semiochemicals but is less effective in the field. It is likely that visual cues interact with olfactory signals (Bernath *et al.* 2012) and enhance the search for oviposition sites, explaining the better performance of traps with open water surfaces in the study. This hypothesis is supported by the findings from field surveys that highly turbid water is consistently colonized by early instars in the field (Herrera-Varela *et al.* 2014) and preferred over less turbid or clear water in semifield choice tests (Okal, unpublished). It is likely that turbid surfaces reflect polarized light especially during dusk and make these habitats more visible in the landscape. Further understanding of the interaction between visual and chemical cues and more effective traps will increase the possibility to efficiently lure vectors into the traps when competing with natural habitats.

#### 8.3.5. Field trials under different eco-epidemiological conditions

Future work needs now to be invested in developing optimal traps that can be baited with synthetic odourants for routine use to test cedrol (and other yet to be identified odourants) under different eco-epidemiological field conditions.

### 8.4. General conclusions

Increasing evidence suggests that existing vector control strategies though effective will not achieve malaria elimination in Africa. These tools focus on the indoor environments and work with a relatively small group of insecticides to impact mosquitoes. For this reason, exophilic mosquitoes and those that are resistant to insecticides survive to maintain transmission even in areas earmarked for elimination of the disease. To target

malaria vectors outdoor there is an urgent need to study mosquito ecology beyond that which directly relates to blood feeding and develop tools that could be used outdoors. This thesis presents useful work in developing tools and advancing the understanding of oviposition substrate selection in *Anopheles gambiae s.s.*. Adoption of the tool kit proposed here for investigating the chemoecology of gravid malaria mosquitoes and the development of efficient formulations based on the compounds identified could lead to new strategies for monitoring and controlling vectors for malaria in Africa.

#### 8.4.1. *Anopheles gambiae s.s.* make informed oviposition substrate choices cued partially by semiochemicals

Results here show that gravid malaria mosquitoes make informed choices when selecting oviposition substrates. These choices are partially mediated by water vapour and volatile organic chemicals. This study extensively demonstrated the response of gravid mosquitoes to water vapour, infusions and putative semiochemicals. It showed that while water vapour attracted mosquitoes to any aquatic substrate, volatile organic chemicals elicited a discriminative response in the malaria mosquito. Infusions and semiochemicals routinely used to attract and sample gravid *Culex* and *Aedes* diseases vectors were avoided by the mosquito giving a hint for the ineffectiveness of such traps and the determinants of niche differentiation among mosquito genera. On the other hand aged soil infusions and one compound identified from them attracted mosquitoes demonstrating olfactory attraction to potential oviposition site.

#### 8.4.2. An optimised toolkit is essential for the effective identification and evaluation of oviposition semiochemical for malaria mosquitoes

A newly developed toolkit comprising of the WHO tube bioassay, a dual port olfactometer, a re-designed egg-count bioassays and a modified BG Sentinel trap enabled the identification and description of many oviposition repellents and an attractant for *An. gambiae s.s.*, *An. arabiensis* and potentially *An. funestus*. No previous study has systematically reviewed common bioassays used for investigating the oviposition behaviour of malaria mosquitoes. The toolkit described here can be equally useful with *Aedes* mosquitoes that lay single eggs and skip-oviposit. It also provides a

guideline for future studies seeking to optimise bioassays for studying the olfactory response of gravid mosquitoes.

#### 8.4.3. Targeting gravid mosquitoes using chemical cues could be a supplementary strategy for monitoring and controlling malaria vectors

This study demonstrated that it is possible to attract and trap gravid mosquitoes outdoors using chemical cues. It was shown that six day old infusions attracted more *An. gambiae s.s.* mosquitoes to modified BG Sentinel traps in the semi-field than did water alone. One chemical identified from the infusions (cedrol) equally consistently attracted more mosquitoes in the semi-field and in the field. In addition to this, cedrol also attracted wild populations of *An. arabiensis* a sympatric species to *An. gambiae s.s.* that is believed to be more exophilic and thus elusive to LLINs and IRS. A third species *An. funestus* was also trapped in the field. These findings indicate that although further work is needed to develop traps, identify more compounds and optimise the release rates and presentation of semiochemicals in traps, research for new tools along this hypothesis is well founded.

## Chapter 9. References

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## Chapter 10. Appendices

### **Appendix A:**

#### **Cage bioassays comparing the oviposition response of *Anopheles gambiae s.s.* to filtered tap water and distilled water in two choice experiments**

Oviposition bioassays need to be replicated in large numbers to account for the variability in responses of gravid females from different egg batches and under different climate conditions. Consequently, a large amount of water is needed as oviposition substrates. Distilled water is frequently used in oviposition bioassays but can be a limiting factor when working at remote field sites. The authors therefore aimed to evaluate whether purified lake water can be used as alternative to distilled water in oviposition bioassays.

**Methods:** Piped non-chlorinated water pumped from Lake Victoria was passed slowly through a sand charcoal gravel filter for purification (referred to as filtered tap water). The aim was to remove large and small particles from the water including the majority of algae and bacteria. Two choice cage bioassays were carried out comparing the oviposition response of 300 individual gravid *Anopheles gambiae s.s.* females to filtered tap water versus double-distilled water. Bioassays were done in 30×30×30 cm cages. The cages had a steel framework founded on a galvanized metallic base and covered with fine mosquito netting. The cage-net also had an insert sleeve for introducing and retrieving oviposition substrates and gravid mosquitoes. Oviposition substrates were offered in 70 mm diameter glass cups (Pyrex®) that were autoclaved and afterwards kept in an oven at 200°C for at least 2 hours before experiments. In each cage two cups were provided in opposite corners one filled with 100 ml filtered tap water (test) and the other cup filled with 100 ml double-distilled water (control). The arrangement of oviposition cups was systematically altered between adjacent cages to adjust for position effect. The test cup was randomly placed in one corner of the first cage and test cups in subsequent cages were moved one corner step in a clockwise direction relative to that of the preceding cup. Corresponding control cups were added in each cage diagonal to the test cup to complete a two choice set up. Adjacent cages were placed on a table a minimum of 30 cm apart. The experiment was carried out under ambient light and temperature conditions in makeshift huts. Two huts were used containing two tables each. Twenty-five cages were placed per table

totalling 100 cages per experimental nights. The experiment was replicated for three rounds using different batches of mosquitoes. Individual mosquitoes were placed in the cages at 18:00h and the response (presence of eggs) per treatment and cage recorded at 8:00h in the morning.

**Data analysis:** Data was analysed with R statistical software version 2.14.2 using the one sample proportions test with continuity correction. This test investigates whether the response rate of individual gravid females towards the two treatments differs significantly from 0.5 hypothesizing that if the two treatments would be equally suitable for oviposition 50% of the females should have laid in the test and 50% in the control.

**Results:** In total 242 out of the 300 females laid eggs (81%). Out of the 242 females 33 (14%) laid in both test and control cups. The remaining 209 females laid either in test or control. Therefore in total 275 responses were recorded. The bioassays were carried out in three rounds. Table A1 shows the results of the proportion tests for the individual rounds and for the pooled data.

	<b>Number of responses (n/N)</b>	<b>Response rate (%) for filtered tap water (95% CI)</b>	<b>p-value*</b>
<b>Round 1</b>	59/105	56 (46-66)	0.242
<b>Round 2</b>	50/89	56 (45-67)	0.289
<b>Round 3</b>	45/81	56 (44-66)	0.341
<b>TOTAL</b>	<b>154/275</b>	<b>56 (49-62)</b>	<b>0.054</b>

**Table A1:** Response of *Anopheles gambiae s.s.* towards the filtered tap water

\*null hypothesis: response rate equals 50%

The test cup with the filtered tap water received 56% of all responses (eggs laid) towards the two treatments both in the individual rounds as well as when all data were pooled for analyses. This only slightly increased proportion was neither significantly different from 50% for the individual rounds nor when the data were pooled at the  $\leq 0.05$  significance level. Nevertheless, when the data were pooled the difference approached significance.



**Conclusion:** Gravid *An. gambiae* s.s. females did not show any strong preference for the filtered tap water over the double-distilled water. The approaching significance level when the data were pooled might indicate a genuine effect likely reflecting a difference in water quality due to the incomplete filtration of algae and bacteria from the water. Nevertheless, the difference between the two water sources was so small that the authors conclude that the filtered tap water does not contain strong oviposition semiochemicals and can be used for studying the oviposition behaviour towards chemical and visual cues as a replacement for distilled water.

## Appendix B

### Short report of the Gas Chromatograph –Electroantennogram (GC-EAD) analysis of *Anopheles gambiae* s.s. electrophysiological responses to putative oviposition semiochemicals

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**Introduction:** Electroantennogram (EAG) responses of *Anopheles gambiae* s.s. to synthetic chemicals potentially involved in affecting oviposition behaviour were measured. Previous such studies have delivered the stimulus by blowing air over a piece of filter paper impregnated with the compound. When testing compounds having different volatilities this approach gives very different, generally unquantified, amounts of the chemicals actually delivered to the insect antenna. To overcome this problem, in this study the same amounts of each compound were delivered to the insect antenna through a gas chromatograph coupled directly to the EAG preparation. In order to correct for variations between different EAG preparations, a reference compound was included in every run. A whole insect preparation was used rather than isolated antennae or heads used previously.

**Materials and Methods:** Female *Anopheles gambiae* s.s. were drawn from a stock colony of Mbita origin maintained at NRI, Chatham, UK. Adult males and females were reared together, provided with a 10% sucrose solution and offered a blood-meal 2-5 days prior to EAG experiments. Females that fed successfully were selected visually for EAG recording. Responses were recorded from whole insects mounted on filter paper using solvent-free correction fluid.

Test compounds were 2-tridecanone, 2-phenylethanol, benzyl alcohol, 3-methylbutan-1-ol, based on the work of Lindh et al. (2008), indole (Lindh et al., 2008; Blackwell and Johnson, 2000; Meijerinck et al., 2000), 3-methylbutanoic acid (Lindh et al., 2008; Cork and Park, 1996), 1-octen-3-ol (Cork and Park, 1996; Blackwell and Johnson, 2000), 6-methyl-5-hepten-2-one and geranyl acetone (Meijerinck et al., 2000). The reference compound was 4-methylphenol which gave a good EAG response in preliminary studies and was also reported as a stimulus by Cork and Park (1996), Meijerinck et al. (2000)

and Blackwell and Johnson (2000). Each run included two or three of the test compounds and 4-methylphenol, all at 10 ng which delivers 5 ng to the insect antenna.

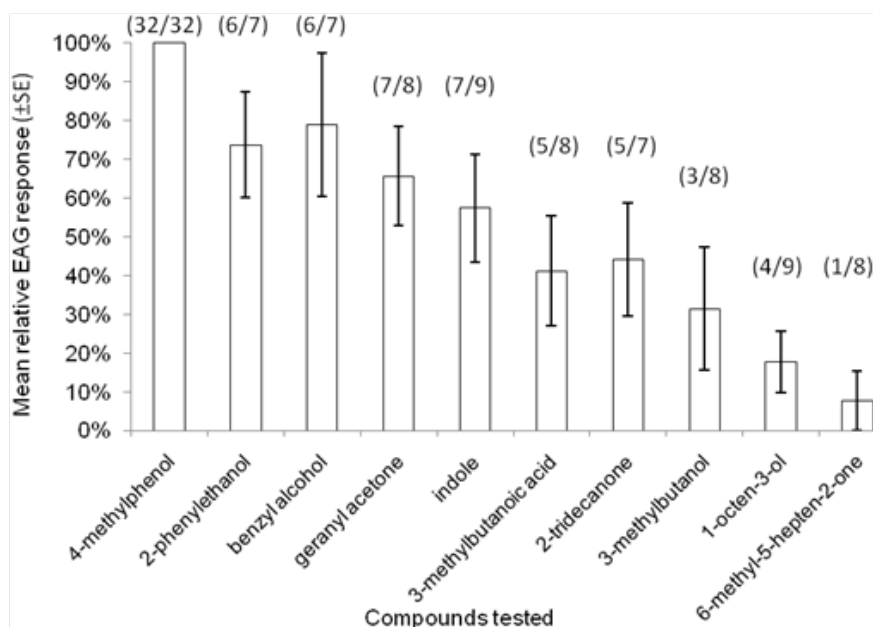
**Results:** Only runs (32) in which the reference compound, 4-methyl-phenol, elicited a clear EAG response were included in the analysis. Table A2 shows both mean EAG responses to test compounds for all the runs and also for only the runs where a significant EAG response to that compound was recorded. The data are shown graphically in Figure A1.

Of the test compounds, geranyl acetone gave a response in 7 out of 8 runs; 2-phenylethanol in 6/7 runs; benzyl alcohol in 6/7 runs and indole in 7/9 runs. A small EAG response to 6-methyl-5-hepten-2-one was only observed in 1/8 runs. No compound gave a higher mean EAG response than the reference compound, 4-methylphenol, and the above four compounds gave the highest mean responses of the compounds tested.

**Discussion:** EAG responses were recorded from female *Anopheles gambiae* s.s. of Mbita origin that were blood-fed and assumed to be mated. Using coupled GC-EAG to deliver accurately a known dose of the test compounds, 4-methylphenol gave the highest and most consistent responses, followed by benzyl alcohol, 2-phenylethanol, geranyl acetone and indole, 2-Tridecanone, 3-methylbutanoic acid, 3-methylbutanol, 1-octen-3-ol and 6-methyl-5-hepten-2-one gave lower and less consistent EAG responses. All these compounds have previously been implicated as semiochemicals affecting host-finding and/or oviposition by mosquitoes.

**Table A2.** Mean EAG responses of mated female *Anopheles gambiae* s.s. to compounds (5 ng) relative to the response to 4-methylphenol, showing means for all runs and for only runs in which a response was observed.

	responses/ runs	Relative EAG response			
		all runs		responses only	
		mean	SE	mean	SE
4-methylphenol	32/32	1.00	-	1.00	-
2-phenylethanol	6/7	0.74	0.14	0.86	0.07
benzyl alcohol	6/7	0.79	0.18	0.92	0.15
geranyl acetone	7/8	0.66	0.13	0.75	0.10
indole	7/9	0.57	0.14	0.65	0.11
3-methylbutanoic acid	5/8	0.41	0.14	0.66	0.28
2-tridecanone	5/7	0.44	0.15	0.62	0.13
3-methylbutanol	3/8	0.31	0.16	0.84	0.12
1-octen-3-ol	4/9	0.18	0.08	0.32	0.09
6-methyl-5-hepten-2-one	1/8	0.08	0.08	0.61	0.08



**Fig. A1.** Mean EAG responses ( $\pm$  standard error) of mated female *Anopheles gambiae* s.s. to compounds (5 ng) relative to the response to 4-methylphenol, showing means for all runs.