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African Insect Science for Food and Health

Larval habitat discrimination by the African malaria
vector *Anopheles gambiae sensu lato*: Observations
from standardized experiments and field studies

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2015

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LONDON SCHOOL OF HYGIENE & TROPICAL MEDICINE

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

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Abstract

Current malaria vector control strategies in Africa target indoor resting and biting mosquitoes and rely heavily on a small number of insecticides. These interventions have led to the selection of insecticide resistance, behavioural adaptations of the vectors and leave naturally exophilic species nearly untouched. Gravid *Anopheles gambiae s.l.* female searching for an oviposition site would be a novel target for vector control. However, little is known about the oviposition-site selection behaviour (criteria) of this mosquito.

The major aim of the presented research was to investigate if gravid *An. gambiae s.l.* make informed choices when selecting an oviposition site and to identify physical, chemical and biological parameters associated with these choices under standardized experimental and natural field conditions.

Standardized field tests and dual-choice oviposition bioassays were used to evaluate responses to soil and rabbit food pellets infusions and habitat water and also to test if bacteria and the volatile chemicals that bacteria produce are relevant to habitat selection. A case–control approach was used to study natural aquatic habitats on Rusinga Island in Lake Victoria during the long rainy season in 2012 to compare the characteristics of habitats colonized (cases) and not colonized (controls) by early instar *Anopheles* larvae. Factors evaluated included biological characteristics of the sites, zooplankton, invertebrate fauna, physical parameters, nutrients, bacteria communities and volatile chemicals released from the water. Multivariate analyses were used to investigate associations between oviposition site characteristics and habitat selection by *Anopheles*.

The experimental work illustrated that wild and caged *An. gambiae s.l.* females discriminate between potential aquatic habitats for oviposition and gravid *An. gambiae s.l.* female select suitable habitats using preferred and avoided chemical cues from water bodies. It furthermore emphasizes that natural infusions can be used to manipulate the oviposition behaviour of *An. gambiae s.l.*. In the field no evidence was found that bacteria from natural habitat water were involved in habitat selection. Although chemical cues were highly diverse analysis suggests that cases and control habitats differ in the headspace volatile profile of the water. High turbidity >200 nephelometric turbidity units (NTU) was the only environmental factor strongly associated with cases. Other risk factors were higher grass coverage (positive association), and the abundance of creeping water bugs of the family *Naucoridae* and fish (negative associations).

This study demonstrates that gravid *An. gambiae* females choose suitable habitats for oviposition using a complex system of chemical and visual cues from water bodies. Habitats preferred by *An. gambiae* exhibited distinct and measurable characteristics that can be potentially exploited to attract and kill gravid females to improve malaria vector monitoring and control.

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OviART team and families. Wayando, Rusinga Island, Western Kenya. December 2013

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Chapter 1 Introduction

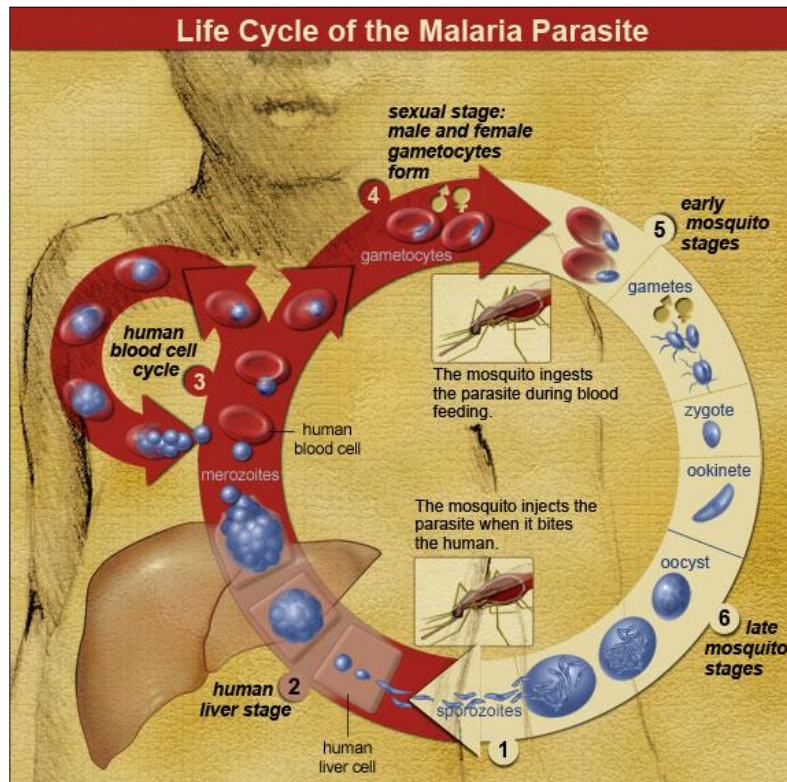
1.1 Malaria

Malaria is among all parasitic diseases in humans the one with the greatest impact on populations worldwide in terms of lives lost and social and economic burden (Witty 2004; Warburg *et al.* 2011). It is widespread in tropical and subtropical regions, including parts of America, Asia, and Africa. The World Health Organization reported 198 million cases globally in 2014 resulting in 584,000 deaths. The biggest burden of malaria is found in sub-Saharan Africa, where an estimated 90% of malaria deaths occur in children under the age of 5, who accounted for 78% of the deaths reported (WHO 2014).

Malaria is caused by *Plasmodium* (Haemosporida: Plasmodiidae) parasites and transmitted by *Anopheles* (Diptera: Culicidae) mosquitoes. Five species of the genus *Plasmodium* cause all malaria infections in human beings. Most cases are caused by either *P. falciparum* or *P. vivax*, but infections can also be caused by *P. ovale*, *P. malariae*, and, in parts of Southeast Asia, by the monkey malaria *P. knowlesi* (Kantele & Jokiranta 2011; White *et al.* 2014). Only around 41 of more than 400 *Anopheles* species are able to transmit malaria between humans at a level of major concern to public health (Harbach 2013).

The most competent malaria vector mosquitoes are found in sub-Saharan Africa and belong to the *Anopheles gambiae* species complex which consists of nine species (Edmondson 1959; Gillies & Coetzee 1987) and the *Anopheles funestus* species complex which also consists of 9 species. Members of a species complex have different behaviour, larval habitat requirements and vectorial capacity to transmit the disease (Gillies & De Meillon 1968; Gillies & Coetzee 1987). *Anopheles gambiae sensu stricto* (*s.s.*) and *An. arabiensis* of the *An. gambiae* complex (*An. gambiae sensu lato* (*s.l.*) and *An. funestus s.s.* of the *An. funestus* complex are the most effective malaria vectors worldwide because they are long lived and robust to environmental change, occur in high densities in tropical climates, breed readily in a large variety of available larval habitats and have a strong preference for biting humans when no control measures stop them from doing so (Gillies & De Meillon 1968; Gillies & Coetzee 1987; Sinka *et al.* 2012). To transmit malaria, a female *Anopheles* must bite a person carrying *Plasmodium* gametocytes and live long enough to allow the parasite to complete its sexual development which ends as sporozoites in the mosquito salivary glands; this sporogonic cycle

takes 10-12 days in *An. gambiae s.l.*. Consequent bites can then infect a healthy person with *Plasmodium* (Figure 1.1).



Source: NIH/NIAID (<http://www.niaid.nih.gov/topics/Malaria/Pages/lifecycle.aspx>)

Figure 1.1. Life cycle of malaria parasite:

(1) A female *Anopheles* mosquito carrying malaria-causing parasites feeds on a human and injects the parasites in the form of sporozoites into the bloodstream. The sporozoites invade liver cells. (2) The sporozoites grow, divide, and produce thousands of haploid merozoites, per liver cell. (3) The merozoites exit the liver cells and re-enter the bloodstream, beginning a cycle of invasion of red blood cells, asexual replication, and release of newly formed merozoites. This multiplication results in illness and complications of malaria. (4) Some of the merozoite-infected blood cells leave the cycle of asexual multiplication and develop into sexual forms, called gametocytes that circulate in the bloodstream. (5) When a mosquito bites an infected human, it ingests the gametocytes. In the mosquito gut, the infected human blood cells burst, releasing the gametocytes, which develop further into mature sex cells called gametes. Male and female gametes fuse to form diploid zygotes, which develop into actively moving ookinetes that burrow into the mosquito midgut wall and form oocysts. (6) Growth

and division of each oocyst produces thousands of active haploid sporozoites. After 10-12 days (for *Plasmodium falciparum*), the oocyst bursts, releasing sporozoites into the body cavity from which they travel to and invade the mosquito salivary glands. The cycle of human infection re-starts when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the human bloodstream (taken from (WHO 1957))

Malaria control measures aim to prevent morbidity and mortality and to reduce the socioeconomic loss due to the disease. Control measures currently used, target either the malaria parasites through early diagnosis and treatment of malaria with effective medicines or they target the mosquito vector with the aim to prevent transmission (WHO 2014).

1.2 Vector control

1.2.1. The history of vector control

Since the discovery of the malaria parasite in human blood by Alphonse Laveran and later the establishment of the role of mosquitoes in malaria transmission by Sir Ronald Ross at the end of the 19th century, numerous regional and global efforts have been carried out in order to control, eliminate and eventually eradicate this disease (Najera 2001). In 1913 Malcom Watson, studying a malaria outbreak in the lowland areas of Malaysia, noticed that not all *Anopheles* mosquitoes were able to carry the parasite and thus to transmit the disease. In particular, he demonstrated that in Malaysia the main carrier of the parasite was *An. umbrosus* which bred in pools of stagnant water in the jungle; he then proved that the abolition of these pools by drainage and cultivation of the land reduced malaria incidence significantly. Furthermore he demonstrated that malaria could be eradicated guided by the study of the ecology of *Anopheles* mosquitoes (Watson 1913). This strategy focused on the mosquito vector and malaria epidemiology was later known as “species sanitation” and was effectively implemented in Indonesia and North Holland (Najera 2001). Early in the 1900’s malaria control was carried out on a regional scale and often as part of larger programs targeting other vector borne diseases. Integrated vector management was frequently practiced in these early programs including draining of swamps, treating standing water with petroleum oils or Paris Green, screening of windows and doors and spraying with pyrethrum extracts (Imbahale *et al.* 2012; WHO 2014).

Early successes and tools were rapidly forgotten after the discovery of dichloro-diphenyl-trichloroethane (DDT) during the second war world (Johannesen, Dunn & Morrell 2014).

DDT was the first insecticide with a long lasting residual effect used to kill mosquitoes resting on house walls. It could be applied every six months overcoming the problem faced with pyrethrum extracts that needed weekly application. This attribute allowed malaria control programs to be cost-effective and to be extended to many rural areas (Najera, Gonzalez-Silva & Alonso 2011). The enormous success in combating vector borne diseases with this insecticide however, led to a decline in the interest for funding studies focusing on mosquito biology and to an increase in supporters of the idea that malaria could be wiped out from every place using a single tool; in this case the spraying of houses with the residual insecticide DDT (Johannesen, Dunn & Morrell 2014).

The World Health Organization (WHO) launched the Global Malaria Eradication Program in 1955. This program relied on the use of DDT for vector control and the use of Chloroquine for the treatment of infected individuals (WHO 1957). These new tools yielded extraordinary results in countries with temperate climates and stable economies where malaria was eradicated but failed in most of the tropical countries that were included in the program i.e. Haiti and Nicaragua. In addition, in countries like India and Sri Lanka the campaign interrupted outbreaks for as long as the program was implemented but once the interventions stopped malaria cases went back to normal and often a recrudescence effect was observed (WHO 1959). Furthermore, this global campaign excluded most of the countries from sub-Saharan Africa. Failure to eradicate malaria in some of the areas where it was attempted was attributed to the emergence of drug resistance in humans, mosquito resistance to DDT and lack of community participation (WHO 1978). In 1977 the goal of eradication was officially abandoned and replaced by the aim of malaria control. It was recognized that one single approach was not advantageous in decreasing transmission and that a profound knowledge of malaria epidemiology (i.e. parasite, human, vector) is required at local levels for tailored attacks of the disease (Sharma 2012).

1.2.2. Current vector control in sub-Saharan Africa relies on strategies targeting

***Anopheles* vectors indoors**

After the eradication campaign the international community was slow to respond and the number of cases of malaria increased worldwide. New research foci were the development of drugs and vaccines and the improvement of health systems. For vector control only insecticide-treated nets (ITNs) were intensively investigated (Lengeler 2004). In 1998 WHO launched The Roll Back Malaria initiative that was further supported by the Millennium

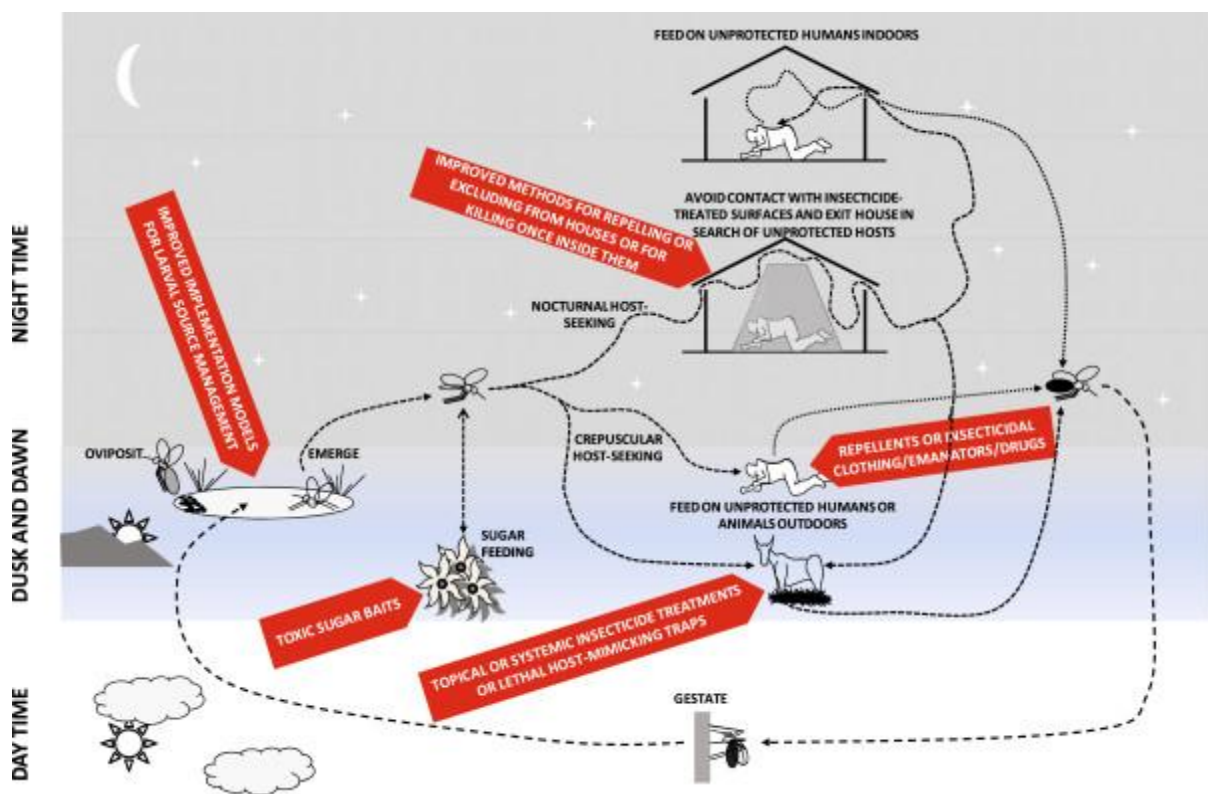
Development Goals with the aim to reduce global malaria cases from 2000 levels by 50% in 2010 and by 75% in 2015 (WHO 1999). This initiative was funded by the Global Fund to Fight AIDS, Tuberculosis and Malaria, the World Bank, and the US President's Malaria Initiative (WHO 2000). The strategy of Roll Back Malaria was based on prompt diagnosis and treatment with artemisinin combination therapies (ACTs) and vector control. Two approaches were recommended for attacking the mosquitoes; insecticide treated nets (ITNs) and indoor residual spraying (IRS) (GMAP 2008). These vector control interventions target exclusively mosquitoes that feed and rest inside human dwellings as is the case with the major African malaria vectors; *An. gambiae s.s.* and *An. funestus s.s.* (Gillies & De Meillon 1968). The result of these interventions has been remarkable in reducing the number of cases and deaths caused by malaria in various parts of the world including a number of countries in Africa (Lengeler 2004; Okiro *et al.* 2007; Protopopoff *et al.* 2007; Pluess *et al.* 2010). As a consequence of this success the enthusiasm for malaria elimination and eradication has reappeared once again on the global agenda (Tanner & de Savigny 2008).

1.2.3 The importance of the development and use outdoor vector control strategies

The current vector control strategies (IRS, ITNs) have shown to be effective in saving lives worldwide however, the same strategies are facing challenges that need to be overcome if malaria elimination is to be achieved. These obstacles include the development of pyrethroid resistance in the major *Anopheles* vectors (Ranson *et al.* 2009; Ranson *et al.* 2011). This is particularly worrying given that this is the only type of insecticide approved for ITNs so far. Behavioural avoidance of the house environment where insecticides are present is another serious concern. Furthermore, vectors show a considerable plasticity in their behaviour which allows them to respond with some flexibility to the vector control measures. It has been shown that historically anthropophilic vectors accept other blood-meal hosts when given no choice, that more outdoor feeding and early evening feeding takes place when people are not protected (Sharma *et al.* 2008; Ferguson *et al.* 2010; Reddy *et al.* 2011; Riehle *et al.* 2011) and that current vector control interventions leave the more exophilic vectors like *An. arabiensis* (Tirados *et al.* 2006; Oyewole *et al.* 2007; Bayoh *et al.* 2010) and secondary vectors like *An. coustani* and *An. rivulorum* less affected by indoor interventions (Ikeshoji 1966; Najera 2001) maintaining low but stable malaria transmission in many areas (Killeen 2014).

Malaria elimination will require the addition of new vector control tools that target the mosquito outside the house (Figure 1.2). To develop these tools however, extensive research

is required to understand the behaviour of adult vectors in the outdoor environment. It is surprising that apart from the blood feeding behaviour little is known about how sub-Saharan *Anopheles* mosquitoes select and exploit other resources like aquatic habitats, mates, sugar sources and resting sites (Ferguson *et al.* 2010). A series of potential new and improved vector control tools tackling multiple points in the mosquito life cycle have been summarized recently by (Killeen 2014). These include spatial repellents, house screening, repellent clothing, insecticide-treated cattle, toxic sugar baits and improved larval source management practices (Figure 1.2).



Source Killeen 2014 (<http://www.malariajournal.com/content/13/1/330>)

Figure 1.2. Life cycle of malaria vector mosquitoes showing different behaviours that enable them to avoid conventional control strategies and targets for novel intervention strategies

1.2.4 Renewed interest in larval source management and selection of aquatic habitats by *Anopheles gambiae s.l.*

Recent field evaluations of larval source management using larvicides under various eco-epidemiological conditions in Africa showed that hand-applied larviciding reduced

transmission by 70-90% where the majority of aquatic mosquito larval habitats were defined and aquatic surface areas not too extensive (Fillinger *et al.* 2008; Fillinger *et al.* 2009b) and that the addition of larviciding to ITNs has a significant added benefit in reducing malaria incidence (Trexler *et al.* 2003b; Fillinger *et al.* 2009a). However, it has also been recognized that larval control programs using larvicides require well established and managed programs, a large and trained labour force and a frequent re-application of larvicides to all aquatic habitats in the target area (Tusting *et al.* 2013).

Field studies have demonstrated that the colonization of aquatic habitats by *An. gambiae s.l.* larvae differ over space and time (Fillinger *et al.* 2009b; Ndenga *et al.* 2011; Imbahale *et al.* 2012). Since not all aquatic habitats have the same chance of being chosen by female vectors for oviposition, individual habitats differ in their capacity for generating adult mosquitoes (Muirhead-Thomson 1945; Muirhead-Thomson 1951; Fillinger *et al.* 2004; Mutuku *et al.* 2006). As a result, researchers have been advocating a more selective approach by targeting anti-larval interventions at larval habitats favoured by *An. gambiae s.l.* to make this intervention more cost-effective (Gu & Novak 2005; Gu *et al.* 2006; Gu, Utzinger & Novak 2008).

At present, however, it is not possible to identify the most productive *Anopheles* larval habitats. Although studies show that clear heterogeneities of productivity exist, the data are not adequate to provide the basis for rational targeting (Killeen *et al.* 2006). We do not know precisely what factors are responsible for these differences nor whether these factors, once identified, would be suitable for directing control activities.

Large-scale longitudinal studies in the floodplains of the Gambia River, rural lowland and highland areas in Kenya and in urban Dar es Salaam in Tanzania (Fillinger *et al.* 2008; Majambere *et al.* 2008; Ndenga *et al.* 2011) have shown that on any given sampling date only 20-50% of aquatic habitats contained anopheline larvae. In these scenarios it is common to find a large number of similar habitats close to human hosts that would seem suitable breeding sites, yet some of them are either consistently free of larvae or their colonization changes over space and time leaving only a small fraction with larvae (Muirhead-Thomson 1945; Muirhead Thomson 1951; Majambere *et al.* 2008).

Risk factor analyses of habitat characteristics identifiable under operational conditions (e.g. habitat type, habitat size, vegetation cover) failed to reveal any factors that could consistently predict sites preferred by *An. gambiae s.l.* (Majambere *et al.* 2008). Nevertheless, site

selection is unlikely to occur at random since laboratory studies indicate that there are several cues which are used by gravid female for oviposition (Bates 1940; McCrae 1984; Bentley & Day 1989; Sumba *et al.* 2004; Sumba *et al.* 2008).

1.3 Oviposition behaviour in mosquitoes

The heterogeneous distribution of larvae is likely to result from a combination of two factors; selection of oviposition site by the female mosquito and the survival of larvae in the aquatic habitat. Whilst a number of studies have investigated risk factors for indoor-biting mosquitoes (Watson 1913; Pinault & Hunter 2012), there are few that have specifically investigated the reverse journey that a gravid female makes from a dwelling to a breeding site.

Oviposition behaviour is associated with a complex interaction of physical and chemical cues emanating from a potential oviposition site that is perceived by gravid mosquitoes when looking for a water body to lay their eggs. This behaviour might be mediated by long-range cues that help the mosquito to identify different habitats; some of these cues (vegetation, shade, light reflection) are probably evaluated visually before the mosquito makes contact with water (Bentley & Day 1989). Short distance cues are probably based on chemicals released from water bodies (attractants and repellents), which guide mosquitoes to locate suitable habitats and make oviposition choices (Takken & Knols 1999).

Early laboratory studies demonstrated that gravid anophelines responded to different light and dark contrasts and brightness (Bates 1940; McCrae 1984). *Anopheles atroparvus*, *An. arabiensis* and *An. gambiae s.s.* were shown to prefer laying eggs on dark backgrounds rather than pale ones (Bates 1940; McCrae 1984; Huang *et al.* 2005; Balestrino *et al.* 2010).

The physical conditions and composition of the substrate are also important. It was shown that *Anopheles gambiae s.s.* preferred to lay eggs in oviposition sites containing calcium (Bates 1940), mud and turbid water (McCrae 1984) and some studies have shown that more eggs were laid in water from a natural breeding site than tap or distilled water (McCrae 1984; Sumba *et al.* 2004). These data suggest that soil factors can also mediate olfactory oviposition behaviour in anophelines, however the evidence is sketchy (Takken & Knols 1999).

1.3.1 The potential role of semiochemicals in oviposition site selection

Olfactory cues are involved in important behaviours of female mosquitoes and there is good

evidence that oviposition is also governed by semiochemicals (Takken & Knols 1999). From all the external signals that a mosquito can perceive, chemicals provide important information about the location, suitability or physiological state of conspecifics, hosts or breeding sites (Logan & Birkett 2007).

Semiochemicals are chemicals that influence the behaviour and physiology of insects (Dicke & Sabelis 1988). Mosquitoes chemosensory organs primarily consist of diverse cuticular extensions called sensillae located primarily on the antennae and maxillary pulps (McIver 1982). Sensillae have porous surfaces that open to an array of olfactory receptor neurons (ORNs), each with unbranched and multi-branched sensory dendrites. The dendrites are suspended in lymph within the sensilla lumen containing odour binding proteins (OBPs). Olfactory molecules that move into the sensilla through the pores bind to OBPs and are carried through to dendrites of ORNs. The ORNs then convey signals to the insects central nervous system (CNS) causing changes in behaviour or physiology of the mosquito (Su, Menz & Carlson 2009). Olfactory receptor neurons can be sensitive to a few chemicals or to a wider range of compounds (Carey *et al.* 2010).

In oviposition, it is hypothesized that the chemicals emanating from a water body guide the female to the breeding site and upon landing and contact with the water surface stimulates the female to lay her eggs (Bentley & Day 1989). Such chemicals can be divided into two broad groups: those that attract or repel mosquitoes from a distance, and those that mediate the actual act of oviposition. Attractants and repellents are volatile compounds acting over a distance whilst those involved in the initiation of oviposition may be non-volatile chemicals. The process of oviposition can be briefly summarized as a pathway of behaviours where the mosquito receives stimulation to take flight, orientates the flight upwind in response to attractants, arrestment and sampling of a site, and finally, oviposition stimulation (Isoe, Millar & Beehler 1995).

Chemical cues can originate from natural water bodies as breakdown products of bacterial or algae origin, from the eggs themselves as an oviposition pheromone or released by other organisms like predators (Laurence & Pickett 1982; Ponnusamy *et al.* 2008a; Silberbush *et al.* 2010). These sources of stimuli might result in the aggregation of eggs in sites suitable for larval development (McCall & Cameron 1995). Until now the evidence for the involvement of semiochemicals in oviposition site selection is limited to very few mosquito species. Numerous compounds have been described as being involved in the oviposition behaviour in

few species of the genera *Culex* and *Aedes* although just a few of them have been confirmed as oviposition attractants (Table 1).

The first and most studied oviposition pheromone was identified in mosquitoes of the genus *Culex*. Initially observations showed the egg rafts attracted gravid females of *Cx. tarsalis*, *Cx. molestus* and *Cx. quinquefasciatus* to lay eggs at the same oviposition sites, later it was found that the increment observed in the oviposition of *Culex* spp. occurred due to droplets present on the egg's apex (McCall & Cameron 1995). Gas chromatography coupled to mass spectrometry revealed that the active compound in the droplets was erythro-6-acetoxy-5-hexadecanolide (AHD) (Laurence & Pickett 1982). The pheromone was synthesized and its efficacy tested in a field site in Tanzania. Pheromone alone caught as many gravid mosquitoes as a standard grass infusion and was active for 9 days (Mboera *et al.* 2000).

In contrast chemical mediation of oviposition site selection in *An. gambiae s.l.* is poorly understood. Gravid female mosquitoes have been shown to detect 3-methyl-indole, indole, p-cresol, o-cresol, phenol, m-cresol 4-methylcyclohexanol and 2-propyl phenol in electrophysiological studies (Blackwell & Johnson 2000). These compounds are therefore thought to be important cues for gravid *An. gambiae s.l.* and are frequently referred as putative attractants. There is however, a huge gap in evidence showing that these chemicals actually elicit a behavioural response and more importantly the type of response they might elicit. Nevertheless, if a better understanding of the chemical cues used by gravid females is gained this would increase the possibilities to implement novel strategies for vector control.

Perhaps the better examples of how the identification of semiochemicals can improve dramatically control of an insect target comes from agriculture. When it became clear that insects used their chemical senses to communicate with conspecific and other species of animals or plants it was possible to develop semiochemical-based pest management that were environmentally and human friendly (El-Sayed *et al.* 2006). These methods have shown to be advantageous because many if not most semiochemicals are relatively nontoxic to vertebrates as well as beneficial insects and also its high selectivity to the targets species. The most successful semiochemical-based-pest management approaches include mass trapping, attractant (lure) & Kill and Push-Pull strategies (de França *et al.* 2013).

Mass trapping uses species-specific synthetic chemical lures (pheromones and host/food attractants), to attract insects to a trap where they would be confined and die. Mass trapping using odour-baited traps is one of the older approaches to direct control of insects for population control and eradication (Steiner 1952; El-Sayed *et al.* 2006). As an extension of the mass trapping strategy, “Attract and Kill” approaches have been created by using sex pheromones or food attractant in combination with a killing agent (insecticide). This is a very efficient system, given that the insect can be directed to a selected point where it will get in contact with the insecticide allowing reducing of amount of insecticide applied i.e. there is no need to cover the whole crop (Charmillot, Hofer & Pasquier 2000). These systems have been successfully used against several pests like the boll weevil, *Anthonomus grandis*; apple maggot, *Rhagoletis pomonella* and the codling moth, *Cydia pomonella* among others (Bostanian & Racette 2001).

One of the major developments in chemical ecology is the understanding that semiochemicals should not be used alone, but in combination with biological control agents. Probably the best example is the Push-Pull strategy, which involves “pushing” the insect away from the economic crops, and “pulling” them onto a trap crop where their population is reduced by a natural enemy (Foster & Harris 1997). This strategy is based in a profound understanding of chemical ecology, agrobiodiversity, plant-plant and plant-insect interactions (El-Sayed *et al.* 2006). In Eastern Africa Push-Pull is effectively used to control pests (Stemborers) and weeds affecting maize and sorghum crops. This strategy is based in the intercropping of the cereal crop with a repellent intercrop such as *Desmodium* (Push), and with an attractive trap plant such as Napier grass (Pull) planted as a border crop and this intercrop (Cook, Khan & Pickett 2007; Hassanali *et al.* 2008). Beside this, push-pull strategy has been applied in the control of *Helicoverpa sp.* in cotton, thrips on chrysanthemums and the Colorado potato beetle *Sitona lineatus* (de França *et al.* 2013).

In mosquitoes, preliminary “push-pull” studies have been done in semi-field systems or in small scale trials with promising results (Barbosa *et al.* 2010; Obermayr *et al.* 2012; Salazar *et al.* 2012), however, the knowledge about the semiochemicals and its role in oviposition behaviour remain incipient.

Table 1.1 Infusions, bacteria and semiochemicals that mediate oviposition response in mosquitoes

Source	Bacteria identified	Chemicals identified	Mosquito species	Response	Authors
Manure infusion			<i>Ae. aegypti</i> var. <i>queenslandis</i>	Attraction	(O'Gower 1963)
Log pond infusion Grass infusion		0-cresol <i>alpha</i> -Ethyl-p-methoxybenzyl alcohol Ethyl methylcarbamate Phenethyl methylcarbamate <i>alpha</i> -conidendrol tetraacetate N-Ethyl-o-veratrylamine 2,6 Dimethoxyphenol-ethylene oxide	<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i>	Attraction No effect	(Gjullin, Johnson & Plapp 1965)
Hay infusion	<i>Aerobacter aerogenes</i>	No identified	<i>Cx. pipiens quinquefasciatus</i>	Attraction	(Hazard, Mayer & Savage 1967)
	Pseudomonacea	Capric acid	<i>Culex restuans</i> <i>Culex pipiens</i> <i>Culex tarsalis</i> <i>Aedes aegypti</i>	Attraction	(Maw 1970)
Breeding site water	<i>Pseudomonas aeruginosa</i>	Intermediate metabolites of Capric and pelargonic acids	<i>Culex pipiens fatigans</i> <i>Culex molestus</i> <i>Aedes aegypti</i>	Attraction	(Ikeshoji 1966) (Ikeshoji, Saito & Yano 1975)
<i>Betula papyrifera</i> infusion		P-cresol	<i>Aedes triseriatus</i>	Attraction	(Bentley <i>et al.</i> 1979)
Purina® Laboratory chow infusion		Acetic acid Propionic acid Isobutyric acid Butyric acid Isovaleric acid Caproic acid	<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i>	Repellence	(Hwang, Kramer & Mulla 1980)
		Fatty acids from C ₅ to C ₁₃ Nonanoic acid*	<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i> <i>Ae. Aegypti</i>	Repellence	(Hwang <i>et al.</i> 1982)
Egg rafts		Erythro-6-acetoxy-5-hexadecanolide (AHD)	<i>Cx. quinquefasciatus</i>	Oviposition pheromone	(Laurence & Pickett 1982)
	<i>Bacillus cereus</i> <i>Pseudomonas aeruginosa</i>		<i>Ae. aegypti</i>	Stimulation/Attraction	(Hasselschwert & Rockett 1988)
Bermuda grass infusion		phenol 4 Methylphenol 4 Ethylphenol	<i>Cx. quinquefasciatus</i>	Stimulation/Attraction	(Millar, Chaney & Mulla 1992)

		indole 3 Methyl indole (skatole)			
Hay infusion Larval rearing water Field collected larval water		3 Methyl indole 4 Methylphenol 4 Ethylphenol indole phenol	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Moderate responses to infusions and chemicals	(Allan & Kline 1995)
Bermuda grass infusion			<i>Cx. quinquefasciatus</i> <i>Cx. tarsalis</i>	Attraction	(Isoe <i>et al.</i> 1995; Isoe & Millar 1995)
Oak leaf infusion			<i>Ae. albopictus</i> <i>Ae. triseriatus</i>	Stimulation/Attraction	(Trexler, Apperson & Schal 1998)
	<i>Bacillus cereus</i> <i>Pseudomonas aeruginosa</i>		<i>Ae. albopictus</i>	Stimulation/Attraction	(Pavlovich & Rockett 2000)
Larval conditioned water		Heneicosane	<i>Ae. aegypti</i>	Oviposition attractant pheromone	(Mendki <i>et al.</i> 2000)
Bacteria filtrates	<i>Bacillus cereus</i> <i>Pseudomonas fluorescens</i> <i>Bacillus thuringiensis</i> <i>Bacillus sphaericus</i> <i>Bacillus megaterium</i> <i>Azospirillum brasilense</i>		<i>Cx. quinquefasciatus</i>	Attraction/No effect	(Poonam, Paily & Balaraman 2002)
Larval rearing water Soil contaminated cotton towels Oak leaf infusion	<i>Psychrobacter immobilis</i> <i>Sphingobacterium multivorum</i> <i>Bacillus species</i>		<i>Ae. albopictus</i>	Attraction	(Trexler <i>et al.</i> 2003b)
		4 Methylphenol 3 Methyl indole	<i>Ae. albopictus</i>	Repellence	(Trexler <i>et al.</i> 2003a)
Grass (<i>Panicum maximum</i>) infusion			<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Stimulation/Attraction	(Santana, Roque & Eiras 2006)
Breeding site	<i>Stenotrophomonas maltophilia</i>		<i>An. gambiae s.s.</i>	Repellence	(Huang <i>et al.</i> 2006a)
Long chain fatty acids		Hexadecyl pentanoate, Tetradecyl heptanoate Tridecyl octanoate	<i>Ae. aegypti</i> <i>Ae. albopictus</i>	Repellence	(Sharma <i>et al.</i> 2008)
Bamboo leaf infusion White-oak leaf infusion	14 spp most of them Gammaproteobacteria 18 spp most of them Gammaproteobacteria	Nonanoic acid Decanoic acid Dodecanoic acid Tetradecanoic acid Tetradecanoic acid, methyl ester Hexadecanoic acid Hexadecanoic acid, methyl ester	<i>Ae. aegypti</i>	Attraction	(Ponnusamy <i>et al.</i> 2008a)

		Octadecanoic acid			
Midguts and breeding sites	<i>Vibrio metschnikovii</i> <i>Proteus sp.</i> <i>Micrococcus sp.</i> <i>Bacillus sp.</i> <i>Exiguobacterium sp.</i> <i>Comamonas sp.</i>		<i>An. gambiae s.s.</i>	Attraction	(Lindh <i>et al.</i> 2008)
Long chain fatty acids		Octadecyl propanoate, Heptadecyl butanoate, Hexadecyl pentanoate, Tetradecyl heptanoate	<i>Anopheles stephensi</i>	Repellence	(Sharma <i>et al.</i> 2009)
Conditioned water with <i>Notonecta maculate</i>		n-heneicosane n-tricosane	<i>Culiseta longiareolata</i>	Repellence	(Silberbush <i>et al.</i> 2010)
Synthetic analyses - Antennal transcriptome profiles		2-propyl phenol 4-methylcyclohexanol	<i>An. gambiae s.s.</i>	Attraction Repellence	(Rinker <i>et al.</i> 2013)

Substrates in bold are recognized as oviposition attractants

1.3.2. Microbial organisms as a source of volatile chemicals released from aquatic habitats

It has long been known that infusions made of manure, grass and food pellets attract gravid females of *Cx. quinquefasciatus* and *Ae. aegypti* to oviposition sites (O'Gower 1963; Hazard, Mayer & Savage 1967; Hwang, Kramer & Mulla 1980). In addition it has been shown that the attraction is mediated by bacteria communities present in the infusion (Hazard, Mayer & Savage 1967). In 1975 Ikeshoji and co-workers discovered that bacteria of Pseudomonaceae family were responsible for production of oviposition attractants for *Ae. aegypti* and *Cx. molestus* through metabolism of capric and pelargonic acid previously added to the water (Maw 1970; Ikeshoji, Saito & Yano 1975).

The importance of bacteria and their volatiles in the egg laying behaviour of some mosquito species has been corroborated in studies investigating the responses of gravid females to odours from bacterial cultures or filtrates. For instance, *Pseudomonas aeruginosa* and *Bacillus cereus* attracted gravid *Ae. aegypti* (Hasselschwert & Rockett 1988), whilst *P. fluorescens* and several different *Bacillus* species were attractive to *Cx. quinquefasciatus*, *Ae. aegypti* and *Ae. albopictus* (Pavlovich & Rockett 2000; Poonam, Paily & Balaraman 2002; Trexler *et al.* 2003a). In addition, indirect evidence for microbial involvement in production of oviposition attractants has been observed by reduction or elimination of the bacteria from test solutions (Benzon, Apperson & Clay 1988).

Again, for *An. gambiae s.l.* our knowledge on the involvement of microbial organisms in the oviposition site selection is extremely poor, limited to a hand full of papers of a restricted number of researchers implemented under artificial laboratory conditions without sufficient replication. Sumba and co-workers showed that gravid females laid 60% more eggs on wet papers above soil and water from natural habitats (containing bacteria and other microbes) than on wet paper above the same substrate that had been heat sterilized (Sumba *et al.* 2004). Otienoburu demonstrated that Lake Victoria water, which is one of the major sources of water in *An. gambiae s.l.* habitats around the shores of Lake Victoria, increased egg numbers compared with distilled water (Otienoburu *et al.* 2007). These studies two support the theory that microbials are involved in the oviposition choice of gravid females. The specific volatiles that mediate the responses have, in most studies, not been determined. Recently, positive responses from *An. gambiae s.s.* have been shown to bacteria isolated from *An. gambiae s.s.* breeding sites or their midguts. Six bacterial species stimulated a positive oviposition response (Lindh

et al. 2008). In contrast, Huang and collaborators tested a mixture of cultured bacteria also isolated from a natural larval habitat against bacteria-free nutrient agar and against expectations, gravid *An. gambiae s.s.* females avoided the bacteria and laid eight times more eggs in bacteria free nutrient agar than in mixed cultures of bacteria. Four bacteria species were presented in the mixture and when tested individually, only one species, *Stenotrophomonas maltophilia*, had a significant impact on the oviposition response by significantly lowering the number of eggs laid in the presence of the bacteria compared with bacteria-free controls (Huang *et al.* 2006a).

In order to test if microbial communities present in soil and water from a vibrant *Anopheles* breeding site produce volatiles chemicals that mediate oviposition behaviour in *An. gambiae s.s.* it is necessary to elucidate the role of microorganisms under standardized laboratory conditions to confirm if previous results can be replicated and under field conditions where it is expected that microbial communities are complex and affected by a large number of water quality parameters.

1.4 Rationale

Current vector control interventions in sub-Saharan Africa are insufficient to achieve malaria elimination in most areas due to residual transmission maintained by vectors with a more exophilic behaviour as well as due to the increasing threat of insecticide resistance (Ranson *et al.* 2011; Killeen 2014). Interventions that target outdoor vector populations without insecticides or with insecticides not used in the indoor environment are urgently needed to complement front-line interventions (Ferguson *et al.* 2010). Understanding the oviposition site selection of the major sub-Saharan malaria vectors of the *Anopheles gambiae* species complex could contribute significantly to the development of new and improved interventions by targeting the gravid females in search of an oviposition site and by targeting the most productive breeding sites rationalizing larval control interventions.

In addition, the identification of oviposition cues opens the possibility of exploring new perspectives to the control of *Anopheles* mosquitoes. The combination of oviposition attractants and larvicides might also be useful for the surveillance and control of vectors that feed outdoor. In this case gravid females would be directed to breeding sites previously treated with a larvicide that will kill the offspring of the egg-laying female. This “Attract and Kill” strategy provides a method of control that avoids traps becoming breeding sites. “Push–pull” schemes could also be implemented that use repellent

compounds to push vectors away from oviposition sites close to their hosts and attractive compounds that would guide them to specific traps for their capture.

To date the understanding of oviposition site selection in *An. gambiae s.l.* is very limited and restricted to experimental work implemented in the laboratory, mostly in small confined cages. Whether the behaviour in the laboratory can be extrapolated to their natural behaviour remains to be proven. In the natural environment of mosquitoes many cues interact. For example visual and chemical stimuli are likely to interact in a way that is unknown in the uncontrolled system. Factors like cage or cup size, different ways of presenting the substrate and the use of different strains of colony mosquitoes may all influence the oviposition response.

The evidence collected previously support the hypothesis that bacteria play a role in the oviposition choice of gravid *An. gambiae s.l.* females. There have been however, very few studies investigating the effect of bacteria and their volatiles on *An. gambiae s.l.* oviposition responses. To date all of the studies have been performed solely on bacteria collected from aquatic habitats containing larvae but no attempt has been made to compare the bacterial community of aquatic habitats that contain *An. gambiae s.l.* larvae with those that do not have larvae present in the water. There is no published work on the chemical headspace emitted from habitat water versus sterilized water or even from natural habitats that could provide evidence that chemical cues (from bacteria or in general) are involved in the habitat selection. Therefore, the study of oviposition behaviour in *An. gambiae s.l.* is fundamental to gain understanding on the ecology of this primary malaria vector and required to understand larvae distribution and survival in the field.

1.5 Overall aim and hypotheses

The aim of this thesis was to investigate the oviposition site selection in *Anopheles gambiae sensu lato*, the major malaria vectors in sub-Saharan Africa under controlled conditions and in field settings in Western Kenya.

The research was driven by the following hypotheses:

Hypothesis 1: Gravid *An. gambiae s.l.* females make choices when looking for a site to oviposit in. Females evaluate the suitability of a habitat using chemical cues from water bodies; these oviposition choices made by a gravid female benefit the offspring and cannot be modified by experience in one generation.

Hypothesis 2: Microbial communities present in soil and water from a vibrant *Anopheles* breeding site produce volatiles chemicals that mediate oviposition behaviour in *An. gambiae s.s.*

Hypothesis 3: Natural aquatic habitats without *Anopheles gambiae s.l.* larvae (controls) differ significantly from habitats that are well colonized by early instar larvae (cases) in their bacteria communities and in their profile of volatile chemicals released from the water.

Hypothesis 4: Specific physical, chemical and biological characteristics of natural aquatic habitats colonised by *Anopheles* larvae can be associated with the bacteria and chemical profiles and can assist in predicting habitat selection by gravid females in natural settings.

Chapter 2 Habitat discrimination by gravid *Anopheles gambiae sensu lato* – a push-pull system



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2.2. Please list the paper's authors in the intended authorship order

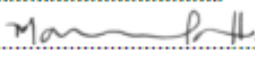
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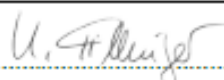
3. For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)

I contributed significantly in the conception of the idea and experimental design. I developed all protocols and implemented the experiments, analysed the data and wrote first draft of the manuscript.

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2.1 Abstract

Background: The non-random distribution of *Anopheles* larvae in natural habitats suggests that gravid females discriminate between habitats of different quality. Whilst physical and chemical cues used by *Culex* and *Aedes* vector mosquitoes for selecting an oviposition site have been extensively studied, those for *Anopheles* remain poorly explored. Here the habitat selection by *Anopheles gambiae sensu lato (s.l.)*, the principal African malaria vector, was investigated when presented with a choice of two infusions made from rabbit food pellets, or soil.

Methods: Natural colonization and larval survival was evaluated in artificial ponds filled randomly with either infusion. Dual-choice, egg-count bioassays evaluated the responses of caged gravid females to (1) two- to six-day old infusions versus lake water; (2) autoclaved versus non-autoclaved soil infusions; and assessed (3) the olfactory memory of gravid females conditioned in pellet infusion as larvae.

Results: Wild *Anopheles* exclusively colonized ponds with soil infusion and avoided those with pellet infusion. When the individual infusions were tested in comparison with lake water, caged *An. gambiae sensu stricto (s.s.)* showed a dose response: females increasingly avoided the pellet infusion with increasing infusion age (six-day versus lake water: odds ratio (OR) 0.22; 95% confidence interval (CI) 0.1-0.5) and showed increasing preference to lay eggs as soil infusion age increased (six-day versus lake water: OR 2.1; 95% CI 1.4-3.3). Larvae survived in soil infusions equally well as in lake water but died in pellet infusions. *Anopheles gambiae s.s.* preferred to lay eggs in the non-autoclaved soil (OR 2.6; 95% CI 1.8-3.7) compared with autoclaved soil. There was no change in the avoidance of pellet infusion by individuals reared in the infusion compared with those reared in lake water.

Conclusion: Wild and caged *An. gambiae s.l.* females discriminate between potential aquatic habitats for oviposition. These choices benefit the survival of the offspring. It could be demonstrated that the choice of habitat is mediated by chemical cues based on both preference and avoidance. These cues, if identified, might be developed for ‘push-pull’ strategies to improve malaria vector monitoring and control.

2.2 Background

Selection of suitable oviposition sites is a critical step in the life history of mosquitoes (Refsnider & Janzen 2010). This is a process whereby individuals select and occupy a non-random set of aquatic habitats. Habitat selection is of major importance for the interpretation of spatial and temporal distributions of populations, and for understanding intra and inter-specific relations that influence the abundance of individuals (Morris 2003; Rejmankova *et al.* 2005). Organisms without any parental care are likely to choose habitats based on a set of innate or learned cues in order to maximize the survival and fitness of their offspring (McCall & Eaton 2001; Rejmankova *et al.* 2005).

Mosquitoes utilize a wide range of aquatic niches for oviposition, including natural ponds, puddles, stream fringes, marshes, tree-holes and plant axils, man-made pits, drains, rice fields and containers (Laird 1988). Field studies have shown that mosquitoes are discriminating in selecting sites for egg deposition (Muirhead-Thomson 1945; Macan 1961) and that oviposition choices made by gravid females are a key factor in determining larval distribution (Bates 1940; Muirhead-Thomson 1940a; Muirhead-Thomson 1940b; Wallis 1954). Although different species are found in the same type of habitat, oviposition site selectivity is considerably species specific (Bentley & Day 1989). Immature stages of *Anopheles gambiae sensu lato*, the major malaria vectors in sub-Saharan Africa, are typically described as inhabiting very small, temporary sunlit pools and puddles without vegetation (Muirhead Thomson 1951; Gillies & De Meillon 1968; Gimnig *et al.* 2001; Minakawa, Sonye & Yan 2005). Reviews of the literature and recent research on larval ecology have however, shown that this is a gross oversimplification of the wide range of habitats colonised by this species (Holstein 1954; Fillinger *et al.* 2004; Fillinger & Lindsay 2011), a fact recognised over half a century ago by Holstein who reviewed the ‘extraordinary diversity of the breeding-places’ of *An. gambiae s.l.* (Holstein 1954). Numerous studies have described how the presence of *An. gambiae s.l.* larvae (Fillinger *et al.* 2004; Majambere *et al.* 2008; Fillinger *et al.* 2009b; Munga *et al.* 2009; Ndenga *et al.* 2011) and the capacity of individual habitats for generating adult mosquitoes (Bates 1940; Muirhead-Thomson 1945; Muirhead-Thomson 1951; Fillinger *et al.* 2004; Mutuku *et al.* 2006) differs markedly over space and time, yet statistical analyses of these surveys failed to reveal any risk factors that could consistently predict sites preferred by *An. gambiae s.l.* (Fillinger *et al.* 2004; Majambere *et al.* 2008; Fillinger *et al.* 2009b; Munga *et al.* 2009; Ndenga *et al.* 2011). This might lead to the conclusion that this species randomly deposits its eggs in a large

range of habitats and that the heterogeneous distribution of larvae results from the survival of larvae in the aquatic habitat (Muirhead-Thomson 1940a; Muirhead-Thomson 1940b) rather than the adults' choice.

Surprisingly, fully gravid malaria vectors looking for suitable larval habitats have been grossly understudied (Ferguson *et al.* 2010). Compared with the wealth of knowledge of the physical and chemical factors used by gravid culicines for selecting an oviposition site (Bentley *et al.* 1979; Hwang *et al.* 1982; Laurence & Pickett 1982; Beehler, Millar & Mulla 1992; Millar, Chaney & Mulla 1992; Beehler, Millar & Mulla 1994; Millar *et al.* 1994; Isoe *et al.* 1995; Isoe & Millar 1995; Mendki *et al.* 2000; Ganesan *et al.* 2006; Ponnusamy *et al.* 2008a; Seenivasagan *et al.* 2009; Ponnusamy *et al.* 2010a) those potentially used by the world's most deadly malaria vector remain almost unexplored. Whereas many publications recognize that the distribution of larvae between seemingly suitable aquatic habitats is probably due to the choice of the gravid female (Bates 1940; Kennedy 1942; Muirhead-Thomson 1945; Muirhead-Thomson 1951; McCrae 1984; Gu *et al.* 2006) and that this choice probably impacts on the fitness of her offspring there is little empirical evidence to support these assertions. Most recent research has evaluated the characteristics of aquatic habitats associated with the presence and absence of larvae (Gimmig *et al.* 2001; Fillinger *et al.* 2009b; Gouagna *et al.* 2012; Kweka *et al.* 2012; Munga, Vulule & Kweka 2013) but the understanding of the behaviour of gravid female *An. gambiae s.l.* when searching for an oviposition site remains, at best, sketchy (Bates 1940; McCrae 1984; Sumba *et al.* 2004; Huang *et al.* 2005; Huang *et al.* 2006b; Huang *et al.* 2007; Lindh *et al.* 2008; Sumba *et al.* 2008; Kweka *et al.* 2011).

Laboratory studies demonstrated that physical conditions of the aquatic habitats influence oviposition site selection in *An. gambiae s.l.* with females preferring dark backgrounds to pale ones, muddy water to clear water and fully hydrated substrates (Bates 1940; McCrae 1984; Huang *et al.* 2005; Balestrino *et al.* 2010). Turbidity has been suggested as an important physical cue for oviposition behaviour in *An. gambiae s.l.* although the evidence for this is contradictory (Ye-Ebiyo *et al.* 2003; Paaijmans *et al.* 2008).

Even less is known about the chemical cues and their interaction with physical factors. Water vapour is itself an attractant to gravid mosquitoes (Okal *et al.* 2013). It has been shown that gravid *An. gambiae s.l.* are sensitive to bacteria-derived odours (Sumba *et al.* 2004; Huang *et al.* 2006a; Lindh *et al.* 2008) which have been associated with increased (Sumba *et al.* 2004; Lindh *et al.* 2008) and reduced (Huang *et al.* 2006a) egg numbers

compared to sterile media in cage bioassays. Whilst over 20 putative oviposition semiochemicals have been suggested in the literature based on the analyses of bacteria- or habitat-derived volatile chemicals and electro-antennogram studies (Blackwell & Johnson 2000; Lindh *et al.* 2008) there is no report of any inducing a behavioural response in gravid females (increasing or decreasing the oviposition response) except for water vapour (Okal *et al.* 2013).

Here the oviposition behaviour of *An. gambiae s.l.* was explored to test the hypotheses that a gravid *An. gambiae s.l.* female evaluates the suitability of a habitat using chemical cues from water bodies, that oviposition choices made by a gravid female benefit the offspring and that this choice cannot be modified by experience in one generation.

Habitat selection by gravid *An. gambiae s.l.* was tested by presenting a choice of two infusions; one made with soil from an area where natural habitats occur frequently, and one made with rabbit food pellets. Rabbit food pellets are frequently used as diet for mosquito larvae in insectaries (Haeger & Provost 1964; Rasgon 2012) and infusions made of grass, hay and other plant material, including rabbit food pellets have shown to be attractive to a range of mosquito species and have been used in gravid traps (Nguyen, Su & Mulla 1999; Jackson *et al.* 2005a; Silver 2008; McPhatter & Debboun 2009). It was aimed to explore whether *Anopheles gambiae s.l.* might also be drawn to rabbit pellets infusion.

Natural colonization and larval survival was evaluated in artificial ponds filled randomly with either infusion. As a consequence of field observations, two choice egg count bioassays were used to explore the pattern of oviposition seen in the field. Experiments were designed to address the following questions: 1) Do gravid *An. gambiae s.l.* females discriminate between different habitats when searching for an oviposition site?, 2) Does the oviposition choice benefit the survival of their offspring?, 3) Are gravid females guided by preference and/or avoidance? 4) Are oviposition choices likely to be based on chemical and/or physical cues? and 5) Is the choice made by a gravid female influenced by her olfactory memory of her larval habitat?

2.3. Methods

2.3.1. Study site

Experiments were carried out at the International Centre for Insect Physiology and Ecology (ICIPE), Mbita, on the shores of Lake Victoria, Western Kenya (geographic coordinates 0° 26' 06.19" South; 34° 12' 53.13" East; altitude 1,137 meters above sea level). Mbita has a typical tropical climate; temperatures oscillate between 18-28 °C and there is an annual rainfall of 1,436 mm (based on data from ICIPE meteorological station for 2010-2012). Two rainy seasons occur annually, the long rainy season between March and June and the short rainy season between October and December. Malaria is endemic in the area and transmitted by three vectors, which are in order of their abundance: *An. arabiensis*, *An. gambiae s.s.*, and *An. funestus* (Minakawa *et al.* 2012).

2.3.2. Mosquitoes

Open-field trials were conducted with wild anopheline and culicine females that oviposited in tubs of water sunk into the ground. These were colonized within three days. Laboratory experiments were carried out with insectary-reared *An. gambiae s.s.* (Mbita strain) supplied by ICIPE's insectary and reared following standard operating procedures. Briefly, larvae were reared in round plastic tubs (diameter 0.6 m) filled with water from Lake Victoria and fed Tetramin® fish food twice daily. Larvae were collected randomly from several tubs on the day of experiment. Gravid mosquitoes were prepared by selecting 300 female and 300 male mosquitoes, two to three days old, from their rearing cages at 12.00 h and keeping them in 30×30×30 cm netting cages at 25-28°C and 68-75% relative humidity. To avoid mosquito desiccation, cotton towels (folded to 25x12 cm) were saturated with tap water and 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 h on the same day. Ethical approval from this procedure was obtained from the Observational/Interventions Research Ethics Committee of the London School of Hygiene and Tropical Medicine (LSHTM Ethics Ref: 8557). After feeding mosquitoes were provided with 6% glucose solution *ad libitum*. A plastic vial (25 ml) with a piece of paper towel folded into a wick was used to provide 10 ml of 6% glucose. The whole procedure was repeated 24 hours later to ensure a high proportion of mosquitoes fully gravid. After the first blood meal unfed female mosquitoes were removed from the cages. Fed female mosquitoes were kept together with males for two days after the second blood meal before using them in an experiment (i.e. females 4 - 5 days after first blood meal). In the afternoon (16.30 h) of

the day of an experiment 45-100 (depending on experiment and availability) visually presumed gravid females, that is with an enlarged, pale white abdomen, were selected from the holding cage. A small proportion of these mosquitoes were probably not gravid because most females needed two blood meals to be fully gravid and some are never gravid even after three feeds (Gillies 1958; Lyimo & Takken 1993). Whilst two meals were provided it could not be guaranteed that two meals were taken by all females. This might be the reason that not all mosquitoes exposed to oviposition medium in experiments laid eggs (responded), therefore the number of responders was smaller than the number tested. Non-responders were excluded from the analyses.

2.3.3. Experimental procedures

2.3.3.1. Do gravid An. gambiae s.l. females discriminate between different habitats when searching for an oviposition site?

To explore natural colonization of habitats by wild mosquitoes 20 artificial habitats were created by implanting 20 plastic tubs (40 cm diameter 20 cm deep) into an open-sunlit field during the long rainy season in May 2011. The tubs were placed in four lines of five tubs each 4 m apart (Fillinger, Knols & Becker 2003). Two different substrates were randomly offered in the artificial habitats. Half of the tubs (10) received 30 g of rabbit food pellets (Scooby® rabbit and rodent food, Nairobi) containing hay and grains from maize, wheat, barley, cotton, sunflower, soya bean meal, and traces of molasses, vitamins and minerals. The remaining half of the tubs (10) received 2 kg of dry soil taken from a field at ICIPE. Soil texture was characterized as a silty clay loam according to the United States Department of Agriculture (USDA) texture triangle (Brown) using the detergent method (Whiting *et al.*) to separate and quantify soil mineral particles of different size. A volume of 15 L of non-chlorinated tap water pumped from Lake Victoria, henceforth referred to as tap water, was added to each tub. The two treatments are henceforth referred to as pellet and soil infusion. To study the oviposition response of wild mosquitoes the tubs were monitored daily between 8.00 and 10.00 h by dipping five times per tub with a standard dipper (350 ml). Two different dippers were used for the two treatments to avoid contamination. Four dips were taken from the edge of the tubs and one from the middle. The content of each dip was emptied into a white plastic bowl and all early instars (1st and 2nd stage larvae) counted and recorded for both culicine and anopheline mosquitoes. All larvae and the water were returned to the respective tub. The tubs were followed for 16 days. Artificial habitats were searched for pupae and collected daily to prevent any emergence of potential disease vectors. Pupae

were allowed to emerge in cages in the laboratory and any anophelines emerging identified to species using morphological keys (Gillies & De Meillon 1968; Gillies & Coetzee 1987) and for specimens of the *An. gambiae* complex using the ribosomal DNA-polymerase chain reaction (PCR) method (Scott, Brogdon & Collins 1993).

Table 2.1. Summary details of dual choice egg-count bioassays to evaluate oviposition choices in *An. gambiae* s.s.

Dual choice cage egg-count bioassays sets	Treatments		No. of rounds (replicates)	Total no. of females that laid eggs for all rounds (total number mosquitoes set up)
	Control	Test		
Set 1: Pellet infusions	tap water	tap water	3	66 (75)
	tap water	2 day old pellet infusion		64 (75)
	tap water	4 day old pellet infusion		67 (75)
	tap water	6 day old pellet infusion		68 (75)
Set 2: Soil infusions	tap water	tap water	9	153 (225)
	tap water	2 day old soil infusion		161 (225)
	tap water	4 day old soil infusion		160 (225)
	tap water	6 day old soil infusion		171 (225)
Set 3: Vision versus olfaction in soil infusions	tap water	tap water	12	186 (220)
	tap water	6 day old soil infusion		150 (220)
	tap water	autoclaved 6 day old soil infusion		157 (220)
	autoclaved 6 day old soil infusion	6 day old soil infusion		169 (220)
Set 4: Olfactory memory – pellet infusions	<i>Tap water reared An. gambiae females</i>			
	tap water	6 day old pellet infusion	1	31 (45)
	<i>Pellet infusion reared An. gambiae females</i>			
	tap water	6 day old pellet infusion	1	37 (45)

2.3.3.2. Does the oviposition choice benefit the survival of their offspring?

Larval survival was assessed by introducing individual insectary-reared first instar *An. gambiae* s.s. larvae in infusions collected from the tubs set up in the field. Infusion samples were taken after 1, 6, 11 and 16 days. One hundred ml of infusion was collected from each of the 10 tubs per treatment and pooled per treatment in a plastic bottle. Tap water was used as a control. First instars were introduced in 100 ml plastic cups containing 50 ml of pellet infusion, soil infusion or tap water. Twenty larvae were exposed individually per treatment per day. Larvae were fed every second day with finely ground Tetramin® Baby fish food. Food was provided with a blunt toothpick that was first wetted in tap water and then dipped quickly, not more than 1 mm deep into the ground food, and then dipped onto the surface of the test water. Larval development was monitored daily and the time of death or time to pupation and emergence recorded. This experiment was implemented under ambient conditions in a semi-field system (80m²) with screened walls and a glass roof (Knols *et al.* 2002).

2.3.3.3. Are gravid females guided by preferences or avoidance?

Based on the analysis of the field data a series of two-choice egg-count bioassays were designed to investigate if the response of wild gravid females observed in the field was based on avoidance or preference of an infusion or both.

Gravid females were selected from insectary cages and transferred individually to 30x30x30 cm cages. In each cage two glass cups (Pyrex®, 100 ml, 70 mm diameter), surrounded by tightly fitting aluminium cylinders, so that mosquitoes could see only the water surface, were filled with 100 ml of either the control or test medium and placed in diagonal corners of the cage. Prior to use cups and cylinders were cleaned with detergent, then autoclaved and kept in an oven at 200°C for at least two hours. The position of oviposition cups containing the test medium was alternated between adjacent cages to control for possible position effect. The placement of the first test cup was randomly allocated for one of the four cage corners in the first cage. Subsequent test cups were rotated in the next possible corners in a clockwise direction relative to the position of the preceding cup. One control cup was added in each cage diagonal to the test cup to complete a two choice set up. The experiments were carried out in makeshift sheds (Figure 2.1) that exposed the mosquitoes to ambient light, temperature and relative humidity but protected the cages from rain.



Figure 2.1. Location of egg-count bioassays. A. Sheds (10 m long \times 5 m wide \times 2.8 m high) with walls made of reed mats and a roof made of translucent corrugated polycarbonate sheets. B. Interior of a shed. In each shed two tables hold up to 25 standard cages each, allowing 40 cm of space between adjacent cages.

Two sets of experiments were carried out consecutively (Table 2.1, Set 1 and 2). In the first set oviposition choice was evaluated for 2, 4 and 6 day old pellet infusions compared with tap water. In the second set the oviposition choice was evaluated for 2, 4 and 6 day old soil infusions compared with tap water. In both sets of experiments internal controls were used to validate the two choice experiment. Here equal numbers of cages were set up where both cups in a cage contained tap water and were labelled randomly as control and test cup, assuming that gravid females lay eggs in both cups in an equal proportion.

Infusions were prepared in a similar way as for the field tests. Fifteen litres of tap water were either incubated with 30 g of pellets to prepare a pellet infusion or incubated with 2 kg of soil to prepare a soil infusion. Infusions were prepared in a plastic tub (40 cm diameter 20 cm depth) 6 days, 4 days and 2 days before the day of experiment in order to test all ages in parallel. Tubs were covered with mosquito proof netting and kept in makeshift sheds at ambient conditions but protected from rain. Experiments were implemented over 3-9 rounds depending on the availability of gravid females and the response rate per round (Table 1) with fresh batches of infusions and different batches of mosquitoes for every round. On the day of experiment infusions were sieved through a clean piece of cotton cloth to remove large debris remaining from the pellets or soil.

Fifteen to 25 replicate cages per treatment were set up per round. A single gravid female was introduced per cage at 17.30 h. The next morning between 8.00- 9.00 h the absence or presence and the number of eggs was recorded for the control and test cup in each cage.

Turbidity, conductivity, dissolved oxygen and pH were measured in five cups per treatment in four different batches of pellet, soil infusions, and tap water using a turbidity meter (TURB 355IR, WTW Germany) and a multimeter (Multi 340i, WTW, Germany). In addition one batch of pellet, soil infusion and tap water was tested for Ammonium (NH_4^+), carbonate hardness, total hardness, nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) content using Aquamerck® test kits from the compact laboratory for water testing (Aquamerck® No.111151, Germany).

2.3.3.4 Are oviposition choices likely to be based on chemical cues?

Soil infusions differed strongly in colour and turbidity from tap water. To assess if the oviposition response observed was based on visual or chemical cues a third set of dual choice egg-count bioassays were implemented with six day old soil infusions (Table 2.1, Set 3) comparing the relative attractiveness of autoclaved and non-autoclaved infusion (Sumba *et al.* 2004; Ponnusamy *et al.* 2011). The experiment followed the same experimental procedures as described above. After filtering the infusion through a cloth on the day of experiment, the infusion was split in two equal volumes and half autoclaved at 120°C for 20 minutes to kill bacteria potentially involved in releasing oviposition semiochemicals (Sumba *et al.* 2004; Ponnusamy *et al.* 2010b) and to reduce the amount of volatile chemicals from the solution whilst maintaining the colour and turbidity of the infusion. After autoclaving the infusion was left to cool to ambient temperature before setting up the cage bioassays. The oviposition choice of individual gravid females was evaluated for six day old soil infusion versus tap water, autoclaved six day old soil infusion versus tap water and for autoclaved versus non-autoclaved six days old infusion.

In order to confirm that autoclaving sterilized the infusion, samples (1 ml) of both infusions were taken for bacterial cultures. Samples were serially diluted (tenfold) two times in distilled water. After dilution, 100 µl of each of the x1 (undiluted), $\times 10^{-1}$ and $\times 10^{-2}$ dilutions was spread separately onto the surface of duplicate Lysogeny Broth (LB) agar-plates (LB Lennox-Fisher Scientific) (Bertani 2004). Plates were incubated overnight at 30°C and the presence of colonies recorded.

The same physical and chemical parameters were measured for the autoclaved infusion as described above for the non-autoclaved pellet and soil infusions.

2.3.3.5. Is the choice made by the gravid female influenced by her olfactory memory of her larval habitat?

A fourth set of experiments (Table 2. 1, Set 4) was designed to assess the possibility that a gravid female's choice for an oviposition site might be influenced by her olfactory memory of her larval habitat, as has been suggested for culicine species (McCall & Eaton 2001; Kaur, Lai & Giger 2003).

To test this, approximately 2000 *An. gambiae s.s.* eggs were dispensed in 1.5 L of two-day old pellet infusion and another 2000 eggs in tap water and reared under the same conditions to the adult stage. The infusion and tap water in the rearing pans was replaced every two days with fresh two-day-old infusion or tap water until all surviving larvae pupated. Larvae were fed with Tetramin® fish food twice daily following routine insectary procedures. Pupae were collected in a cup with 100 ml of rearing water and placed in 30x30x30 cm cages for emergence. Gravid females for cage bioassays were obtained as described above.

Dual choice cage bioassays were carried out in parallel with gravid *An. gambiae s.s.* reared in the infusion and gravid *An. gambiae s.s.* reared in tap water. A single mosquito was offered a choice between six-day-old pellet infusion or tap water. Forty-five replicates were set up in parallel for both treatment groups as described above.

2.3.4. Sample size considerations

The sample size (number of responders) in the four sets of cage experiments differed for a number of reasons. Due to adverse climate conditions affecting the mosquito supply during the pellet infusion bioassays the production of colony-reared mosquitoes was low. Nevertheless, two-sample comparison of proportions power calculation showed that 66 responders in each arm in the pellet infusion bioassays (Table 2.1, set 1) was sufficient to detect a 23% increase or decrease in the proportion of eggs laid in the treatment compared with the tap water only experiment with 80% power at the 5% level of significance. The effect of the pellet infusion observed on oviposition response was much stronger than 23%. In the soil infusion experiments (Table 2.1, Set 2 and Set 3), a minimum of 150 responders in each arm were analysed. This was sufficient to detect a 15% increase or decrease in the proportion of eggs laid in the treatment as compared to the tap water only experiment at the same power and significance level. This level of

accuracy was deemed appropriate for investigating significant behavioural cues affecting the oviposition choice. The evaluation of olfactory memory required the mosquitoes to be reared in pellet infusion where larval mortality was nearly 98%. Therefore, only 45 females could be tested, out of which only 31 and 37 responded in the two treatments. (table 2.1, Set 4). The hypothesis for this experiment was that the preference of gravid females could be changed and therefore at least double the proportion of eggs laid in pellet infusion by infusion reared females as compared to the tap water reared females. With 31 responders in each arm the experiment was powered (80%) to detect a change in the proportion of 35%.

2.3.5 Statistical analyses

All data were analysed in R statistical software version 2.13.1 (R Development Core Team 2011). The one sample proportion test function was used to estimate the 95% confidence intervals (CI) for the proportion of larvae surviving in pellet infusion, soil infusion and tap water. Pupation time of larvae exposed to different treatments was calculated using the following formula: $(Ax1)+(Bx2)+(Cx3)...(Gx10)/\text{Total number of pupae collected}$, where A, B, C...G are the number of pupae collected on day 1,2,3 to 10. Dual choice egg-count bioassays were analysed using generalized linear models (glm-function) with a quasibinomial distribution fitted to account for the overdispersion. In the first three sets of experiments the proportion of eggs laid in test cups in the cages with equal treatments (tap water in both cups) were compared with the proportion of eggs laid in test cups in cages with two different treatments. It was hypothesised that gravid females presented with an identical treatment would lay in both cups in an approximately equal proportion ($p=0.5$). The statistical analysis aimed to reveal if the test treatment of interest (e.g. infusions of different age) received an increased or decreased proportion of the total number of eggs laid when compared to the tap water only treatment. Therefore, the treatment choice (e.g. tap water only cages, cages with infusion versus tap water) and the round of experiment were included as fixed factors to analyse their impact on the outcome (proportion of eggs laid in test cup). A similar analysis was used for the fourth set of experiments to compare the proportion of eggs laid in test cups (pellet infusion) by gravid females that were reared in hay infusion during their larval development compared to the proportion of eggs laid in test cups by gravid females that were reared in tap water. The mean proportion of eggs laid in test cups in different treatments and their 95% CIs were calculated as the exponential of the parameter estimates for models with no intercept included. Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

2.4 Results

2.4.1. Gravid *An. gambiae s.l.* females discriminate between habitats when searching for an oviposition site

Mosquitoes oviposited in the artificial ponds shortly after they had been set up since early instar larvae were found from day three and larvae hatch approximately 24-48 hours after eggs are laid. Ponds with pellet infusion were colonized exclusively and in high densities by culicine mosquitoes. No anopheline larvae were detected over the 16 day observation period. In sharp contrast, early instar anophelines were consistently found from day 3 to day 16 in the soil infusion ponds (Figure 2.2). Based on the pattern of larval abundance, peak oviposition occurred 6-10 days after setting up the ponds. Anophelines nearly always occurred in higher densities than culicines. Anopheline larval densities are naturally relatively low, with approximately 1-3 larvae/dip in natural habitats in the study area (Fillinger *et al.* 2004). In the present study an average of 10 (95% CI 5-18) early instar larvae/dip was recorded, indicating that the soil infusion ponds were a highly favourable habitat.

All pupae collected from the artificial habitats belonged to the *Anopheles gambiae* complex. PCR-based species analysis revealed that nearly all the wild *An. gambiae s.l.* were *An. arabiensis* (98%, 49/50).

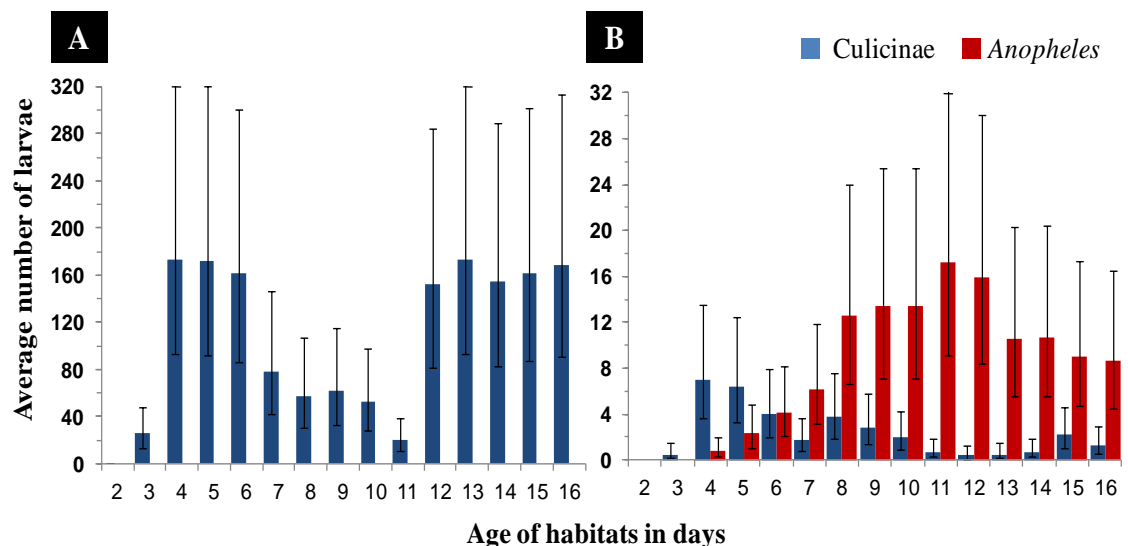


Figure 2.2. Natural colonization of artificial habitats. Daily average of early instar larvae in (A) Pellet infusions (B) Soil infusions.

2.4.2. The female's choice of oviposition site benefits the survival of her offspring

Anopheles gambiae s.s. larvae survived equally well in soil infusion and tap water irrespective of the age of the infusion. In contrast, larvae placed in pellet infusion only survived in the one day old infusion in similar numbers but survival was reduced by over 60% ($P<0.001$) in pellet infusions six days and older compared with tap water or soil infusions of the same age (Figure 2.3). Mean pupation time for survivors did not significantly differ between treatments or ages of the infusion and was on average 7.5 days (95% CI 6.6-8.3).

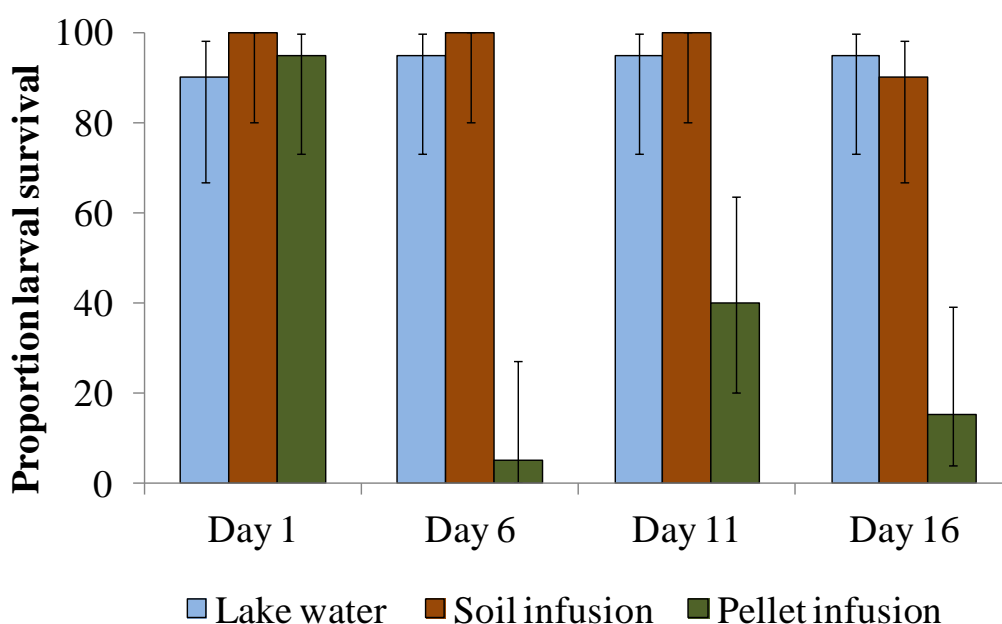


Figure 2.3. Survival of *An. gambiae s.s.* larvae to the pupal stage kept in different infusions or tap water. Error bars show 95% confidence intervals.

2.4.3. Gravid female *An. gambiae s.s.* show avoidances and preferences when selecting an oviposition site

An approximately equal proportion of females laid eggs in test and control cups when a choice of tap water in two separate cups was provided. Fewer females laid their eggs in pellet infusion as it aged, whilst for soil infusion the opposite was the case with an increasing proportion of females laying eggs in soil infusion as it aged.

Similar results were seen for the proportions of eggs laid. The distribution of eggs between tap water and two day old pellet infusion did not significantly differ from the distribution between the two cups with tap water only. Pellet water however became unattractive from day 4 (Figure 2.4). It was 85% less likely for an egg to be laid in the test cup in the treatments that contained six-day-old pellet infusion versus tap water than it was when both cups contained tap water. In contrast to the pellet infusion, larger proportions of eggs were laid in the test cups with increasing age of the soil infusion. An egg was more than twice as likely to be laid in the test cup in the treatments that contained six-day-old soil infusions compared with tap water than it was when both cups contained tap water (Figure 2.4).

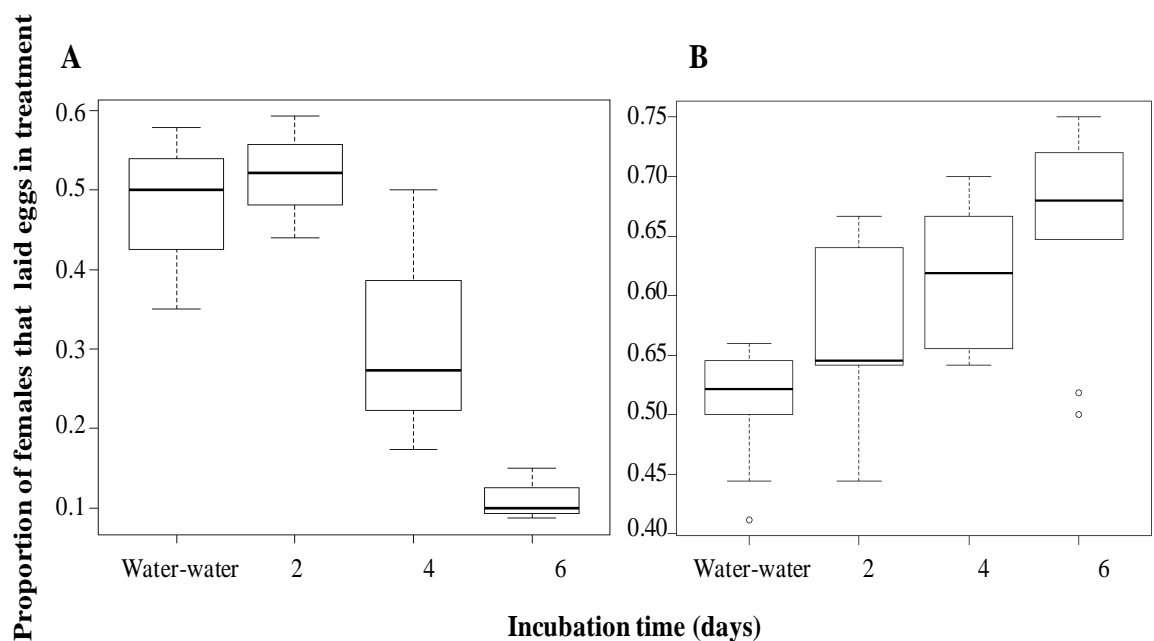


Figure 2.4. Proportion of gravid *An. gambiae* s.s. laying eggs in infusions of different ages compared with control water. (A) Pellet infusion experiment (B) Soil infusion experiment. Dark line in the middle of the boxes represents the median response rate of the gravid females to the test cup.

On average, individual females laid 63 eggs (95% CI 60-65) (Figure 2.5) irrespective of the experiment and treatment. Notably, 18% (95% CI 11-26%) of gravid females laid eggs in both cups provided in a cage, a behaviour known as skip oviposition in other mosquito species (Colton, Chadee & Severson 2003) but rarely reported for *An. gambiae* s.s. (Chen, Fillinger & Yan 2006). The average number of eggs laid by skip-ovipositing females and by females that chose only a single cup was similar. Whilst the percentage of skip-ovipositing females was similar in all treatments with two equal tap water

choices and in all soil infusion treatments this behaviour was affected by the pellet infusion. Only a few *An. gambiae s.s.* females skip-oviposited in the four and six day old pellet infusion treatments (6%, 95% CI 3-9%).

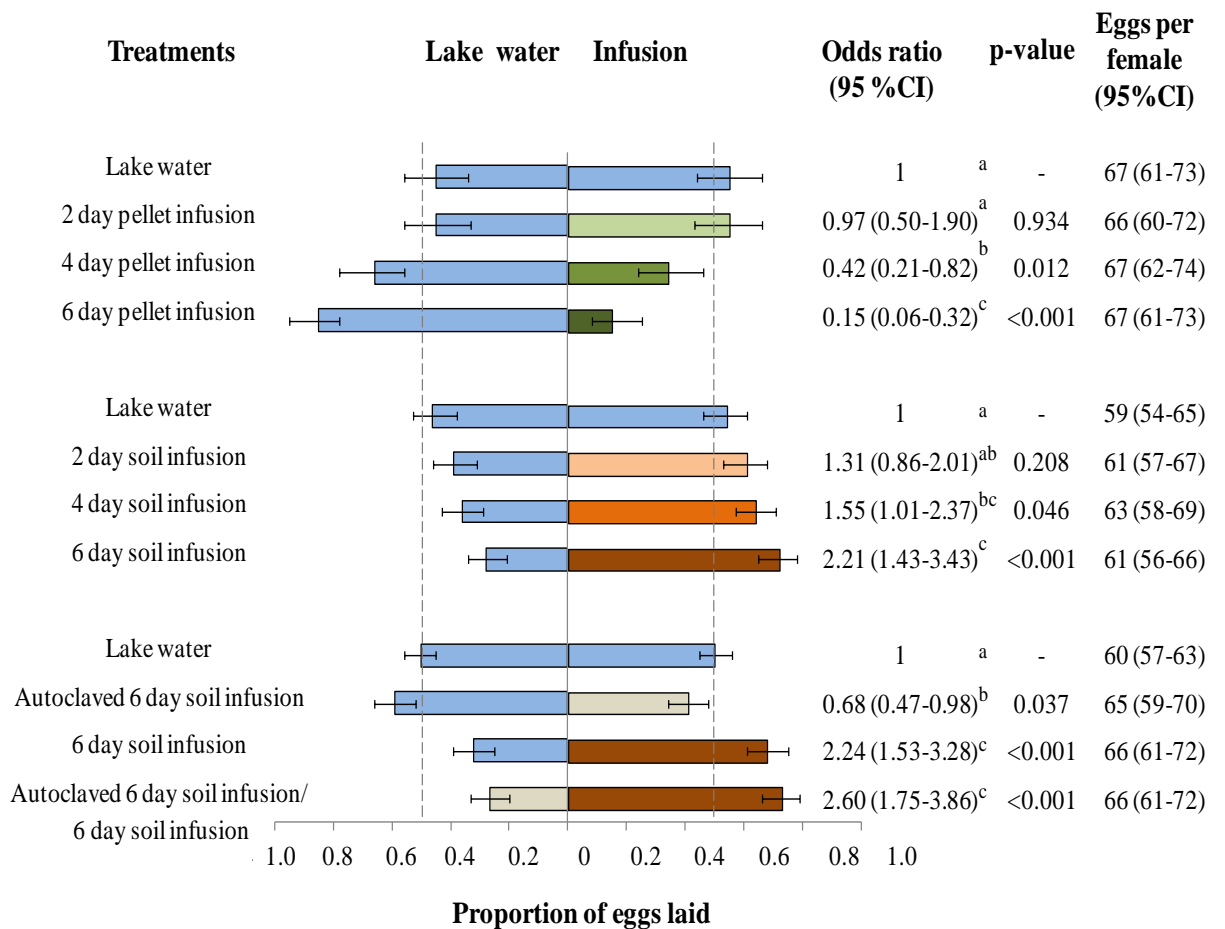


Figure 2.5. Oviposition response of caged *An. gambiae s.s.* to pellet and soil infusions of different incubation times and non-autoclaved and autoclaved 6 day soil infusion. P values are given for comparisons between treatments containing lake water in both cups with treatments that contained infusion in the test cup. Multiple comparison of treatments for each set of experiments: Treatments denoted with the same letter are not significantly different.

Pellet and soil infusions differed in key physical and chemical parameters. All pellet infusions had a strong smell to the human nose, were low in turbidity and had a slightly green colour but differed little in appearance compared with tap water in the oviposition cups. In contrast, soil infusions did not have any smell to the human nose, were light brown in colour and turbid, providing a strong visual contrast to the tap water. Pellet infusions were also characterized by relatively high conductivity, low pH and oxygen deprivation. In contrast conductivity of soil infusions was approximately half that of

pellet infusions, was saturated with dissolved oxygen and had a higher pH (Table 2.2). The variability of these measures between infusions of different incubation times within a treatment group was relatively low and does not appear to explain the differences in the behavioural responses. The only factor that changed over time was turbidity in the soil infusion and notably the most preferred six day old infusion was less turbid than the others.

The increased carbon and total hardness of the pellet infusion corresponded with the increased conductivity levels and the high ammonium and phosphate levels compared with the soil infusion (Table 2.2).

2.4.4. Chemical cues from the infusions are responsible for the oviposition choice in cage bioassays

Since soil infusions differed in appearance from the tap water, an additional set of experiments was carried out to evaluate whether the attractiveness of this infusion was due to visual cues. Cage experiments with two equal choices of tap water confirmed an equal distribution of eggs between control and test cup. Notably, when gravid females had a choice between tap water and autoclaved soil infusion a slight avoidance of the autoclaved infusion was observed (Figure 2.4). The autoclaving may have altered some chemical properties of the infusion accounting for this slight repellent effect.

The oviposition preference for six day old soil infusion compared with tap water was also confirmed in this set of experiments with nearly identical odds ratios as before of 2.2. The preference for the six day old infusion was confirmed when given a choice between autoclaved and non-autoclaved infusions of similar colour and turbidity. The odds of an egg being laid in the six day old infusion when provided with autoclaved infusion of the same age as an alternative choice was approximately 40% higher (2.6) than it was when the alternative was tap water (2.2). This increase can be explained by the repellent effect of the autoclaved infusion which in addition to the pulling effect of the soil infusion now adds a pushing effect of the autoclaved infusion. Nevertheless, the difference in the two odds ratios was not significant.

Table 2.2. Physical and chemical properties of pellet and soil infusions

Parameter	Oviposition substrates in choice experiments							
	Tap water	Pellet infusions			Soil infusions			
		2 days	4 days	6 days	2 days	4 days	6 days	Auto-claved
Turbidity (NTU)	1 (0.6-1.4)	22 (20-23)	17 (14-19)	25 (20-29)	222 (197-248)	97 (92-102)	73 (61-84)	137 (108-166)
Conductivity (uS/cm)	107 (105-110)	477 (462-491)	553 (547-559)	543 (532-555)	173 (171-176)	207 (203-212)	237 (233-242)	266 (258-274)
Dissolved oxygen (mg/l)	4 (2.7-5.3)	0.3 (0.21-1.4)	0.7 (0.9-1.4)	0.3 (0.2-0.4)	5.3 (5.0-5.6)	6 (5.7-6.3)	6.3 (6.0-6.6)	4.8 (4.6-5.1)
pH	8.1 (7.9-8.1)	6.3 (6.2-6.3)	6.7 (6.5-6.9)	7.4 (7.3-7.5)	7.7 (7.6-7.7)	7.9 (7.8-7.9)	8 (7.9-8.0)	8.9 (8.8-8.9)
Ammonium (mg/l)	0				5			0
Nitrate (mg/l)	10				10			10
Nitrite (mg/l)	0				0			0.025
Phosphate (mg/l)	0				3			1.5
Carbonate hardness (mmol/l)	0.1				3.9			2.7
Total hardness (mmol/l)	0.1				1.5			0.7

Results from this set of experiments suggest that chemical cues are involved in the oviposition responses observed. If the preference for the 6 day old soil infusion over tap water was based on turbidity and/or colour of the infusion alone a similar response in the choice tests with autoclaved versus non-autoclaved infusion should have been seen as in the choice tests with tap water versus autoclaved infusion. Due to the slight repulsion of the autoclaved infusion the odds of finding an egg in the non-autoclaved six day old infusion should have been approximately 1.3 (decreased choice for autoclaved infusion by 30% or increased choice of fresh infusion by 30%). Nevertheless, the remaining odds of 2.2 can only be explained by chemical cues being either involved in attracting the female from a short distance or stimulating the female to lay eggs on contact with water. Physical and chemical water parameters were similar for autoclaved and non-autoclaved soil infusions. Bacteria cultures from autoclaved infusions confirmed that samples did

not contain any bacteria that could grow on LA plates as opposed to the non-autoclaved infusion where colonies of at least 3 different morphologies were observed.

2.4.4. The oviposition choice of the gravid female is not influenced by her olfactory memory of her larval habitat

Rearing *An. gambiae s.s.* in two to four day old pellet infusion did not alter their oviposition response towards the infusion ($P=0.392$). Gravid females reared in tap water and gravid females reared in pellet infusion show an equally strong avoidance of the six day old pellet infusion provided in choice experiments.

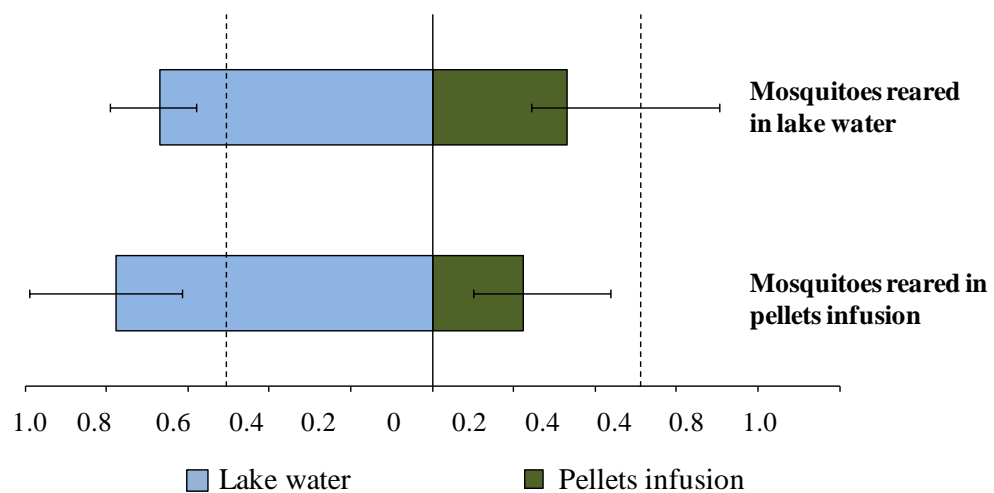


Figure 2.6. Egg laying responses of *An. gambiae s.s.* reared in tap water or in pellet infusion to tap water and pellet infusion.

2.5 Discussion

The results confirm that wild and caged *An. gambiae s.l.* females can discriminate between potential aquatic habitats for oviposition and make clear choices when presented with contrasting oviposition media. These choices benefit the survival of the offspring. Although the experimental design does not allow to conclude whether the stimuli acted over a distance (attractants and repellents (Dethier, Barton & Smith 1960)) or on contact with the oviposition medium (stimulants and deterrents (Isoe, Millar & Beehler 1995)) it could be demonstrated that the choice of breeding site is guided by both avoidance and preference. The exclusive way however, in which the artificial ponds were chosen in the field, where they were set up relatively close to each other, suggest

that these characteristics were detected by both culicines and anophelines from a distance rather than on contact.

Muddy water has previously been suggested to increase oviposition response of gravid *An. gambiae* s.s. in cages when offered together with clear water (McCrae 1984), however here presented cage experiments could not confirm this observation. The difference in turbidity between tap water and infusions cannot explain the avoidance and preference observed at short range in the cages. Two day and 6 day old pellet infusions did not differ in their turbidity but significantly differed in the oviposition response they elicited. Similarly, all soil infusions should have elicited equally strong responses from gravid females if turbidity was an important oviposition cue at short range. On the contrary, the six day old soil infusion remained equally preferred for oviposition when tested against a turbid and autoclaved infusion than when tested against clear tap water. The results suggest that chemical and not visual cues were responsible for the responses observed in the cage experiments. The previously published preference of muddy water (McCrae 1984) may have been based on chemical cues associated with the muddy water which was taken from a natural habitat. The possibility however, that visual cues played a role in the selection of oviposition sites by wild mosquitoes in field experiments especially when searching for water bodies from a distance cannot be entirely excluded.

It is likely that the chemical cues used by mosquitoes to avoid pellet infusions and to prefer soil infusions were of microbial origin, which is supported by the lack of attraction of the autoclaved soil infusion compared to clear tap water. It is most likely that the observed oviposition choices were rooted in the water quality of the habitat and consequently the associated microorganisms and chemicals in the water. The physical and chemical water parameters measured for the two infusions suggest that they represented aquatic habitats in different stages of decay. Contrary to the expectation at the onset of the experiment, pellet infusions created a habitat type in a severe state of decomposition. High ammonia and phosphate levels are characteristic of recently inundated organic material (Palmer 2002). The odour of the pellet infusion is associated with fermentation of organic material by facultative and anaerobic bacteria. This leads to depletion of the oxygen supply and a decrease in pH as a result of accumulation of organic acids in the water (Gerhardt 1959; Sigeo 2005; Cunha *et al.* 2010). The soil infusion had the characteristics of a less nutrient rich habitat containing relatively little organic matter. This limits the removal of oxygen by aerobic heterotrophic microorganisms and hence the water column will stay aerobic (Sigeo 2005).

Anaerobic fermentation products of organic matter have been previously shown to be highly attractive to a number of gravid culicine species such as *Culex stigmatosoma* (formerly *peus*) (Gerhardt 1959), *Culex quinquefasciatus* (Kramer & Mulla 1979; Millar, Chaney & Mulla 1992; Mboera *et al.* 1999; Burkett-Cadena & Mullen 2007; McPhatter & Debboun 2009), *Culex pipiens* (Jackson *et al.* 2005a), *Aedes aegypti* (Santana, Roque & Eiras 2006; Ponnusamy *et al.* 2010b) and *Aedes albopictus* (Trexler, Apperson & Schal 1998; Zhang & Lei 2008). These infusions have been associated with a range of bacteria such as *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* (Hazard, Mayer & Savage 1967; Ikeshoji, Saito & Yano 1975; Hasselschwert & Rockett 1988) and volatile chemicals produced by them including 4-methylphenol, 3-methylindole, carboxylic acids and their methyl esters (Millar, Chaney & Mulla 1992; Allan & Kline 1995; Trexler *et al.* 2003a; Ponnusamy *et al.* 2008a). It is likely that similar factors were responsible for the strong repellent/deterrent effect on gravid *An. gambiae s.l.* females.

Most stagnant water bodies will show increasing signs of decomposition over time but the speed and extent of this will depend largely on habitat quality (Ruppel, Setty & Wu 2004). Therefore, it is argued that habitat age or permanence alone is not a good predictor for the oviposition response of *An. gambiae s.l.* as has been suggested (Munga, Vulule & Kweka 2013). For example the content and input of organic matter, source of water and frequency of fresh water inflow will affect the composition of the biotic community and chemical and physical characteristics of an aquatic habitat (Palmer 2002; Ruppel, Setty & Wu 2004; Sigeo 2005). This might explain why in some environments semi-permanent and permanent habitats are just as well colonised as temporary habitats traditionally thought to be the preferred *An. gambiae s.l.* habitats (Gillies & De Meillon 1968; Fillinger *et al.* 2004; Minakawa *et al.* 2012). Habitats made of pellet infusion were avoided by anophelines from an early habitat age, whilst interestingly, the highest preference of the soil infusion was recorded on day six in the laboratory and between day six and 10 in the field after the habitats were well established, contradicting the idea that *An. gambiae s.l.* is a pioneer species colonizing temporary habitats immediately after their occurrence (Gillies & De Meillon 1968).

Typically, it is reported that *An. gambiae s.l.*, although largely a generalist, is not found in heavily polluted waters (Symes 1940; Holstein 1954). Hancock (Hancock 1930) further observed that *An. gambiae s.l.* avoided water with a low pH when it was also

accompanied with high organic matter content. Addition of freshly cut vegetation (i.e. grass cuttings) to aquatic habitats has also been shown to prevent the larval development of *An. gambiae s.l.* [16]. The results from the experiments with pellet infusions support these observations. On the other hand, there have been recent reports of *An. gambiae s.l.* colonizing polluted habitats especially in urban areas (Awolola *et al.* 2007; Castro *et al.* 2010; Kudom, Mensah & Agyemang 2012). Clearly, the degree of avoidance or acceptance of a polluted habitat by *An. gambiae s.l.* depends on the extent and nature of pollution (Holstein 1954). Results show that two-day-old pellet infusions were not rejected by anophelines and even four-day-old infusions still received a considerable proportion of the oviposition responses despite their adverse water characteristics. This supports the idea that *An. gambiae s.l.* has a very high tolerance level of what they accept as oviposition sites, especially in the absence of better alternatives in close vicinity as is often the case in urban environments and in contrast to the here presented field experiment where good habitats were offered right next to the unfavoured ones.

Importantly, *An. gambiae s.l.* appears to have an innate propensity to avoid specific chemical cues that were emitted from the pellet infusion. Rearing *An. gambiae s.s.* from egg to pupae in this infusion did not alter this behaviour. Gravid females that had experienced the pellet infusion during larval development avoided the infusion for oviposition as much as the females that had no prior experience of it. This suggests that the environment in which *An. gambiae s.s.* develop as larvae does not determine the preferred oviposition site when they return to lay eggs. This is in contrast to published work on *Cx. quinquefasciatus* where it was demonstrated that rearing the larvae in an infusion made from guinea-pig faeces cancelled their innate preference for a hay infusion (McCall & Eaton 2001).

The cage bioassays with individual gravid females allowed a number of interesting observations that are rarely reported since the majority of studies with *An. gambiae s.s.* have been done with groups of mosquitoes where the actual number of females laying per cage is unknown (McCrae 1984). The occurrence of skip oviposition in gravid *An. gambiae s.s.* and how this is affected by chemical cues was demonstrated. Furthermore, the design revealed that the mean number of eggs laid per female in a cage was similar irrespective of the experiment and treatment; only the distribution between cups differed when two different choices were presented. This indicates that gravid females did not retain their eggs in the presence of an unfavoured substrate when they were offered a suitable alternative choice. It also shows that the preferred soil infusion did not stimulate

individual females to lay more eggs than they would do in tap water. Testing individual females also excludes potential aggregation effects. Whilst from the field experiments it might have been possible that gravid females selected habitats that already received eggs from conspecific females, cage bioassays with individual females showed the same avoidance and preference behaviour than observed in the field confirming that conspecifics alone cannot explain the observed choice.

The potential involvement of microbial activity in breaking down organic matter and producing semiochemicals that impact on the oviposition responses of gravid *An. gambiae s.s.* was deduced partly by the lack of attraction of *An. gambiae s.s.* to a sterile soil infusion. This must however be interpreted with caution since autoclaving the infusion might not only have killed the microbes but affected the chemistry of the resulting infusion, possibly altering the response of gravid mosquitoes by chemical changes rather than biological changes (Ponnusamy *et al.* 2010b).

Batch-to-batch variations were recorded in the response of gravid mosquitoes to the infusions, resulting for example in some rounds showing a high preference and other rounds only a moderate preference for the soil infusion. This variation can be attributed to differences in the quality and amounts of odorants released from the infusions and stochastic events. Fresh infusions were prepared for every test round with different batches of pellets and soil. Especially, for the soil it is highly likely that there were differences in the soil condition as well as differences in the species composition of the microbial community associated with the natural materials over time. It has been previously shown that natural infusions can be an inconsistent source of odorants for oviposition site seeking mosquitoes and therefore every batch needs to be verified to be behaviourally active before it can be used for subsequent experiments (Ponnusamy *et al.* 2010b). Ideally, if semiochemicals were to be used for monitoring and/or controlling gravid malaria vectors specific chemically defined oviposition cues would be preferred over natural infusions to ensure a consistent response in gravid females either pushing them away from human population (Seenivasagan *et al.* 2010; Siriporn & Mayura 2012) or pulling them towards a gravid trap (Seenivasagan *et al.* 2009; Seenivasagan, Sharma & Prakash 2012).

Whilst the observed avoidance behaviour towards the organically rich pellet infusion was strong and in the same range as reported for other species in response to unfavourable chemical cues (Seenivasagan *et al.* 2010; Siriporn & Mayura 2012;

Tennyson *et al.* 2012), the observed preference in the cages for the soil infusion was relatively weak and it is questionable whether it could compete with other suitable habitats from a larger distance. Nevertheless, consistent response derived from over 150 replicates in two experiments represents likely a genuine effect. Further investigations are in progress to characterize the bacteria communities associated with the infusions and the volatile chemicals emitted from the infusions and detected by gravid *An. gambiae s.s.* using gas-chromatography coupled to mass-spectrometry and coupled gas chromatography-electroantennogram detection.

It must be cautioned that not all soils and all rabbit food pellets will lead to the same physical and chemical parameters than the here presented infusions. Therefore the two infusions of this study only serve as specific examples for two highly contrasting media. Further work is needed to screen other soil samples to see if the observed response is a response common for all soil infusions prepared under standard conditions and if the same bacteria and chemical profiles can be detected, or, which is more likely, that there are significant differences depending on the source of the soil.

2.6 Conclusion

This work illustrates that a gravid *An. gambiae s.l.* female selects a suitable habitat for oviposition using chemical cues from water bodies. It furthermore emphasises that natural infusions can be used to manipulate the oviposition behaviour of *An. gambiae s.l.*. Soil infusions have the potential to be used to bait gravid traps for the collection of *An. gambiae s.l.*, although further work must be carried out to determine whether the observed preference was based on the specific soil type tested or whether similar responses can be achieved with any soil. The low *An. gambiae s.l.* catching efficacy reported for gravid traps operationally used for *Culex* and *Aedes* monitoring might partly be explained by the infusions routinely used in these traps i.e. fermented hay infusions, rabbit food pellet and cow manure infusions (Lewis 1974; Lampman & Novak 1996; Jackson *et al.* 2005b). The identification of the chemicals responsible for the preference of the soil infusion might be exploited to bait gravid traps specifically for the collection *An. gambiae s.l.*.

Chapter 3 Do volatile chemicals of microbial origin from natural *Anopheles gambiae* s.s. breeding site increase oviposition responses?



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I developed all protocols and implemented the experiments, analysed the data and wrote first draft of the manuscript.

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3.1 Abstract

Background: Metabolites of bacterial origin serve as chemical cues for some *Aedes* and *Culex* species in their selection of aquatic habitat as oviposition site. Consequently, it has been suggested that similar cues are used by Afro-tropical malaria vectors however the empirical evidence is limited. Here we sought to test the hypothesis that the presence of microbial organisms in a larval habitat and the volatile chemicals they produce are used by gravid *Anopheles gambiae sensu stricto* (*s.s.*) for habitat selection.

Methods: Egg-laying response of gravid females was compared in two choice egg-count bioassays to (i) substrates made of distilled water and fresh soil versus distilled water and versus sterilized soil and distilled water (sterilization was done by autoclaving in polyethylene (PE) bags and in glass for comparison) and (ii) to fresh and sterilized water from a natural *Anopheles* breeding site. Dynamic headspace collection and solid phase micro extraction were carried out to trap volatile chemicals released from test substrates. Gas-chromatography coupled with mass-spectrometry was used to identify volatile compounds and to analyse any association between behavioural observations and chemicals released from the oviposition substrates.

Results: Eight times more *Anopheles gambiae s.s.* females laid eggs in substrates prepared from fresh soil when given a choice to lay in soil autoclaved in PE bags ($P < 0.001$). No preference was however, shown when infusions from fresh soil and soil sterilized in glass were offered as oviposition choices ($p = 0.496$). When offered a choice between soil sterilized in PE bags and distilled water, seven times more females laid eggs in distilled water ($p < 0.001$), while the females did not show any preference for either distilled water or infusion with soil autoclaved in glass flasks ($p = 0.057$). Gravid females did not show any preference for fresh habitat water and laid equally in fresh and sterilized water ($p = 0.379$). Headspace analyses showed that autoclaving soil in PE bags nearly completely removed volatiles from the headspace, whilst autoclaving in glass added a large number of volatiles compared to the fresh sample. Similarly, micro-filtered water contained a larger number of volatile compounds in the headspace than the fresh water sample. Comparison of the chromatograms did not suggest that key chemicals were removed from the sample by removing micro-organisms.

Conclusions: Whilst the volatile headspaces of oviposition substrates were modified by the sterilization method, no evidence was found that the removal of microorganisms changed the volatiles released from the substrates and consequently the oviposition

response. Elimination of microorganisms from soil and water collected from a natural *Anopheles* larval habitat did not affect the oviposition choice of gravid *An. gambiae s.s.*. However, soil substrates prepared from soil autoclaved in PE bags were avoided for oviposition possibly due to the absence of any chemical cue released from this substrate.

3.2 Background

Knowledge of the oviposition behaviour of mosquitoes is restricted to a few species of the genera *Culex* and *Aedes* that have been extensively studied due their important role in arbovirus transmission (Sardelis *et al.* 2001; Turell *et al.* 2001; Goddard *et al.* 2002). Gravid traps have been successfully developed for surveillance of these species that are particularly attracted to oviposition sites with high organic matter content (Reiter 1983; Reiter 1986; Allan & Kline 2004). Consequently, a range of fermented infusions made from various plants were identified to attract gravid females (Gjullin, Johnson & Plapp 1965; Millar, Chaney & Mulla 1992; Allan & Kline 1995; Isoe *et al.* 1995; Santana, Roque & Eiras 2006). Evidence has been provided that volatile microbial metabolites are the cues responsible for the attraction (Hazard, Mayer & Savage 1967; Bentley *et al.* 1979; Hwang, Kramer & Mulla 1980; Ponnusamy *et al.* 2010a).

Subsequently, it has been hypothesized that the African malaria vector *Anopheles gambiae s.l.* also selects oviposition sites based on metabolites produced by microbes living in the water or soil of a potential larval habitat. However, little data exists to support this hypothesis (Sumba *et al.* 2004; Huang *et al.* 2006a; Lindh *et al.* 2008).

The first study investigating this question (Sumba *et al.* 2004) compared the oviposition response in two choice cage bioassays of gravid *An. gambiae s.s.* females to sterile and non-sterile substrates made from soil and water collected from a semi-permanent *Anopheles* breeding site on the shores of Lake Victoria in Western Kenya. On average mosquitoes laid 3 times more eggs in unmodified substrates than in sterilized ones. The authors conclude “that microbial populations in breeding sites produce volatiles that serve as semiochemicals for gravid *An. gambiae*” (Sumba *et al.* 2004). The authors however could not restore the attractiveness towards a sterile substrate after inoculation with bacterial suspensions isolated from the water and soil. Later, nine species of bacteria isolated from the soil and water used in the previous study were identified (Lindh *et al.* 2008). These bacterial isolates were tested individually in two choice cage bioassays to assess the oviposition preferences of *An. gambiae s.s.* In these assays higher egg numbers were reported in response to five of the nine isolates (Lindh *et al.* 2008).

These two studies are to date the only published evidence that bacteria derived volatile chemicals are involved in attracting gravid females or stimulating egg-laying in *Anopheles gambiae s.s.*

When however the previous isolates from Lindh (Lindh *et al.* 2008) stored in glycerine were tested recently, the original response could not be replicated, conversely some of those previously described to induce increased egg-laying were now avoided (Lindh, personal communication). A third study, also conducted in western Kenya (Huang *et al.* 2006a), tested a mixture of cultured bacteria also isolated from a natural larval habitat against bacteria-free nutrient agar to quantify the oviposition response of *An. gambiae s.s.* Against expectation, gravid females avoided the bacteria and laid eight times more eggs in bacteria free nutrient agar than in mixed cultures of bacteria. Four bacteria species were presented in the mixture and when tested individually, only one species, *Stenotrophomonas maltophilia*, had a significant impact on the oviposition response by significantly lowering the number of eggs laid in the presence of the bacteria compared with bacteria-free controls. Whilst these results support the proposition that gravid *An. gambiae s.s.* females are receptive to bacteria derived volatile chemicals, it does not provide evidence that the site selection is mediated by bacteria present in this habitat.

Based on the limited and inconsistent data available, it was sought to closely replicate previous work to accept or reject the hypothesis that microbial communities present in soil and water from a vibrant *Anopheles* breeding site produce volatiles chemicals that mediate oviposition behaviour in *An. gambiae s.s.*

Two-choice cage bioassays were used to quantify the oviposition response of gravid *An. gambiae s.s.* females to non-sterile and sterile substrates from a natural habitat that contained early instar *Anopheles* larvae at the time of substrate collection. In addition, substrates were tested in comparison to a control solution, distilled water. The volatile chemical headspace was collected from all oviposition media to identify any association between behavioural observations and chemicals released from the substrates.

Experiments were carried out to address the following questions:

1. Do gravid *An. gambiae s.s.* females prefer to lay eggs in substrates made from fresh soil from a natural breeding site (containing microorganisms) compared with substrates made from autoclaved soil or distilled water only (sterile)?

2. Do gravid *An. gambiae s.s.* females prefer to lay eggs in habitat water from a natural breeding site (non-sterile) as compared with micro-filtered water or distilled water (sterile)?
3. Do sterile and non-sterile test substrates differ in their chemical profile of their headspace, therefore suggesting that the removal of microbes removes oviposition cues?
4. Are the volatile chemicals released from the test substrates associated with the oviposition response of gravid *An. gambiae s.s.*?

3.3 Materials and methods

3.3.1 Study site

All experiments were done at the International Centre for Insect Physiology and Ecology (ICIPE), Mbita, on the shores of Lake Victoria, Western Kenya (geographic coordinates 0° 26' 06.19" South; 34° 12' 53.13" East; 1,137 meters above sea level).

Soil and water for the experiments were collected from a semi-permanent water body, 380 m from the shores of Lake Victoria in Lwanda Nyamasare village (0°29'089"South, 34°17'848" East) 10 km northeast of ICIPE-Mbita (Figure 3.1.A.), approximately at the same location where samples were taken for the study by Sumba (Sumba *et al.* 2004; U. Fillinger, personal communication). Prior to the experiment and on every day of sample collection it was confirmed by dipping 10 times (Standard 350 ml dipper, Clarke Mosquito Control Products, USA) that the aquatic habitat was colonized by early instar *Anopheles* larvae (>5 larvae/dip), as a proxy indicator for oviposition.

3.3.2. Preparation of soil samples

Approximately 20 kg of soil was dug up from the damp but not immersed edge of the breeding site. The vegetation was carefully removed with a shovel and then the bare soil layer dug up not deeper than 10 cm. The soil was then transported to ICIPE-Mbita where it was spread on a cement floor and dried in the sun for one hour (Figure 3.1.B.). The soil was then divided in two equal parts: 10 kg was kept in a cool and dry place (fresh habitat soil) and the remaining 10 kg sterilized by autoclaving as described below. Fresh soil samples were collected for every round of cage bioassays.



Figure 3.1. Water and soil collection and preparation of two choice tests. A. *Anopheles* spp. breeding site in Lwanda Nyamasare, western Kenya. **B.** Drying of soil after collection at ICIPE-Mbita **C.** Two autoclaving methods: lumpy soil in polyethylene (PE) bag and macerated soil in glass flask. **D.** Two-choice experimental cage, oviposition cup with filter paper to minimize the visual difference of the oviposition substrates.

The procedures of autoclaving soil are rarely described in detail in experimental papers, including that of Sumba et al 2004. Searching the world wide web for standard operating procedures, most used plastic biohazard bags to autoclave soil (Razavi-darbar & Lakzian 2007). Some microbiology and phytology research papers however, reported autoclaving soil in a thin layer spread in a glass container (Balkwill & Casida 1979; Paolillo 1984). Both methods were used here for comparison in two sets of experiments (Figure 3.1.C.).

Method 1: Soil samples were autoclaved in Fisherbrand™ polyethylene biohazard autoclave bags (10 kg of soil per bag) at 121°C and 1.4kg/cm² pressure for 20 minutes (Vertical autoclave C 120L, Webeco, Germany). After sterilization, samples were allowed to cool without opening the bag before being used to prepare oviposition media.

Method 2: Soil samples were macerated and passed through a net (mesh 2 mm) to achieve a homogeneous size of soil particles. Soil samples were then divided in three equal parts and placed in three conical glass flasks of 5 L (Figure 3.1.C.). The soil was then autoclaved in the flasks as described above.

3.3.3. Preparation of water samples

Ten litres of habitat water was collected from the surface of the habitat using a clean plastic jar. Water was passed through a cotton cloth to remove water organisms including mosquito larvae and poured into 5 L jerry cans before transportation to the laboratory. Half of the sample (5 L) was used unmodified for the experiment and the other half (5 L) sterilized by micro-filtration. Large particles were first removed by passing the habitat water through a filter paper (Whatman no.1) with the help of a vacuum suction pump and supernatant water was then filtered through a cellulose nitrate membrane pore size 0.20 µm (Nalgene™) filter in a sterile disposable filter unit.

In order to confirm that autoclaving and micro-filtration sterilized the media, samples of both substrates were taken for bacterial cultures. Soil samples were moisturized with sterile distilled water (1g in 5 ml). After homogenization, 100 µl of each substrate was spread separately onto the surface of duplicate Lysogeny Broth (LB) agar-plates (LB Lennox-Fisher Scientific; Bertani 2004). Additionally, 100 µl of distilled water was spread as a control. Plates were incubated overnight at 30°C and the presence of colonies recorded. Bacteria cultures from autoclaved soil, micro-filtered water and distilled water confirmed that samples did not contain any bacteria that could grow on LB plates as opposed to fresh soil where colonies of at least seven different morphologies were observed.

Distilled water was prepared daily at the ICIPE-Mbita laboratories in the morning of the experiments with a GFL glass water distiller (GFL Glass Mono Distiller 2208, Germany). The raw water used for distillation was chlorinated tap water. Tap water at ICIPE-Mbita is pumped from Lake Victoria approximately 50 m from the shore and approximately 10 m deep into a settlement tank. From there, water goes to a treatment tank to be chlorinated before it is pumped into a water tower for domestic and laboratory use.

3.3.4 Two choice egg-count bioassays

Cage bioassays were carried out under semi-field conditions in make-shift sheds (10 m long × 5 m wide × 2.8 m high) as previously described (Herrera-Varela *et al.* 2014). The cages were protected from rain but otherwise exposed to natural fluctuations of temperature, relative humidity, wind and light.

Experiments were carried out with insectary-reared *An. gambiae s.s.* (Mbita strain) supplied by ICIPE's insectary 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 h and keeping them in 30×30×30 cm netting cages at 25-28°C and 68-75% relative humidity. To avoid mosquito desiccation, cotton towels (folded to 25x12 cm) were saturated with unchlorinated tap water and placed over the cages. Mosquitoes starved of sugar for seven hours were allowed to feed on a human arm for 15 minutes at 19.00 h on the same day. After feeding mosquitoes were provided with 6% glucose solution *ad libitum*. A plastic vial (25 ml) with a piece of paper towel folded into a wick was used to provide 10 ml of 6% glucose. This procedure was repeated the following day. After the first blood meal unfed females were removed from the cages while fed females were kept together with an equal number of males for 72 hours before using them in an experiment. On the day of an experiment 150 visually presumed gravid females (enlarged, pale white abdomen) were selected at 16.30 h from the holding cage. It has been shown (Gillies 1958; Lyimo & Takken 1993) that most females need two blood meals to reach full gravidity and some do not reach gravidity even after three feeds.

Females were transferred individually to 30x30x30 cm cages. In each cage two glass cups (Pyrex®, 100 ml, 70 mm diameter), surrounded by tightly fitting aluminium cylinders, were filled with 100 ml or 100 g of either the control or test medium and placed in diagonal corners of the cage. A round filter paper (Whatman No. 1) was placed on top of each cup allowing contact with the oviposition substrate. The purpose of the aluminium collars and filter paper were to exclude visual differences between the two test substrates (Figure 3.1. D.). Position of oviposition cups containing the test medium was alternated between adjacent cages to control for any position effect. The placement of the first test cup was randomly allocated for one of the four cage corners in the first cage. Subsequent test cups were rotated in the next possible corners in a clockwise direction relative to the position of the preceding cup. One control cup was added in each cage diagonal to the test cup to complete a two choice set up. Each cup contained

either 100 g of fresh habitat soil or 100 g autoclaved habitat soil or 100 ml of habitat water or 100 ml micro-filtered habitat water, or 100 ml distilled water. Soil substrates were moistened with 50 ml of distilled water so that a thin layer of water covered the soil. All glassware used in the bioassays was cleaned with detergent, autoclaved at 121°C for 20 minutes and afterwards kept in a drying oven (Heraeus T5050 EK, Atlanta-Georgia) at 200°C for at least two hours before use in an experiment.

In order to validate the two choice bioassays, cages were set up with both oviposition cups containing equal treatments following a previously described approach (Herrera-Varela *et al.* 2014). Both cups were filled with 100 ml unchlorinated-tap water. The underlying assumption is that gravid females presented with an identical substrate in both cups are equally likely to lay in either cup ($p=0.5$) but stochastic events will lead to a certain variability of the 1:1 outcome. This is considered the baseline or reference. If one treatment is preferred over the other treatment in the same cage, a significant diversion from the 1:1 distribution is expected. Table 3.1 summarizes the experiments implemented and the comparisons made in two choice bioassays.

Table 3.1. Experiments carried out with sterile and non-sterile substrates in two choice cage bioassays

Experiments	Experimental cages		Rounds (Replicates)	Number of females that laid eggs (Total of mosquitoes exposed)
	Control cup	Test cup		
Baseline/Reference	tap water	tap water	4	153 (225)
Oviposition bioassays with soil from breeding site				
Soil sterilization by autoclaving in PE bags	sterile soil	fresh soil	5	102(150)
	sterile soil	distilled water	5	101(150)
Soil sterilization by autoclaving in glass	sterile soil	fresh soil	4	61(120)
	sterile soil	distilled water	4	88(120)
Habitat soil vs. distilled water	distilled water	fresh soil	6	136(180)
Oviposition assays with water from breeding site				
Habitat water vs. micro- filtered habitat water and distilled water	sterile water	Fresh water	4	69(100)
	distilled water	Fresh water	4	75(100)
	distilled water	sterile water	4	70(100)

3.3.5 Analysis of volatile organic chemicals released from oviposition substrates

Volatile organic chemicals released from the oviposition substrates were trapped by dynamic headspace collection also referred to as air-entrainment (Agelopoulos & Pickett 1998) for aqueous and soil samples. In addition, solid phase micro extraction (SPME) was carried out for selected soil samples. All glassware used during volatile collections was first washed with an odourless detergent (Teepol, general purpose detergent, Teepol, United Kingdom) rinsed in water and acetone and then placed in an oven kept at 200°C for at least two hours before use.

3.3.5.1 Dynamic headspace collections

Dynamic headspace collections were used to collect volatiles from 300 ml samples of habitat water, sterile-filtered habitat water and distilled water. Additionally, 45 g of sodium chloride (NaCl) were added to the samples to improve the release of volatile chemicals (Mozuraitis, Buda & Borg-Karlson 2010). Each sample was entrained in duplicate. Test substrates were placed in 500 ml gas wash bottles (MF 29/3/500, Scilabware Ltd. UK) which were tightly closed with a quick fit head (MF 27/3/13, Scilabware Ltd. UK; Figure 4.2). Flexible polytetrafluoroethylene (PTFE) tubing (1/8" O.D.) was connected on each of the two glass tubes; one served as inlet for purified air and the other as outlet where the chemicals were trapped. Air was purified by passage through an activated charcoal filter pumped at a flow rate just above 0.1 L/min. Air was removed at 0.1 L/min through a Tenax trap. The Tenax traps were made out of 25 mg of Tenax® TA (mesh size 60-80, Supelco™, Bellefonte, PA, USA) packed with small amounts of glass wool (Supelco™, Bellefonte, PA, USA) to keep the Tenax in place in a GERSTEL-Twister desorption glass liners (GERSTEL, Muelheim an der Ruhr, Germany).

Tenax traps were conditioned before use by washing with 3-5 ml methyl tert-butyl ether (MTBE) and placed in an oven at 50°C for >2 h for the solvent to evaporate. Volatiles were entrained for 20 hours from each sample. Once the entrainment was completed, the filters were sealed with PTFE tape, wrapped in aluminium foil, packed in a Teflon bag and stored at -70°C until they were sent to the Royal Institute of Technology (KTH), Sweden, for analysis by gas chromatography coupled to mass spectrometry (GC-MS).

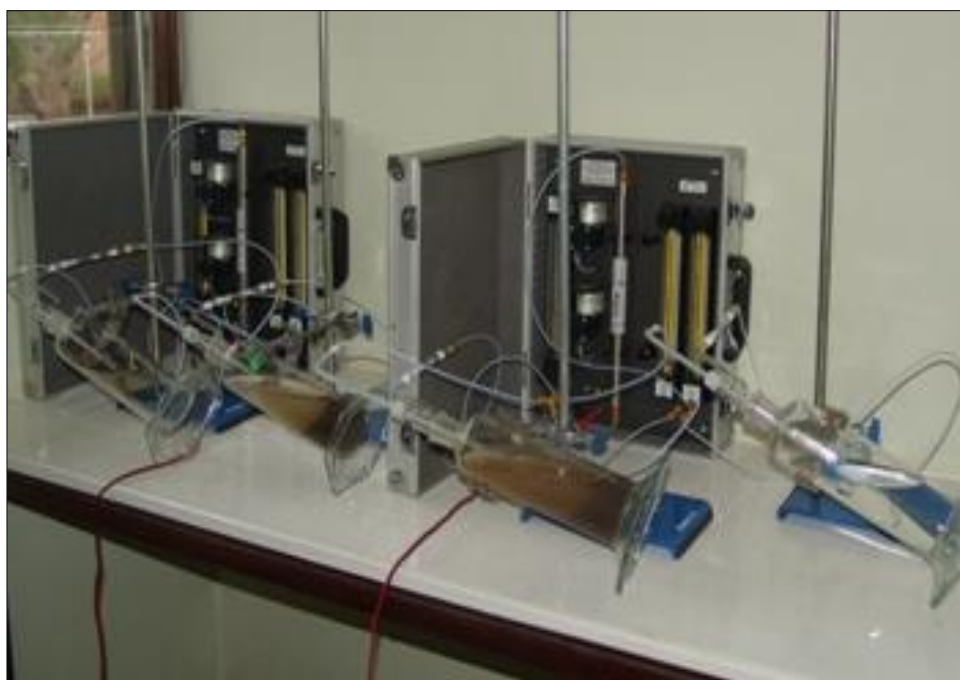


Figure 3.2. Air-entrainment system used to collect volatile compounds from oviposition substrates. Gas-wash bottles were tilted to increase the surface area of the aqueous substrates.

3.3.5.2 SPME-collections

In addition to the dynamic headspace collection, SPME was used to collect volatiles from a single round of soil experiments. Duplicate collections were made from fresh habitat soil, soil autoclaved in PE bags, soil autoclaved in glass flasks and from distilled water. Soil samples were prepared by adding 100 g of fresh soil, or sterile soil to 200 ml of distilled water. These samples were then transported to the Chemical Ecology Laboratory at ICIPE, Nairobi, Kenya. Test substrates were added to 500 ml conical flasks (Quickfit, England) which were sealed with a double piece of aluminum foil. The aluminum foil cover was pierced with the needle of the SPME holder and the 65 μ m polydimethyl siloxane-divinyl benzene (PDMS-DVB) fibre (Supelco™, Bellefonte, PA, USA) exposed to the headspace above the sample. The SPME fibers were conditioned in the GC-injection port at 220°C for 5 min just before sampling. Volatiles were collected for 16 hours after which they were immediately analyzed by GC-MS.

3.3.5.3 Gas chromatography coupled to mass spectrometry (GC-MS) analysis

The GC-MS system used consisted of a 7890A GC (Agilent Technologies, Santa Clara, CA) fitted with a 30 m long HP-5MS column (Agilent Technologies) with an inner diameter of 0.25 mm and 0.25 μ m film thickness. The GC was coupled to a 5975C MS

(Agilent Technologies) with electronic ionization at 70 eV and the ion source kept at 230 °C and the quadrupole at 150 °C. Masses were scanned from 30-400.

Tenax traps were thermally desorbed in a thermal desorption unit (TDU, GERSTEL, Muelheim and der Ruhr, Germany) initially held at 20 °C and then increased at 120 °C /min to 250 °C, the end temperature was held for 5 minutes. The volatiles were then transferred in splitless mode to a cooled injection system (CIS) injector fitted with a Tenax trap (GERSTEL). The CIS injector was held at 10 °C during the TDU program and was then heated at a rate of 12 °C/sec to 260 °C during which the volatiles were transferred to the column. Helium was used as carrier gas with a flow of 1.2 ml/min at a pressure of 34 psi. The temperature of the GC oven was held at 40 °C for 1 min and then increased by 4°C/min to 260 °C and kept there for 3 minutes. Heptyl acetate (35 ng, SAFC, Sigma-Aldrich, Steinheim, Germany) was injected as external standard with each sample.

For SPME analysis masses were scanned from 38-550. The GC injector was kept at 250°C in a splitless mode, helium with a flow of 1.2 ml/min was used as carrier gas. The oven temperature was held at 40 °C for 3 minutes, then programmed to increase at 5°C/min to 260°C and maintained at this temperature for 3 minutes for a total running time of 50 minutes.

3.3.6 Data analysis

Bioassay data were analysed in R statistical software version 3.0.2 (R Development Core Team 2011). Two choice egg-count bioassays were analysed using generalized linear models with a quasibinomial distribution fitted to account for overdispersion. The distribution of eggs in the baseline cages (equal substrates) representing the natural variability in the system was compared with the distribution of eggs in experimental cages (control-test substrates). Experiment (e.g. baseline cages, habitat soil vs. distilled water cages) and the round of assay were included as fixed factors in the model to examine their impact on the outcome (proportion of eggs laid in the test cup).

The mean proportion of females and eggs laid in test cups in different experiments and their 95% confidence intervals (CI) were calculated as the exponential of the parameter estimates for models with no intercept included. Multiple comparisons of experiments were calculated based on the model parameter estimates.

Since non-parametric tests have been used for analysis of egg-laying preferences in previously published work (Sumba *et al.* 2004; Lindh *et al.* 2008) data were also

analyzed using Wilcoxon signed rank test for paired samples (i.e. control cup versus test cup from each experimental cage) for comparison and discussion of outputs.

For the comparison of volatile chemical profiles from the headspaces of substrates, the retention parameters from the chromatograms were integrated using the Chem station integrator and auto-integration function in Chem Station software (MSD ChemStation E.02.01.1177). Mass spectra fragments were recorded manually for each compound integrated. Compounds were grouped according to retention time, MS fragments, and given a unique volatile identification number (ID). Initially, duplicate samples were compared with each other and a volatile profile created for each sample only if compounds were present in both duplicates. A database was created with information about the abundance of each volatile (area of the peak) in the samples and used for analyses. Two databases were created one for Tenax samples and one for SPME samples.

Principal component analysis (PCA) was used to examine the relationships between volatile chemicals and liquid oviposition substrates. PCA was performed in CANOCO5 for windows version 5 (Ter Braak & Šmilauer 2012). PCA was chosen as exploratory ordination method since data was compositional (i.e. same measurement units) and all volatile gradients were 1.9 standard deviation units long assuming a linear response model.

3.4 Results

3.4.1. *Anopheles gambiae* s.s. females do not show a preference for oviposition substrates containing microorganisms from habitat derived soil when compared with sterile substrates

3.4.1.1 Proportion of females responding to the soil samples

When females were presented with a choice between two identical substrates, an equal proportion of females laid in either cup. In comparison, eight fold more *Anopheles gambiae* s.s. females laid eggs in fresh soil infusion when the second choice was to lay in sterile soil infusion that was autoclaved in PE bags ($p < 0.001$). Importantly however, this preference was not observed when fresh soil and soil sterilized in glass were offered in choice tests ($p = 0.496$) suggesting that the autoclaving method altered the substrates differently in addition to sterilizing it.

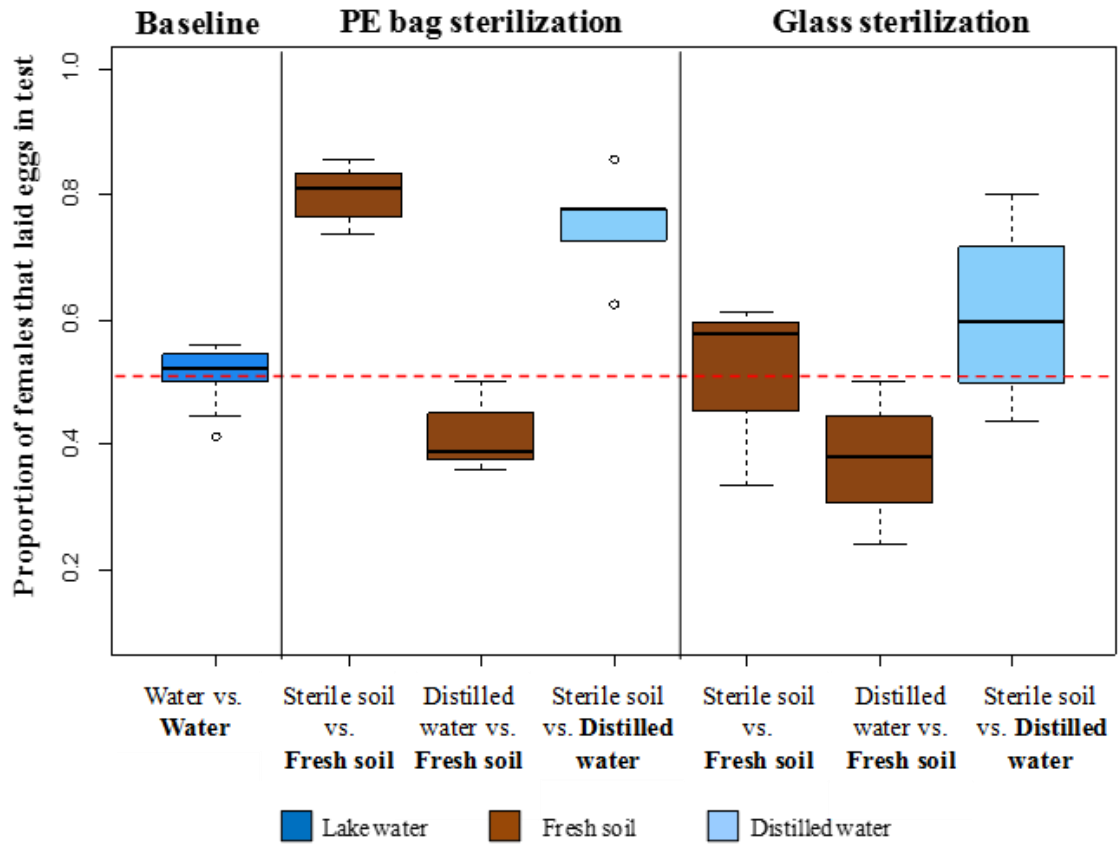


Figure 3.3. Box-and-Whisker plot showing the median proportion of females laying eggs in the test substrates in two choice bioassays (control vs. test). Baseline (Lake water as control and test), experiments including autoclaved soil in polystyrene bags and soil autoclaved in Erlenmeyer flasks. Substrates in test cup are indicated in bold. Colours (blue=lake water, brown=fresh soil, light blue=distilled water) also present the substrate in test cup. Red line indicates no preference for either solution.

Preferred egg laying in fresh substrates could be for two reasons, the preferred substrate attracts or stimulates the egg-laying or the avoided substrate actually repels or deters the female. In order to investigate this, fresh soil was tested in comparison to distilled water and the two sterile soil media were also tested in comparison to distilled water. Tests offering a choice between fresh habitat soil and distilled water were done twice, first in parallel to bioassays with soil sterilized in PE bags and second in parallel to bioassays with soil sterilized in glass. The results from both were very similar, showing unexpectedly a preference of gravid females for the distilled water over the fresh habitat

soil ($p= 0.0178$, Figure 3.3). When a choice of two sterile substrates was presented (sterilized soil versus distilled water) females strongly preferred laying in distilled water over laying in soil sterilized in PE bags ($p<0.001$) whilst they showed only a weak preference for distilled water when presented with soil sterilized in glass ($p=0.057$, Figure 3.3).

3.4.1.2 Proportion of eggs laid in soil substrates

Most published work on mosquito oviposition does not analyse individual mosquitoes' choices but rather count eggs of groups of mosquitoes as a proxy of oviposition preference. For comparison, egg counts were also analysed here. Similar results were found when analysing the proportion of eggs laid in test over control substrates. Mosquitoes offered a choice of equal substrates exhibited a balance distribution of eggs between cups. This distribution differed significantly from the distribution of eggs in tests where a choice was given between distilled water and fresh habitat soil with a higher proportion of eggs laid in distilled water (Figure 3.4). Significant differences in egg distribution to the reference were also found when the choice tests include soil that was autoclaved in PE bags. Females preferred to lay in the alternative substrate offered, either fresh habitat soil or distilled water, with more than three quarters of the eggs laid in those substrates (Figure 3.4). When however, the habitat soil was sterilized in glass, only a slight preference for distilled water was detected ($p=0.027$). The distribution of eggs in cages comparing autoclaved soil in glass against fresh soil did not differ from the distribution of eggs in the reference with equal treatments. Inferences were the same whether the data were analysed with a generalized linear model or with the non-parametric Wilcoxon test for paired samples (Figure 3.4).

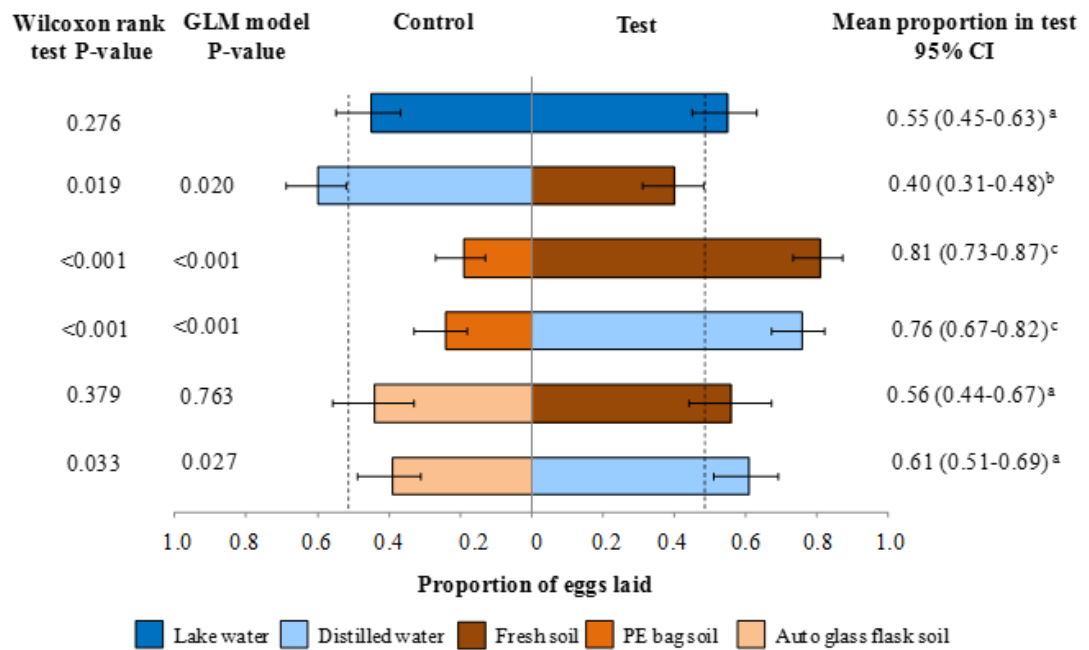


Figure 3.4. Mean proportion of eggs laid in control and test cups in choice tests with fresh habitat soil versus sterile soil or distilled water. Error bars present the 95% confidence intervals of the means. Multiple comparisons of treatments based on the generalized linear model parameter estimates: treatments with same letter are not significantly different at 0.05 level.

3.4.2. *Anopheles gambiae* s.s. females do not show a preference for larval habitat water containing microorganisms as compared with sterile water

3.4.2.1. Proportion of females responding to water samples

Gravid *An. gambiae* s.s. did not show any preference for either of the water substrates tested in either of the tests, however, a slight preference for distilled water was observed when compared with sterile habitat water (Figure 3.5)

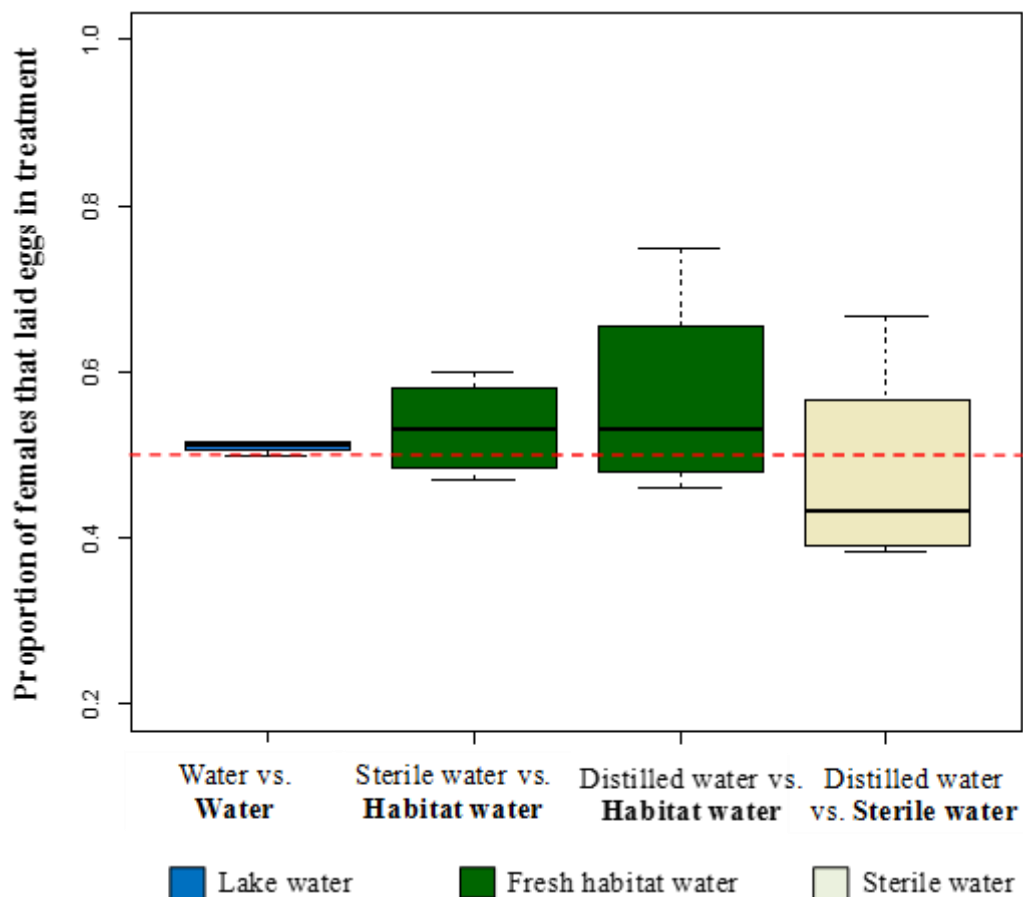


Figure 3.5. Box-and-Whisker plot showing the median proportion of females that laid eggs in test substrates in two choice bioassays (control vs. test): Internal control (equal substrates), treatments including habitat water and sterile water. Test substrates are indicated in bold. Red line indicates 0.5 distributions.

3.4.2.2 Proportion of eggs laid in water substrates

Equal proportions of eggs were laid in sterile habitat water and fresh habitat water. A slight preference for fresh habitat water was seen when offered in comparison with distilled water. This difference was significant using the non-parametric test for paired samples but not when the distribution was compared with the baseline with a generalized linear model (Figure. 3.6).

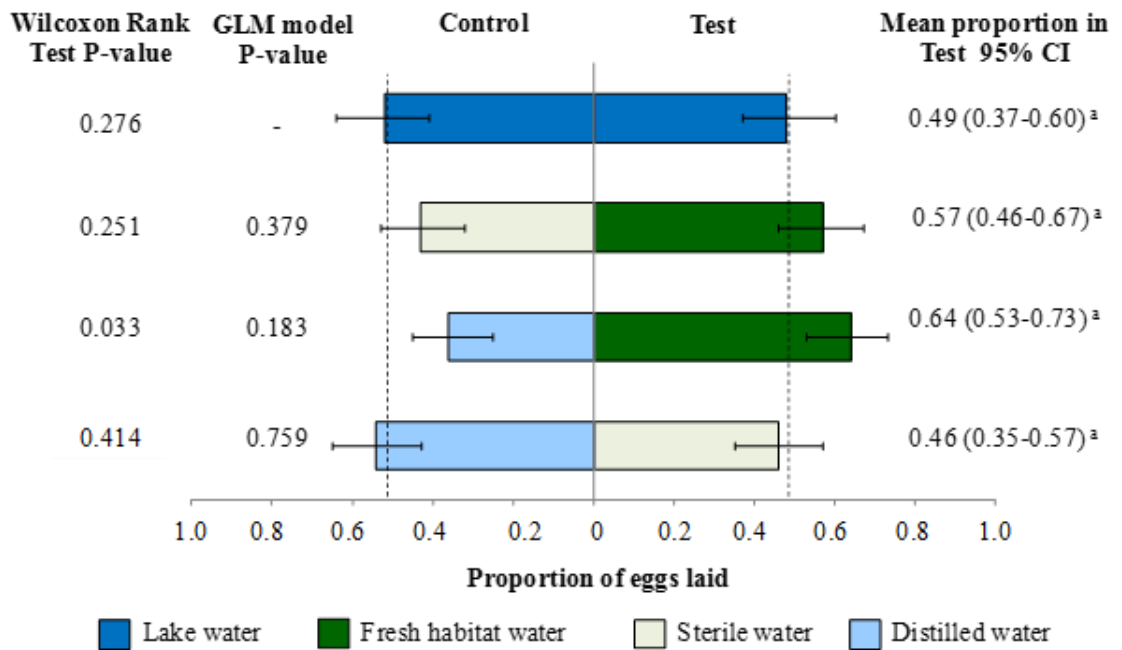


Figure 3.6. Mean proportion of eggs laid in control and test cups in choice tests with fresh habitat water versus sterile habitat water or distilled water. Error bars present the 95% confidence intervals of the means. Multiple comparisons of treatments based on the generalized linear model parameter estimates: treatments with same letter are not significantly different at 0.05 level.

3.4.3. The absence of volatile chemicals in the headspace of soil samples autoclaved in PE bags is associated with the avoidance of this substrate for oviposition

Results presented below for the chemical headspace of substrates tested in the soil bioassays are based on one round of bioassays only due to the loss of all Tenax samples that were injected through auto-injection in a faulty GC-MS. Figure 3.7 shows the chromatograms of the duplicate SPME collections taken from fresh habitat soil, soil autoclaved in PE bags, soil autoclaved in glass and distilled water. The most conspicuous result from this comparison is the absence of volatiles released from the soil autoclaved in PE bags, which is in stark contrast to all other samples.

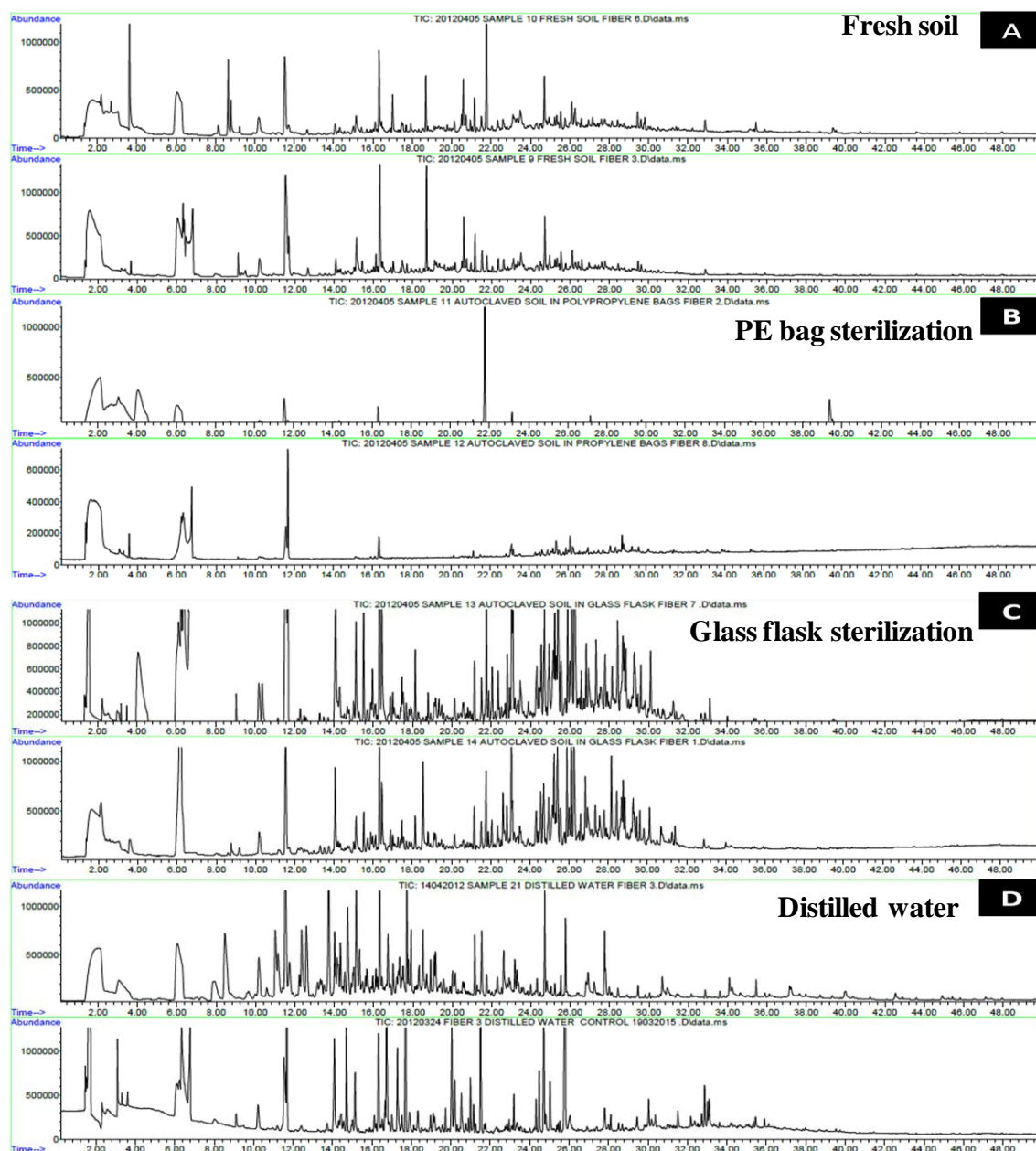


Figure 3.7. Chromatograms of volatiles profiles collected with SPME from soil substrates: A. Habitat soil, B. Soil autoclaved in PE bags, C. Soil autoclaved in glass flasks, D. Distilled water. All substrates are presented with two separate samples.

The headspace of fresh soil contained 32 compounds of which 18 were unique to the sample (i.e. there were not identified in any other sample), another six compounds were shared only with the soil sample autoclaved in glass and four with distilled water. Only four compounds were detected from soil autoclaved in PE bags and these compounds were present in all the other substrates. Their retention time and mass spectra suggest that they were probably contaminants from the GC column. It therefore appears that

autoclaving in PE bags removed all compound released from the fresh soil. In contrast autoclaving soil in glass flasks increased the number of detectable volatiles. Seventy-three compounds were collected in the headspace above soil autoclaved in glass flask; 58 of these were unique to the sample. Eighteen compounds were detected from the headspace of distilled water, eight of them were also detected from fresh and glass autoclaved soil, eight others were unique to the sample. These differences in headspace were not associated with any behavioural differences. Only the absence of volatiles from the headspace of soil autoclaved in PE bags corresponds to a strong shift in the oviposition behaviour of gravid females (Figure 3.3 and 3.4).

4.3.4. Headspace analysis of water substrates

Headspace analysis was implemented for three rounds of habitat water bioassays. In total, 286 compounds were integrated of which half (144) were only detected in a single round. For comparison of substrates only compounds that were present in at least two of the three rounds were included in the analysis. Comparisons of the retention parameters of the chromatograms (Figure 3.8) showed large similarities in the chemical profiles of fresh habitat water, sterile habitat water and distilled water. Seventy volatile chemicals were detected in the headspace of fresh habitat water and 49 (70%) were still present when the water was micro-filtered. Fresh habitat water also shared 48 (69%) volatiles with distilled water. All three substrates had 40 compounds (57%) in common. Micro-filtration of habitat water increased the number of volatile chemicals released from the headspace; a total of 99 volatile chemicals were integrated of which 35 were unique for the micro-filtered habitat water.

Removing microorganisms from the habitat water might or might not have been responsible for the 21 volatile chemicals that were removed from the headspace of fresh habitat water by micro-filtration, however their removal did not lead to a significantly reduced oviposition response.

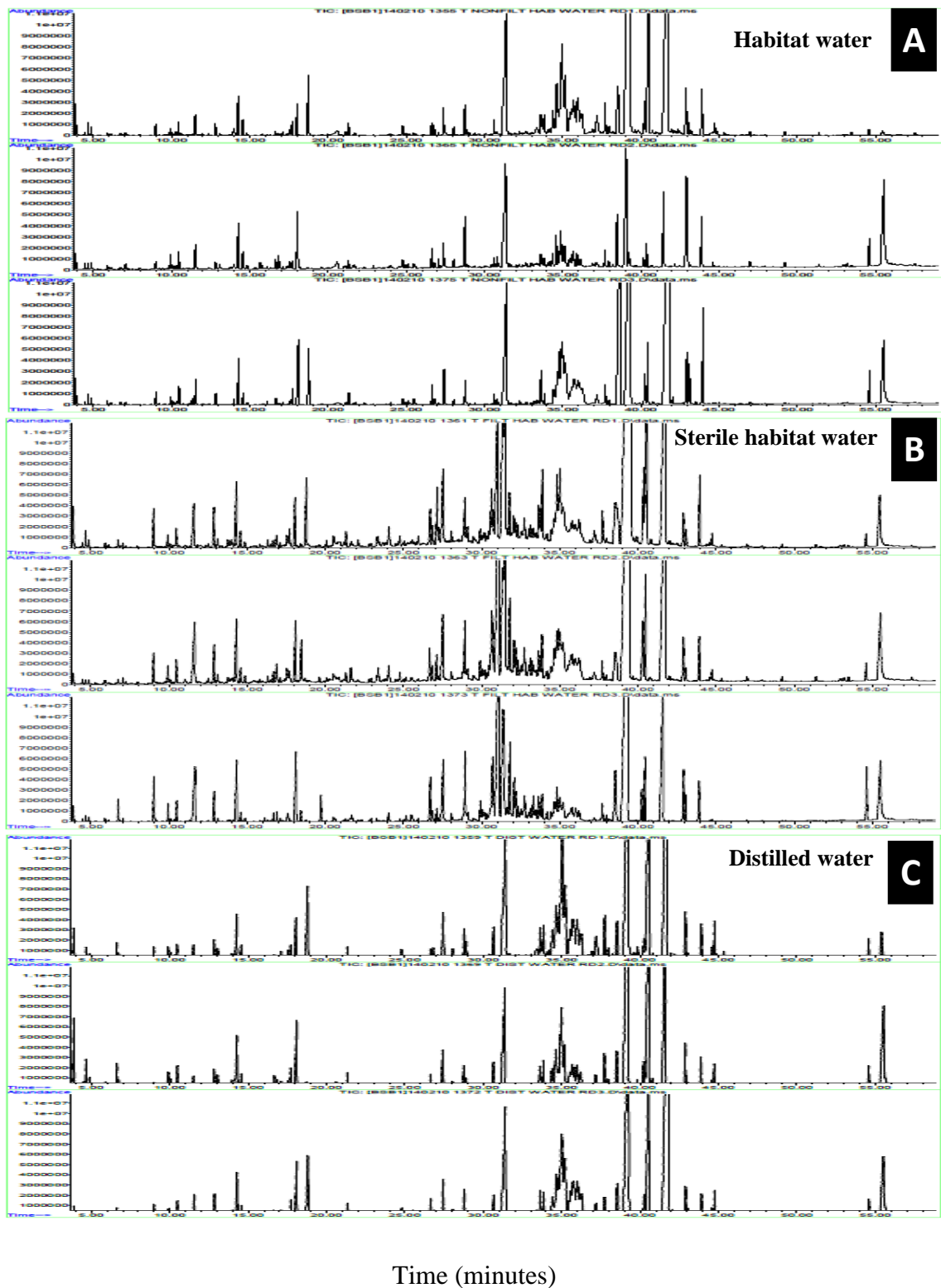


Figure 3.8. Chromatograms of volatiles collected by dynamic headspace collections on Tenax traps from aqueous oviposition substrates: A. Habitat water, B. Sterile habitat water, C. Distilled water. Every chromatogram is an independent replicate.

PCA analysis reveals that volatiles released from the oviposition substrates can explain 70% of the variability observed in the distribution of the samples. It also shows that volatiles can be used to differentiate between habitat water, sterile water and distilled water (Figure 3.9.1). In addition, the diversity plot shows that there is not a large variation between individual replicates and confirms that even though chemicals present in only one replicate were removed from the analysis consistently more chemicals were detected in sterile water samples.

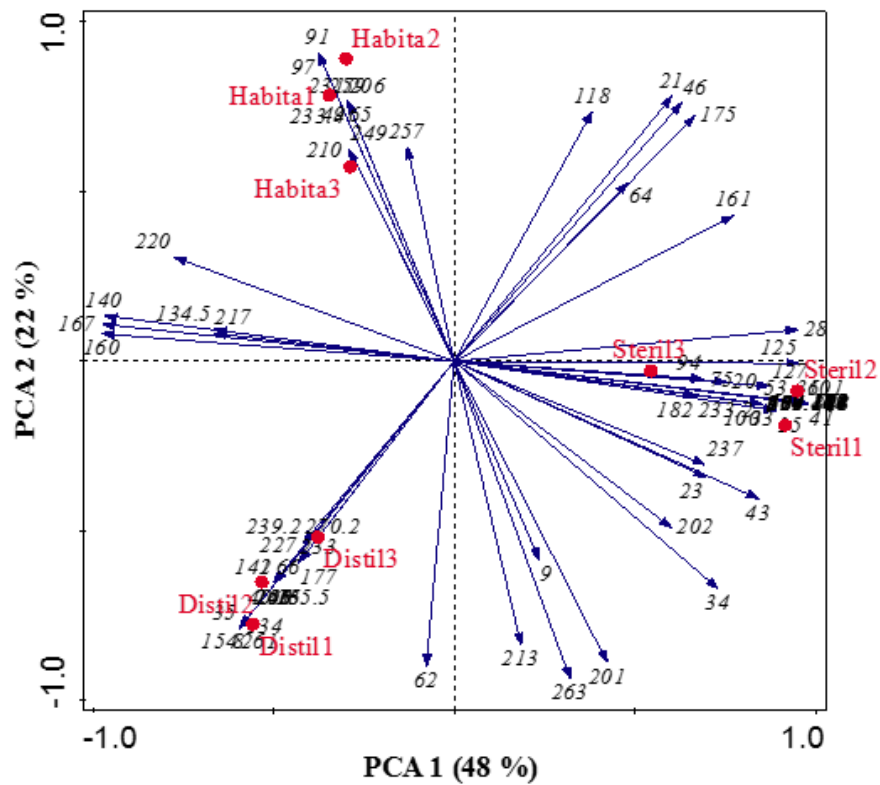


Figure 3.9.1. Principal Component Analysis (PCA) plot of volatile profiles of water oviposition substrates: Fresh habitat water (Habita1,2,3), Sterile water (Steril 1,2,3) and Distilled water (Distil 1,2,3).

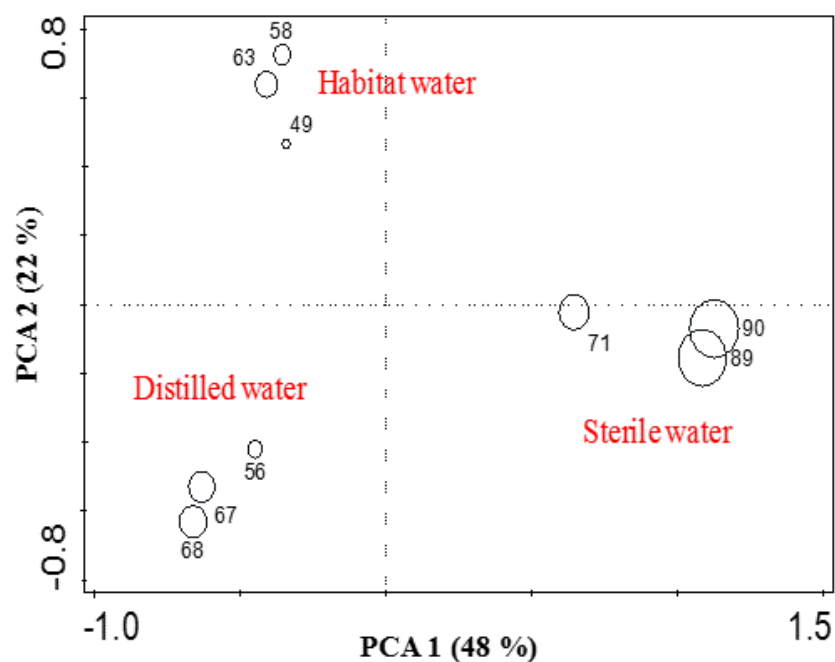


Figure 3.9.2. Diversity diagram of volatile chemicals. Circle sizes and labels indicate the numbers of volatiles chemicals detected in each sample. Distance between the circles approximates the similarity between chemical compositions.

3.5 Discussion

The presence of living microorganisms in soil and water from an *Anopheles* larval habitat did not increase the oviposition response of gravid *Anopheles gambiae* s.s. when compared with the same but sterilized substrate. The sterilization process affected the chemical composition of substrates and those changes influenced the oviposition response of gravid mosquitoes.

Sterile soil samples obtained by autoclaving in PE bags received significantly less eggs compared with any other substrate. In contrast, sterile samples produced by autoclaving in a glass flask received equal proportion of eggs as the fresh habitat soil. These differences in oviposition response illustrate the impact of the autoclaving process rather than the presence of microorganisms. Headspace analyses revealed that autoclaving in closed PE bags removed all detectable volatiles from the sample. On the other hand when autoclaving took place in open glass flasks the number of detectable volatiles increased possibly due to contamination from the autoclave or due to different breakdown products forming in the open flasks.

Given these results it is likely that in this study gravid mosquitoes orientate to substrates that display an array of chemicals, therefore preferences recorded towards fresh soil and distilled water when compared with PE bag soil may have been triggered by sole presence of chemical cues that indicate the existence of a habitat rather than its suitability. Since the bioassay utilised cannot distinguish between deterrent and repellent responses and knowing that only volatile chemicals could be detected with the methods used it cannot however be excluded that non-volatile chemicals functioning as deterrents were present in the soil samples autoclaved in PE bags.

Notably, the glass sterilized soil sample and the fresh soil sample had only a small proportion of volatiles in common yet the oviposition response did not differ. Furthermore, sterile distilled water was preferred by gravid females for oviposition over fresh habitat water. Whilst volatile chemical cues might have been involved in the oviposition site selection, these findings corroborate the conclusion that these volatiles are not produced in situ by life microorganisms.

In the case of water samples, mosquitoes did not exhibit any preferences for fresh or sterilised water. Analyses of the headspace of the substrates showed that they shared the majority of volatile chemicals identified from fresh habitat water. Sterilized habitat water however had consistently more compounds and it must be assumed that these were added in the process of micro-filtration. Nevertheless, these compounds did not affect the oviposition behaviour, nor did those that were removed from the sample through the process. This and the slight preference for distilled water shows that the presence of microorganisms was not used as oviposition cue by gravid *An. gambiae s.s.*.

The fact that distilled water had a similar volatile profile as the fresh habitat water may indicate that the volatile organic compounds released from distilled water are directly related with the source of the water used for distilling. Distilled water is used as a standard control in the majority of mosquito oviposition experiments and in similar experiments with other organisms (Huang *et al.* 2007; Otienoburu *et al.* 2007; Panigrahi *et al.* 2014) in the study of chemical ecology. Rarely is the source of distilled water reported in scientific papers and chemical profiles of headspaces from distilled water are usually not presented creating the impression that distilled water does not contain any more cues than water vapour and that distilled water is a comparable medium between studies. The quality of distilled water depends however, on the quality of the raw water and the distilling process (GFL 2014). Hence the volatile profile of distilled water may

be highly variable between research centres or laboratories and even show high day-to-day variation.

The discrepancy between our findings and similar experiments previously published (Sumba *et al.* 2004) could be attributed to several factors. There is an intrinsic challenge when testing soil and water from a natural breeding site. Larval habitats are exposed to changing climate conditions and human activity that may affect the quality of a habitat over time. As previously shown (Herrera-Varela *et al.* 2014), gravid *An. gambiae s.l.* can distinguish between oviposition substrates with different water quality preferring those resembling a flourishing ecosystem with low organic matter content, low ammonium and an aerobic water column over those that emulate a habitat in decay. However, *An. gambiae s.s.* is an opportunist (Fillinger *et al.* 2004; Minakawa *et al.* 2005; Impoinvil *et al.* 2008) and when choices are rare, colonizes water that might under experimental conditions be less preferred (Awolola *et al.* 2007; Castro *et al.* 2010). Therefore it cannot be excluded that the habitat we collected water from was not optimal, even though it had a thriving early instar *Anopheles* larval population during the sampling period.

Differences in the abiotic and biotic characteristics of the habitats tested in the two studies might be responsible for the differences seen. Perhaps even more important might be the difficulty in replicating the sterilization process since the previous publication lacks a detailed protocol of the autoclaving method, which according to our results affects the volatile chemical headspace of the substrate and consequently the acceptability of the oviposition substrate by mosquitoes. Similarly, the source of distilled water was not reported and the headspace of samples not analyzed. In a scenario, where the autoclaving might have removed all the chemical volatiles and where distilled water was used with an equally limited chemical profile, it would have been shown that the fresh (volatile containing) soil would be preferred over the sterile (non-volatile containing) substrate, and when the two sterile (non-volatile containing) substrates would have been compared a 1:1 distribution of eggs would have been demonstrated as by Sumba *et al.* (Sumba *et al.* 2004). Consequently, it is likely that in previous work volatile chemicals were responsible for the substrate selection but if they were produced *in situ* by live microorganism remains unproven. This might be supported by the fact that restoration of attractiveness of sterile substrates after inoculation with bacterial suspensions was not achieved (Sumba *et al.* 2004). Not only bacteria but all microorganisms are killed/removed by autoclaving and micro-filtration and one of the

reasons why restoration was not achieved could have been that the oviposition cues were generated by other microbes. Aquatic ecosystems are composed of highly diverse groups of microbes including bacteria, microalgae, actinomycetes, fungi, and protozoa (Sigeo 2005). Therefore it is not possible to tell with this methodology if there is a particular group or the synergistic action of several organisms that are responsible for producing the oviposition cues if any (Huang *et al.* 2006a) .

3.6 Conclusion

Previous reports that substrates containing microbes from vibrant larval habitats are preferred over the same substrates when sterilized cannot be confirmed. Elimination of live microorganisms from soil and water collected from a natural larval habitat does not necessarily influence the oviposition choices of gravid *An. gambiae s.s.* mosquitoes. Furthermore, there is no evidence that volatiles produced *in situ* by these microbes are involved in the oviposition choice. It was shown however that volatile profiles of oviposition substrates can be modified by sterilization and the avoidance of substrates without any volatile headspace suggest that volatile chemicals play a role in habitat selection. Chemicals that were removed from fresh habitat soil and water by sterilizing that might have been metabolites from live microorganisms did not affect the oviposition choice. The remaining volatile chemicals might have been produced by microbes and remained in the water or might have been derived from plants and other organic matter in the habitat. Further research is required to investigate if microorganisms are involved in habitat selection of *An. gambiae s.s.*

Chapter 4 Oviposition site selection of *Anopheles gambiae sensu lato* in Rusinga Island, Western Kenya: A case-control approach



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I contributed significantly in the formulation and experimental design.


I developed all protocols and implemented the experiments except the zooplankton identification and GC-MS analyses. I analysed the data and

wrote first draft of the manuscript.

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SUPERVISOR/SENIOR AUTHOR'S SIGNATURE (3 above) 

4.1 Abstract

Background: Gravid *Anopheles gambiae s.l.* make informed choices when looking for a place to lay eggs, however, the signals used by gravid females to discriminate between aquatic habitats remain unknown. Here we investigated physical, chemical and biological factors associated with oviposition of *An. gambiae s.l.* mosquitoes in Rusinga Island, Western Kenya.

Methods: A cross-sectional survey of all aquatic habitats was done during the long rainy season March to July 2012 to compare the characteristics of habitats colonised (cases) and not colonized (controls) by early instar *Anopheles* larvae. Biotic factors evaluated included zooplankton, invertebrate fauna and bacteria communities; abiotic factors included, physical water parameters, nutrients and volatile chemicals released from the water.

Results: The presence of early instar *Anopheles* larvae used as a proxy measure for oviposition was highly associated with high densities of late instar *Anopheles* larvae ($p=0.002$) and increasing turbidity ($p=0.025$). Aquatic habitats with turbidity > 200 NTU were over 80% likely to be colonized by *An. gambiae s.l.*. In habitats with turbidity < 200 NTU the odds of being selected by gravid females increased significantly with a unit increase of cladocerans of the family Moinidae (RR=7, 95% CI (4-11)) and emergent vegetation (RR=1.55, 95% CI (1.02-2.37)). Conversely, the odds were reduced with a unit increase in Naucoridae (RR=0.13, 95% CI (0.09-0.20)) and fish (RR=0.33, 95% CI (0.22-0.50)). Chemical cues from natural habitats were highly diverse however, preliminary analyses showed that a group of hydrocarbons were linked with habitats colonized by early instar larvae. No evidence was found that bacteria communities were involved in habitat selection.

Conclusion: The relationship between environmental characteristics, biotic and abiotic factors that characterize natural oviposition sites of *Anopheles gambiae s.l.* in Rusinga Island, western Kenya are intricate. Further studies are required to investigate chemical cues released from vibrant larval habitats and their interaction with potential visual cues like turbidity investigated. Turbidity and volatile chemicals might be potentially exploited to attract and kill gravid females as a novel intervention for vector surveillance and control.

4.2 Background

The spatial distribution of *Anopheles* larvae in the environment depends initially on the oviposition choices of the gravid mosquito and subsequently on the survival of the larvae in the breeding sites (Bates 1949; Muirhead Thomson 1951; McCrae 1984; Minakawa *et al.* 1999). Traditionally, breeding sites containing *Anopheles* larvae have been characterized in order to determine the factors that regulate larvae abundances and to determine factors that could be used to predict the most productive mosquito habitats in order to design cost-effective vector control strategies targeting immature stages (Minakawa *et al.* 1999; Gimnig *et al.* 2001; Shililu *et al.* 2003; Minakawa, Sonye & Yan 2005; Mwangangi *et al.* 2007; Muturi *et al.* 2008). Consequently, these studies focus either on the late instar larvae as a proxy measure for adult productivity or do not distinguish between early and late instars. Frequently, it is cited that *An. gambiae s.l.* prefers to breed in temporary, shallow, sunlit, bare-edged pools and puddles (WHO 1978; Mereta *et al.* 2013), however, there are usually only two references cited that base their summary on a very limited set of studies (Muirhead Thomson 1951; Gillies & De Meillon 1968). These attributes alone failed to predict the most productive habitats that maintain malaria transmission throughout the year in many routine vector control programs and research studies (Minakawa *et al.* 1999; Killeen *et al.* 2006; Majambere *et al.* 2008).

Consequently, there has been a renewed interest in the larval ecology of afro-tropical malaria vectors over the past 15 years. Studies set out to identify and quantify additional physicochemical and biological factors that can regulate larvae abundance and distributions in natural settings (Fillinger *et al.* 2004; Majambere *et al.* 2008; Fillinger *et al.* 2009b; Mala & Irungu 2011; Ndenga *et al.* 2011; Gouagna *et al.* 2012; Gilbreath *et al.* 2013; Mereta *et al.* 2013). Although most of the studies claim the importance of environmental (i.e. pH, dissolved oxygen, water temperature, turbidity) and biological factors (i.e. emergent vegetation, algae, invertebrate fauna) in the survival of mosquito larvae, the strength and direction of these associations are often ambiguous (Mwangangi *et al.* 2007; Muturi *et al.* 2008; Fillinger *et al.* 2009b; Kenea, Balkew & Gebre-Michael 2011; Mala & Irungu 2011; Ndenga *et al.* 2011; Mereta *et al.* 2013). As an example, it has been implied that the presence of carnivorous aquatic insects is always to the detriment of *Anopheles gambiae s.l.* larvae populations and therefore high densities of larvae are associated with small and temporary habitats where the risk of predation is reduced (Muturi *et al.* 2008; Gouagna *et al.* 2012; Mereta *et al.* 2013; Munga, Vulule &

Kweka 2013). However, this is not always the case. For example, increasing invertebrate diversity has been significantly associated with the most productive breeding sites in The Gambia (Fillinger *et al.* 2009b) and coleopterans of the Haliplidae family with high abundances of *Anopheles* larvae in rice ecosystems in Kenya (Muturi *et al.* 2008).

The impact of different environmental and biological factors on the oviposition site selection of gravid *Anopheles gambiae s.l.* has not been systematically explored under natural field conditions, which might in part be because of the difficulty to sample single eggs of *Anopheles* mosquitoes in the field and in part because although choices can be seen based on the presence or absence of larvae the mechanisms underlying those choices are often intelligible (Bates 1949). In the case of the members of the *Anopheles gambiae* species complex, very few field studies have been carried out to detect their oviposition preferences. Early in the 1950s Muirhead-Thompson established that gravid *An. gambiae s.l.* was not deterred from egg-laying in shaded habitats covered by a horizontal screen located above the surface of the habitat. Furthermore, he also observed that *An. gambiae s.l.* avoided laying eggs in brackish water collected from sub-littoral areas of Nigeria and Sierra Leone (Muirhead Thomson 1951). Apart from these studies all what we know about oviposition behaviour in *An. gambiae s.l.* has been drawn from laboratory experiments performed in small cage settings.

It has been hypothesized that oviposition behaviour alike host seeking is governed by an ample sort of physical and chemical cues that guide gravid females in the process of searching, selecting and laying of their eggs in a suitable aquatic habitat (Bentley & Day 1989; Clements 1999; Takken & Knols 1999). Caged *Anopheles gambiae s.l.* responds to physical cues like light contrast and brightness and have been shown under some circumstances to prefer laying eggs on dark backgrounds rather than in pale ones. Darkening the bottom of a dish could increase egg laying up to three fold (McCrae 1984; Huang *et al.* 2007). Furthermore, gravid females lay more eggs in muddy water and water from breeding sites than tap or distilled water in choice tests in small cages (McCrae 1984; Sumba *et al.* 2004; Huang *et al.* 2005). It has been suggested that preferences could be related to microorganisms present in the water or volatile organic compounds released from the water substrates (Sumba *et al.* 2004; Lindh *et al.* 2008). In the case of chemical cues it has been demonstrated that caged gravid females are receptive to water vapour (Okal *et al.* 2013), bacteria-derived odours (Sumba *et al.* 2004; Huang *et al.* 2006a; Lindh *et al.* 2008) and predator-released kairomones (Warburg *et al.* 2011). Whilst over 20 putative oviposition semiochemicals have been suggested in the

literature (Blackwell & Johnson 2000; Lindh *et al.* 2008) there is only one report (Rinker *et al.* 2013) of two chemicals inducing a behavioural response in caged *An. gambiae s.s.* gravid females (one increasing and one decreasing the oviposition response). Although these controlled experiments have given important insights on *An. gambiae s.l.* oviposition preferences it remains to be proven if these responses can be extrapolated from the laboratory to the natural ecosystems where visual and chemical stimuli are likely to interact in a way that is unknown in the uncontrolled systems (Clements 1999).

To date all studies involving substrates from breeding sites (i.e. bacteria, water, mud) have been performed solely on samples collected from aquatic habitats containing larvae but no attempt has been made to compare natural habitat characteristics of habitats that are colonized by early instar *An. gambiae s.l.* larvae (as a proxy measure for recent oviposition events) with those that do not contain early instar larvae. More importantly, although bacteria and volatile chemicals have been implicated as being responsible for habitat choice under controlled conditions (Sumba *et al.* 2004) bacteria and chemical profiles have never been analysed and compared for natural habitats to verify the hypothesis.

Here I test the following hypotheses:

- (1) Natural aquatic habitats without *Anopheles gambiae s.l.* larvae (controls) differ significantly from habitats that are well colonized by early instar larvae (cases) in their bacteria communities and in their profile of volatile chemicals released from the water.
- (2) Specific physical, chemical and biological characteristics of these habitats colonised by *Anopheles* larvae can be associated with the bacteria and chemical profiles and can assist in predicting habitat selection by gravid females.

To test these hypotheses I aimed to comprehensively characterize aquatic habitats with high abundance of early instar *Anopheles* larvae and aquatic habitats without early instar *Anopheles*. Specifically, I aimed to determine environmental attributes, water chemistry, vertebrate fauna, zooplankton, bacterial communities and chemical profiles.

4.3 Material and Methods

4.3.1. Study area

Larval habitat surveys were implemented on Rusinga Island, Lake Victoria, Western Kenya (0°35'–0°44' South; 34°11'–34°22' East; altitude 1,100 m) during the long rainy season from March to July 2012.

Rusinga is the second largest island in Lake Victoria. Since 1983 it has been connected to the mainland through an approximately 200 m long and 40 m wide causeway (Figure 4.1). It has an area of 42 km² and approximately 24,000 inhabitants. Fishing and small scale farming of maize, millet and animal husbandry are the main economic activities (Weckenbrock & Oldesloe 2005; Kaneko, Mushinzimana & Karama 2007).

Rusinga Island has a rocky and hilly terrain of volcanic origin with scarce vegetation cover. There are several seasonal rivers that contain water only during the rainy season so the lake is the main source of water for the human population and livestock. Two rainy seasons are typically described for the area, the main rainy season between March and June and the short rainy season between October and December (Opiyo *et al.* 2007). Malaria is endemic in the area but transmission intensity fluctuates seasonally having the main peak at the end of the long rainy season. Immature stages of mosquitoes have been found in a variety of natural and man-made breeding sites several of which occur near to human settlements (Fillinger *et al.* 2004; Mukabana *et al.* 2006). The three major African malaria vectors occur in the area; in the order of abundance these are *Anopheles arabiensis*, *Anopheles gambiae s.s.* and *Anopheles funestus* (Minakawa *et al.* 2012).

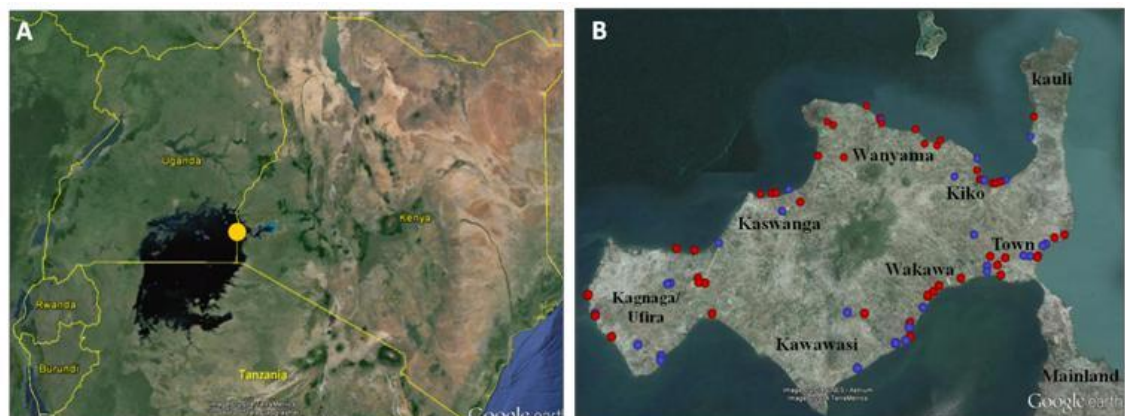


Figure 4.1. Study area A) Lake Victoria Region, East Africa; Yellow dot = Location of Rusinga Island, Kenya B) Map of Rusinga Island showing the 8 administrative zones and the distribution of habitats not colonized (controls=blue dots) and colonized by *Anopheles* larvae (cases=red dots).

4.3.2. Larval habitat mapping

In order to identify factors associated with the presence of early *Anopheles* larvae a case-control approach was used to compare biological, physical and chemical characteristics of aquatic habitats highly colonized by early instar *Anopheles* larvae (cases) with habitats without early instars (controls). Larval surveys were done three days a week from March to July. Every sampling day another location on the island was surveyed. In a previous study (Mukabana *et al.* 2006; Opiyo *et al.* 2007) Rusinga Island was divided into eight administrative zones (Figure 4.1 B.). Each zone was further divided in six sub-zones consisting of two villages and larval surveys were implemented regularly by local community members from each sub-zone (Malaria Surveillance Team (MST)). These community members were mobilized for this study to assist in mapping of all aquatic habitats per sub-zone per day. The sub-zones that were to be visited during one week were randomly selected from the list of all sub-zones a week prior to the surveys using a random-number generator. Each sub-zone was only visited once, all sub-zones had been surveyed at the end of the study. With help of the MST all aquatic habitats in a sub-zone were located in the morning and their position recorded using a Global Positioning System, GPS (GPS 12XL, Garmin, 15 meters accuracy, Schaffhausen - Switzerland). Every habitat received a unique identification number.

4.3.3. Sweep-net method for habitat sampling

Habitats were sampled using the sweep-net method (Silver 2008; Ndenga *et al.* 2011). This method was considered better than the more commonly used dipping method since it provides data on diversity and abundance (numbers per surface area) of the majority of aquatic invertebrates and is more effective in collecting early instars and pupae of mosquitoes since a larger surface area and volume of water is sampled in much shorter time (De Klerk & Wepener 2011). The sweep net was made of a cotton cloth, 30 cm wide, 15 cm high and 40 cm long fixed on a 150 cm long handle. In order to invest a similar collection effort in small and large habitats, the method was first calibrated by sampling twenty-nine habitats from three different subzones. Ten sweeps were taken from each habitat. The net was submerged at an angle of 45° at the margin of the habitat and the habitat swept 1 m in length. The content of every sweep was kept separately in a white tray and the number of early (1st and 2nd instar) and late (3rd and 4th instar) *Anopheles* larvae counted. The area of every habitat was measured and used to estimate the minimum sampling effort (number of sweeps) required per square meters until no

mosquito larva was caught in a sweep anymore. Two to three sweeps were sufficient to collect all larvae per square meter. Consequently, habitats $\leq 1\text{m}^2$ were swept three times. Two sweeps per square meter were done in habitats with an area between 1 and 20m^2 . When a habitat was bigger, a 20m^2 section was purposely selected (highest probability of finding larvae) and 40 sweeps taken.

4.3.4. Selection of cases and controls

Every aquatic habitat identified during the mapping was measured for its size and sampled using the sweep-net method described above. The content of each sweep was emptied in a white tray and the number of early and late instar *Anopheles* and culicines were counted. Other invertebrates present in the net content were also counted and determined in order, suborder or family level as follow: Odonata (Zygoptera and Anisoptera), Ephemeroptera, Coleoptera (adult and larvae stages counted separately). Heteroptera (Notonectidae, Naucoridae, Nepidae). Fish and tadpoles were also recorded. After completing the count, all 4th instar *Anopheles* larvae were collected in ethanol for molecular identification of the members of the *An. gambiae* species complex using polymerase chain reactions PCR (Scott, Brogdon & Collins 1993). The remaining content of the trays was returned into the habitat.

A habitat was eligible to be a case if ≥ 20 early instar *Anopheles* larvae were sampled from a habitat. A habitat was eligible to be a control if no *Anopheles* larvae were collected from the habitat. Habitats with more than 1 and less than 20 early *Anopheles* larvae were designated as intermediate and were not eligible for random selection and further characterisation.

Once all the habitats of a subzone were surveyed, a maximum of two cases and two controls were randomly selected (lottery by drawing a slip of numbered paper) in order to carry out more intensive habitats surveys and to collect samples to analyze zooplankton, bacteria communities and volatile chemical profiles.

4.3.5. Characterization of cases and control habitats

Each aquatic habitat was classified into one of six habitat types: Swamp, puddle, fish pond, cemented-lined pit, borrow pit or drainage (Figure 4.2). These categories represent the diversity of aquatic habitats within the study area (Fillinger *et al.* 2004; Minakawa *et al.* 2012).

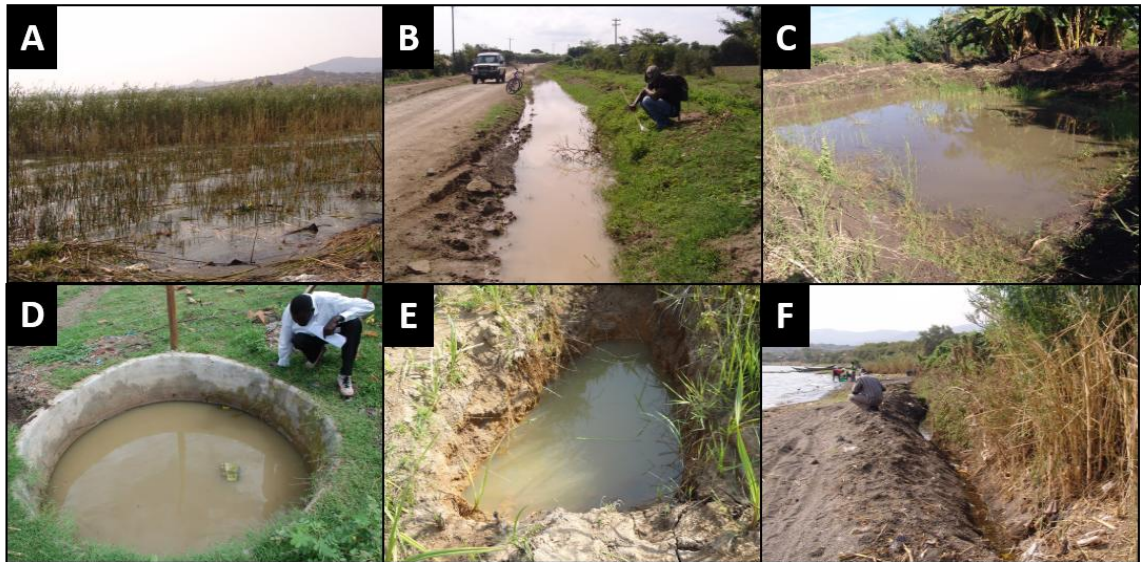


Figure 4.2. Common aquatic habitat types recorded on Rusinga Island. A) Swamp: Area along the lake shore where water is permanent to semi-permanent. Vegetation is often characterized by tall grasses (reeds) and/or floating plants. B) Puddle: Natural and shallow depression (less than 0.5 m deep) that collects rainwater. C) Fish pond: Large man-made pool (>1m deep, >5m long and wide) used for fish farming. D) Cement-lined pit: Pit serving as water reservoir in building sites. E) Borrow pits: Man-made holes in any ground (for taking soil, for getting stones, for building a pit latrine), that can collect rain water or be filled by ground water. F) Drainage: Long narrow excavation in the earth for carrying off excess water or sewage.

Data on size (perimeter), soil type (sand, loam, clay, artificial), water origin (rain, lake, ground), land cover (floodplain of the lake, agricultural fields, compound/bush), presence of floating vegetation (e.g. *Eichornia spp.*, *Lemna spp.*) and presence of algae film on the surface or any other debris were recorded for every habitat. The proportion of habitat covered with emergent vegetation on the surface and on the edge and the proportion of shade were visually estimated. Water depth was measured with a wooden meter at the centre of the habitat when possible and for larger habitats at several points and averaged. Distances to the nearest house and to the lake were measured using the GPS trekking function.

Conductivity, pH, dissolved oxygen and water temperature were recorded with a multimeter (Multi 340i, WTW Germany). Turbidity was measured with a turbidity meter (TURB 355IR, WTW Germany).

Habitat water was tested for ammonium (NH₄⁺), carbonate hardness, total hardness, nitrate (NO₃⁻), nitrite (NO₂⁻), and phosphate (PO₄³⁻) content using Aquamerck® test kits from the compact laboratory for water testing (Aquamerck®No.111151, Germany).

Water samples for bacteria and chemical headspace analyses were collected as follows. First four sub-samples of water of approximately 350 ml were taken in different depths of the habitat using a standard dipper (350 ml dipper, Clarke Mosquito Control Products, USA), two on the ground and two on the surface ensuring that different sections of the habitat are represented (i.e. shade/no shade, vegetation/no vegetation). The sub-samples were sieved with a clean cotton cloth and mixed gently in a plastic basin that was washed several times before with habitat water. From the mixed sample, 100 ml of water were collected in two new sterile Falcon™ type tubes (50 ml) to carry out DNA bacteria analysis; 250 ml of water were collected in wide mouth polypropylene bottles (250 ml) for collection of the chemical headspace (Agelopoulos & Pickett 1998) and 50 ml of water was taken in an amber polyethylene terephthalate (PETE) tube (50 ml) with a Teflon lid to determine the biological oxygen consumption (BOC) for every habitat. All samples were placed in a cooler box and transferred to the laboratory facilities at ICIPE, Thomas Odhiambo Campus (ICIPE-TOC) located 2-9 km from the sampling sites. Water samples for bacteria analyses were stored at -70°C freezer (C340 New Brunswick Scientific, USA) until use. Water samples for chemical headspace analyses were prepared for volatile trapping by adding 37.5 g of NaCl to each sample (Mozuraitis, Buda & Borg-Karlson 2010). The bottles were completely full and did not contain any air. They were tightly closed and sent to the Chemical Ecology Laboratory at ICIPE-Nairobi on the day of sampling and analyzed within 16 hours of sampling. BOC was estimated by subtracting the dissolved oxygen content measured in day 5 post sampling from dissolved oxygen content measured in day zero (sampling day).

Water samples for identification of zooplankton were taken according to the size of the habitat: 2 L for water bodies >10 m², 1 L for water bodies between 1-10 m² and 0.5 L for water bodies <1 m². Water was collected with a standard dipper (350 ml dipper, Clarke Mosquito Control Products, USA) from different spots of the habitat and passed through a sieve with a 50 µm mesh size. Organisms in the sieve were washed with 70% ethanol into a sampling glass (20 ml). Samples were protected against UV radiation in a cooler

box and transported to the laboratory for further identification. Samples were stored at room temperature until the organisms were counted and classified under the microscope (Zeiss, Germany) to family level for Cladocera, Suborder for Copepoda and Class for Ostracoda (Edmondson 1959; Witty 2004).

4.3.6 Bacteria community analysis

DNA extraction, amplification and purification: Water sample (50 ml) duplicates were thawed at room temperature and centrifuged at 4000 g for 30 min at 6°C. The resulting pellet was transferred into a 1.5 ml eppendorf tube and the total nucleic acid was extracted using the protocol supplied with the DNeasy® Blood & Tissue Kit (Qiagen, Hilden – Germany). Crude DNA was used as a template to amplify the 16S rDNA region with universal bacteria primers 968f gc: (5' CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC 3') and 1401r: (5' CGG TGT GTA CAA GAC CC 3'). DNA extracted from water samples was amplified using Ready-To-Go PCR beads (GE healthcare, Little Chalfont - United Kingdom) 1µl of each primer (10 pmol/µl), 1µl of DNA template and nuclease free water (Bioline, London, United Kingdom) were added to achieve a final reaction volume of 25µl. After an initial denaturation of double stranded DNA for 5 minutes at 94°C, the following protocol was used: denaturation at 94°C for 30 seconds, touchdown annealing 58-48°C (decreasing in 1°C /cycle) for 30 seconds, extension 72°C for 30 seconds ; 20 cycles consisting of denaturation 30 seconds at 94°C, annealing 30 seconds at 50°C, extension 30 seconds at 72°C; and final extension 72°C for 5 minutes. In order to concentrate the samples, two PCR reactions (each 25 µl) were carried out per sample, pooled and purified in a final volume of 10 µl using a MinElute® PCR Purification Kit (Qiagen, Hilden – Germany). PCR products were separated by electrophoresis in a 1.5% agarose gel stained by ethidium bromide. Amplicon sizes were determined by comparison to the molecular weight provided by Hyperladder™ V (Bioline, London-United Kingdom).

Denaturing Gradient Gel Electrophoresis (DGGE): PCR products and DNA ladders were analyzed using a Dcode™ Universal Mutation Detection System (Bio-Rad, Hercules, CA - USA). The DNA fragments were separated on a polyacrylamide gel with 8% (wt/vol) acrylamide, containing a linear gradient of denaturant that ranges from 30% to 60% (with 100% denatured defined as 7M Urea plus 40% formamide). Approximately, 10 µl of PCR product from each sample were loaded in a separate well of a DGGE gel. The gel ran at 70V for 16.5 h and 60°C. After completion of

electrophoresis, gels were incubated for 90 minutes shaking at 60 rpm in a staining solution containing 3X GelRed™ in 0.1M NaCl. Digital photos were obtained immediately after staining in an InGenius LHR (Syngene, Cambridge, United Kingdom).

PCR products of cases and controls were grouped according to the habitat type and were run simultaneously on the same gel. Hyperladder™ V (Bioline, London-United Kingdom) was run in parallel with the samples and was used as a reference lane to estimate molecular weights (MW) and to construct the intensity profile of each lane.

Analysis of DGGE gel images: Gel images were analyzed using Image Lab™ software version 5.1 (Bio-Rad Lab, Hercules, CA - USA) to calculate bacteria abundance and diversity of each aquatic habitat. Initially lanes and bands were detected automatically by the software, and then the sensitivity of each lane and additional bands were assessed and edited manually. Hyperladder V was selected as the reference lane and it was used by the software to calculate the molecular weights (positions) for each band. Band number two (300pb) in the hyperladder V was also selected as the reference band to calculate the relative quantity of each band. Data were exported to an Excel spreadsheet and sorted according to molecular weights. Bands with the same molecular weight were assigned a unique band number. Every analysis was done separately for every gel. Information regarding habitat type, habitat ID and habitat status (case/control) was also added in the database.

Each band was considered an operational taxonomic unit (OTU). Banding patterns of each gel were analyzed to assess bacterial species diversity as described by Ponnusamy et al. (Ponnusamy *et al.* 2008b).

Diversity of bacterial species: The relative quantity data were used to calculate Shannon-Weaver diversity indices (H'):

$$H' = -\sum P_i \log P_i$$

n_i = OTU intensity (relative quantity) for i th band, N = Summed intensities of all OTUs in a lane, P_i = Proportion of total diversity represented by the i th species (OTUs) $P_i = n_i/N$

Evenness index (E) was estimated for the number of bacterial species in each lane as follow

$$E = H'/\text{Log}S$$

S = Number of DGGE OTUs in each lane.

4.3.7. Chemical headspace analysis

Volatile organic compounds released from the habitat water were trapped by solid phase micro extraction (SPME). Trapping was done in parallel for cases and controls collected on the same date. In addition the chemical headspace was collected from a bottle containing distilled water, stored and transported the same way as the field samples and from an empty bottle that served as control for background volatiles.

Water samples were added into 500 ml conical flasks (Quickfit, England) which were covered with a double piece of aluminum foil. The aluminum foil cover was pierced with the needle of the SPME holder and the 65 μ m polydimethylsiloxane / divinylbenzene (PDMS-DVB) fiber (Supelco™, Bellefonte, PA, USA) exposed to the headspace above the sample. The SPME fibers were conditioned in the GC-injection port at 220°C for 5 minutes just before sampling. Volatiles were collected for 16 hours after which they were immediately analyzed by gas chromatography coupled to mass spectrometry (GC-MS).

Compounds trapped on SPME fibres were analysed on a GC-MS system comprising a 7890A GC (Agilent Technologies, Santa Clara, CA) fitted with a 30 m HP-5MS column (Agilent Technologies) with an inner diameter of 0.25 mm and 0.25 μ m film thickness. The GC was coupled to a 5975C MS (Agilent Technologies) with electronic ionization at 70 eV and the ion source kept at 230°C and the quadropole at 150°C. Masses were scanned from 38-550. The GC injector was kept at 250°C in a splitless mode, helium with a flow of 1.2 ml/min was used as carrier gas. The oven temperature was held at 40°C for 3 minutes, then programmed to increase at 5°C/min to 260°C and maintained at this temperature for 3 minutes for a total running time of 50 minutes.

For the comparison of volatile chemical profiles from the headspaces of aquatic habitats, the retention parameters from the chromatograms were integrated using the Chem station integrator and auto-integration function in Chem Station software (MSD ChemStation E.02.01.1177). Mass spectra fragments were recorded manually for each compound integrated. Compounds were grouped according to retention time, MS fragments, and given a unique volatile identification number (ID). A database was

created with information about the abundance of each volatile (area of the peak) in the samples and used for analyses.

4.3.8 Data analyses

Generalized linear models (GLM) with a negative binomial distribution were used to estimate means and 95% confidence intervals for each factor measured in the cases and control groups. Factor that differed significantly between cases and controls were further included in a predictive GLM to evaluate their impact on the presence of early instar *Anopheles* larvae. Analyses were performed in SPSS statistical software version 20.

Principal component analyses (PCA) were used to explore possible associations of bacteria communities, chemical profiles and case and control habitats in reduced dimension plots. This analysis allows to visualize the data without constraint of an initial hypothesis concerning the relationships between bacteria, volatile chemicals and aquatic habitats either control or case.

Bacteria and chemical profiles were only analysed for 60 habitats which presented the extreme ends of the cases and controls in terms of early instar larval abundance. The 30 cases with the highest early instar larval abundance (>43 early instar larvae per m^2) were selected for analyses. These were compared with 30 controls that were randomly selected (Random Sorter in Microsoft Excel) from the controls. Bacteria communities' analyses were carried out separately for every habitat type. In each analysis relative densities of OTUs were centred and standardized. For volatile chemical analyses data were centred and standardized by relative amount of volatile chemical. PCA was chosen in all ordination analyses given that all data were compositional and gradient lengths were less than three standard deviation units long. PCA was performed in CANOCO 5 for windows version 5 (Ter Braak & Šmilauer 2012).

4.4 Results

In total 219 habitats were sampled across the eight zones of Rusinga Island during the long rainy season of 2012. Of those 44 habitats were included in the analyses as controls and 72 as cases. One hundred and three intermediate habitats with more than 1 but less than 20 early *Anopheles* larvae were excluded from the analyses. All specimens that amplified during PCR mosquito identification (168/182) were *An. arabiensis*.

Presence of aquatic habitats colonized and not colonized by *Anopheles* larvae was strongly influenced by rainfall. At the onset of the rainy season aquatic habitats were

scarce and only a small fraction was positive for *Anopheles* larvae. In addition, habitats were colonized in low density which is evidenced by the high proportion of intermediate habitats found. As soon as the rains progressed the number of larval habitats increased and also the density of early instar larvae per habitat leaving very few habitats free of mosquito larvae during between early June and early July. The number of aquatic habitats without *Anopheles* larvae increased again at the end of July following a month without rain (Figure 4.3).

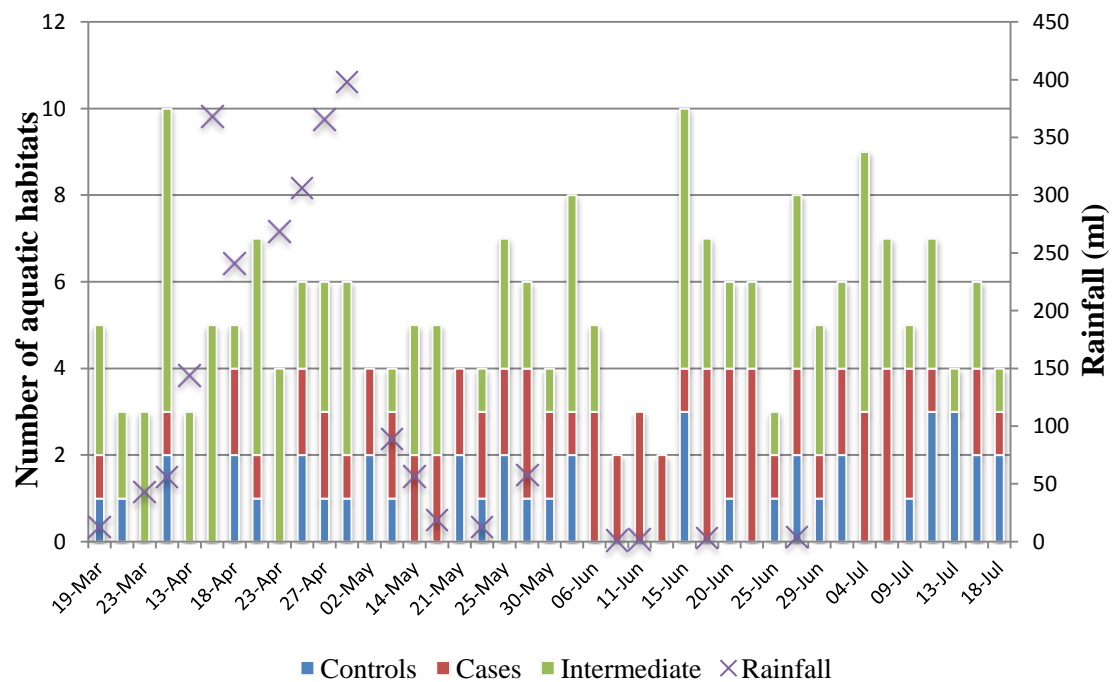


Figure 4.3. Frequency of occurrence of controls and cases during sampling dates and their relation with rainfall.

4.4.1 Differences in environmental and biological factors between control and case habitats

Initially, modelled means and 95% confidence intervals (CIs) were calculated for every factor measured during the habitat surveys and compared between control and case habitats. Ten characteristics differed significantly in their means between these two groups (Table 4.1) and were used to construct a predictive multivariate model that could explain the differences in mosquito colonization between the two groups. Out of ten factors included in the model only the abundance of late instar *Anopheles* larvae and turbidity remained significantly associated with the presence of early instar *Anopheles* larvae (Table 4.1).

Oviposition (as expressed by high numbers of early instar *Anopheles* in cases) was strongly and positively associated with the abundance of conspecific late instars. One unit increase in late instar larvae increased the odds of an aquatic habitat being a case four times (Table 4.1).

Table 4.1 Differences of biological and environmental factors between control and case habitats

Factor	Means (95% CI)		Odds ratio ^a (95% CI)	GLM P-value
	Controls	Cases		
<i>Anopheles</i> late/ m ²	0.24 (0.18-0.32)	7.17 (5.69-9.04)	4.078 (1.683-9.880)	0.002
Naucoridae/m ²	1.50 (1.12-2.02)	0.31 (0.25-0.39)	0.760 (0.300-1.926)	0.563
Coleoptera adults/m ²	1.14 (0.85-1.53)	2.32 (1.84-2.92)	1.227 (0.951-1.584)	0.116
Fish/m ²	1.19 (0.89-1.60)	0.31 (0.24-0.38)	0.882 (0.607-1.281)	0.509
Cyclopoida/L	74.8 (55.7-100)	189 (150-238)	0.998 (0.994-1.002)	0.407
Nauplius/L	280 (208-376)	620 (492-781)	1.000 (0.999-1001)	0.629
Moinidae/L	2.48 (1.85-3.34)	149 (118-187)	1.011 (0.981-1.042)	0.479
Turbidity (NTU)	56.2 (41.8-75.6)	205 (163-259)	1.005 (1.001-1.010)	0.025
Ammonium (mg/L)	0.20 (0.15-0.27)	0.06 (0.05-0.08)	0.312 (0.029-3.399)	0.339
Nitrite (mg/L) ^b	0.01 (0.01-0.02)	0.04 (0.03-0.04)	-	-

^aoutput of the multivariate GLM modeling the probability of a habitat being a case based on the listed explanatory variables

^bNitrite is a redundant parameter excluded from the model. Only 16% of the habitats had a value different from zero for nitrites.

Turbidity was the only environmental factor associated with the presence of *Anopheles* mosquitoes, every unit (NTU) increase in turbidity augmented the probability of a habitat to be colonized by *Anopheles gambiae s.l.* by 0.4 % (Table 4.1). Exploratory data analysis (Figure 4.4) showed that if turbidity of a habitat was > 200 NTU the habitat was most likely a case (22 cases/26 habitats >200 NTU). Only four habitats measured >200 NTU were not colonised by mosquitoes; these water bodies had exceptionally high coverage of vegetation, one of them had a complete surface layer of floating duck weed (*Lemna* sp.). Consequently, the turbid surface of the water may have not been visible (or otherwise detectable) for the gravid females or the unfavourable environmental conditions might have overwritten the preference for high turbidity.

Ninety habitats exhibited turbidity < 200 NTU and of those 55% (50/90) were colonised by *Anopheles*.

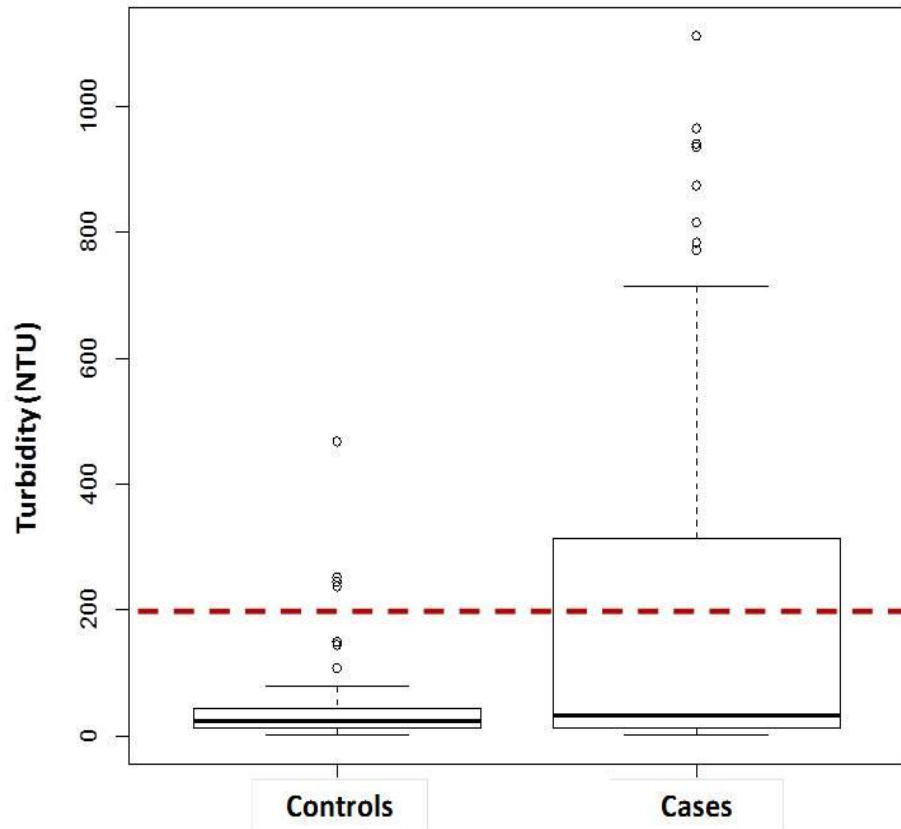


Figure 4.4. Box-and-Whisker plots showing the median turbidity and interquartile range for control and case habitats. Red line at 200 NTU.

Based on this results two major questions arose:

- (1) What differences other than turbidity exist between habitats with < 200 NTU turbidity (henceforth called clear habitats) and habitats with >200 NTU turbidity (henceforth called turbid habitats) that are colonized by *Anopheles* mosquitoes (cases)?
- (2) Are there any differences between clear habitat controls and clear habitat cases that might explain why these habitats have been chosen or not chosen for oviposition by *An. gambiae s.l.*?

To explore these questions the habitat data were divided in three groups according to turbidity levels: clear controls (n=40), clear cases (n=50) and turbid cases (n=22). The four control habitats with >200 NTU were excluded from the analysis.

4.4.2 Differences between clear and turbid case habitats

Clear and turbid case habitats were equally chosen for oviposition as expressed by the nearly identical abundance of early instar *Anopheles* larvae (22-23 early *Anopheles*/m²) in the two groups (Table 4.2). When comparing the explanatory variables for clear and turbid case habitats significant differences were found regarding invertebrate fauna composition, zooplankton composition and environmental factors. Turbid cases had twice as many late instar *Anopheles* larvae than clear cases. In addition, turbid cases were strongly associated with high densities of Cladocerans of the family Moinidae, copepods of the order Cyclopoida and Rotifera, which were rare in the clear cases. On the contrary, clear cases exhibited higher abundances of Odonata, Heteropterans of the family Notonectidae, and of cladocerans from the families Daphnidae, Chyboridae and Macrothricidae, than turbid cases. These results may indicate that although both clear and turbid habitats were chosen by gravid *Anopheles* females to oviposit turbid habitats provided an additional advantage to the larvae increasing their survival perhaps due to lower density of predators or due to a mutualism with the organisms in the same trophic level i.e. cladocerans of the Moinidae family. Turbid cases were on average four times bigger in area than clear cases or controls. (Table 4.2). Turbid cases were also characterized by four times higher phosphate content than clear cases. Phosphate concentrations were highly correlated with turbidity (Spearman's rho 0.57, $p < 0.001$).

All aquatic habitats on Rusinga Island irrespective of case or control were found within 100 to 300 meters from the next house and therefore blood hosts. However, clear cases were strongly associated with the lake, being never further away from the shore more than 200 meters, whilst turbid cases and controls could be found much further away from the lake (Table 4.2).

Turbid cases were found in all habitat types but being most common in the category of puddle/fish ponds. These habitats are constituted for abandoned fish ponds that keep rain water forming a puddle. Most of the turbid cases were located close to agricultural fields, filled with rain water and composed by loamy soil substrate. In contrast, the majority of clear cases were situated in the floodplains of the lake with equally distribution of habitats filled with rain or lake water and sand as primary substrate (Figure 4.5).

Table 4.2 Comparison of biological and environmental factors between clear (<200 NTU) control habitats, clear (<200 NTU) case habitats and turbid (>200 NTU) case habitats. Red indicates significant differences between clear habitats (controls versus cases) and blue indicates significant differences between clear and turbid cases.

Aquatic organisms*	Mean (95% CI)			RR (95% CI, p (in reference to control <200NTU))	
	control <200NTU	case <200NTU	case >200NTU	case <200NTU	case >200NTU
	N=40	N=50	N=22		
<i>Anopheles</i> early instars	0	22.2 (16.8-29.3)	23.1 (15.2-35.1)	p<0.001	p<0.001
<i>Anopheles</i> late instars	0.26 (0.19-0.35)	5.11 (3.87-6.74)	11.87 (7.82-18.02)	20.0 (13.1-30.2), p<0.001	46.3 (27.5-78.0), p<0.001
Ephemeroptera	2.65 (1.94-3.61)	3.93 (2.92-5.28)	0.46 (0.28-0.75)	1.48 (0.97-2.27), p=0.070	0.17 (0.10-0.31), p<0.001
Odonata	0.27 (0.20-0.37)	0.35 (0.27-0.47)	0.02 (0.01-0.03)	1.30 (0.85-1.96), p=0.220	0.08 (0.05-0.14), p<0.001
Notonectidae	7.24 (5.31-9.87)	7.64 (5.79-10.08)	0.74 (0.49-1.12)	1.06 (0.70-1.60), p=0.798	0.10 (0.06-0.17), p<0.001
Naucoridae	1.64 (1.21-2.24)	0.22 (0.17-0.29)	0.51 (0.34-0.78)	0.13 (0.09-0.20), p<0.001	0.31 (0.19-0.53), p<0.001
Corixidae & Nepidae	2.24 (1.65-3.03)	2.37 (1.79-3.12)	2.26 (1.55-3.58)	1.05 (0.70-1.60), p=0.803	1.05 (0.62-1.77), p=0.854
Coleoptera larvae	0.75 (0.55-1.02)	0.95 (0.72-1.25)	0.59 (0.39-0.89)	1.27 (0.84-1.93), p=0.255	0.78 (0.47-1.32), p=0.361
Coleoptera adults	1.23 (0.90-1.67)	2.52 (1.91-3.32)	1.86 (1.22-2.83)	2.05 (1.35-3.11), p=0.001	1.52 (0.90-2.55), p=0.117
Daphniidae	6.15 (4.40-8.59)	6.96 (5.17-9.36)	0	1.13 (0.72-1.77), p=0.590	p<0.001
Chydoridae	1.13 (0.73-1.72)	7.62 (5.67-10.23)	0	6.77 (4.03-11.37), p<0.001	p<0.001
Bosminidae	0.18 (0.08-0.39)	0	0	p<0.001	p<0.001
Sididiidae	9.60 (6.90-13.30)	0.06 (0.02-0.19)	0.22 (0.08-0.6)	0.01 (0.002-0.021), p<0.001	0.02 (0.01-0.07), p<0.001
Macrothricidae	12.50 (9.06-17.25)	37.66 (28.44-49.87)	0.77 (0.41-1.46)	3.01 (1.97-4.62), p<0.001	0.06 (0.03-0.13), p<0.001
Haparticoidea	0.65 (0.40-1.07)	0.60 (0.38-0.94)	0.00	0.92 (0.47-1.80), p=0.815	p<0.001
Moinidae	2.45 (1.68-3.51)	17.28 (12.99-22.98)	448 (295-681)	7.13 (4.47-11.35), p<0.001	184 (105-322), p<0.001
Ostracoda	59.97 (43.70-82.30)	64.90 (49.08-85.81)	10.46 (6.75-16.19)	1.08 (0.71-1.65), p=0.714	0.17 (0.10-0.30), p<0.001
Cyclopoida	70.70 (51.74-96.60)	58.68 (44.37-77.61)	487 (320-740)	0.83 (0.54-1.26), p=0.383	5.88 (4.09-11.61), p<0.001
Naupilius	243 (178-332)	258 (195-340)	1444(951-2194)	1.06 (0.70-1.61), p=0.788	5.93 (3.52-9.99), p<0.001
Rotifera	2217 (1626-3023)	953 (722-1258)	1390 (915-2112)	0.43 (0.28-0.65), p<0.001	0.63 (0.37-1.06), p=0.079
Fishes	1.31 (0.96-1.79)	0.43 (0.33-0.57)	0.02 (0.01-0.03)	0.33 (0.22-0.50), p<0.001	0.01 (0.01-0.02), p<0.001
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Turbidity (NTU)	32 (23-44)	35.14 (27-47)	593 (390-901)	1.11 (0.73-1.69), p=0.643	65 (11.05-31.48), p<0.001
Phosphates (mg/L)	0.63 (0.35-0.90)	0.60 (0.35-0.84)	2.25 (1.88-2.62)	0.97 (0.67-1.40), p=0.876	5.08 (3.21-8.04), p<0.001
conductivity (mS/sec)	366 (268-499)	466 (353-615)	620 (408-942)	1.28 (0.84-1.93), p=0.252	1.70 (1.01-2.85), p=0.047
pH	8.08 (7.86-8.30)	8.12 (7.93-8.32)	8.54 (8.25-8.84)	1.04 (0.78-1.41), p=0.753	1.59 (1.10-2.30), p=0.013
Nitrates mg/L	14.37 (10.43-19.81)	15.60 (11.72-20.76)	14.32 (9.29-22.06)	1.09 (0.71-0.67), p=0.709	0.99 (0.58-1.70), p=0.988
Dissolved oxygen (ppm)	5.90 (1.01-4.22)	7.38 (1.11-5.49)	6.91 (1.58-4.42)	1.25 (0.80-1.96), p=0.326	1.17 (0.66-1.05), p=0.580
Total hardness (mmol/L)	1.50 (1.15-1.84)	1.67 (1.35-1.98)	1.47 (1.01-1.94)	1.19 (0.76-1.90), p=0.463	0.98 (0.55-1.76), p=0.953
Carbonate hardness (mmol/l)	5.55 (3.96-7.77)	5.86 (4.34-7.91)	7.00 (4.48-10.94)	1.06 (0.67-1.66), p=0.813	1.26 (0.72-2.21), p=0.416
Habitat size (m ²)	6.60 (4.70-9.20)	6.50 (1.00-9.00)	25.60 (16.70-40.00)	0.99 (0.64-1.55), p=0.981	3.89 (2.26-6.67), p<0.001
Habitat shaded (%)	26 (19-35)	17 (12-22)	13 (8-19)	0.64 (0.42-0.99), p=0.043	0.49 (0.29-0.83), p=0.009
Edge grass cover (%)	47 (34-64)	66 (50-88)	52 (34-80)	1.42 (0.93-2.16), p=0.103	1.12 (0.66-1.89), p=0.677
Surface grass cover (%)	24 (18-33)	37 (28-50)	18 (12-28)	1.55 (1.02-2.37), p=0.042	0.76 (0.44-1.30), p=0.320
Distance to next house (m)	127 (93-173)	158 (120-209)	211 (139-322)	1.25 (0.82-1.89), p=0.300	1.67 (0.99-2.81), p=0.054
Distance to the lake (m)	318 (233-434)	148 (112-195)	378 (249-574)	0.46 (0.31-0.70), p<0.001	1.18 (0.71-2.00), p=0.517
water depth (cm)	32 (24-45)	23 (17-31)	20 (13-30)	0.71 (0.46-1.08), p=0.114	0.61 (0.36-1.04), p=0.069
* means for insects and fishes are per square metre, means for zooplankton are per litre					

RR are based on univariate modelling of the single explanatory factor where means are compared.

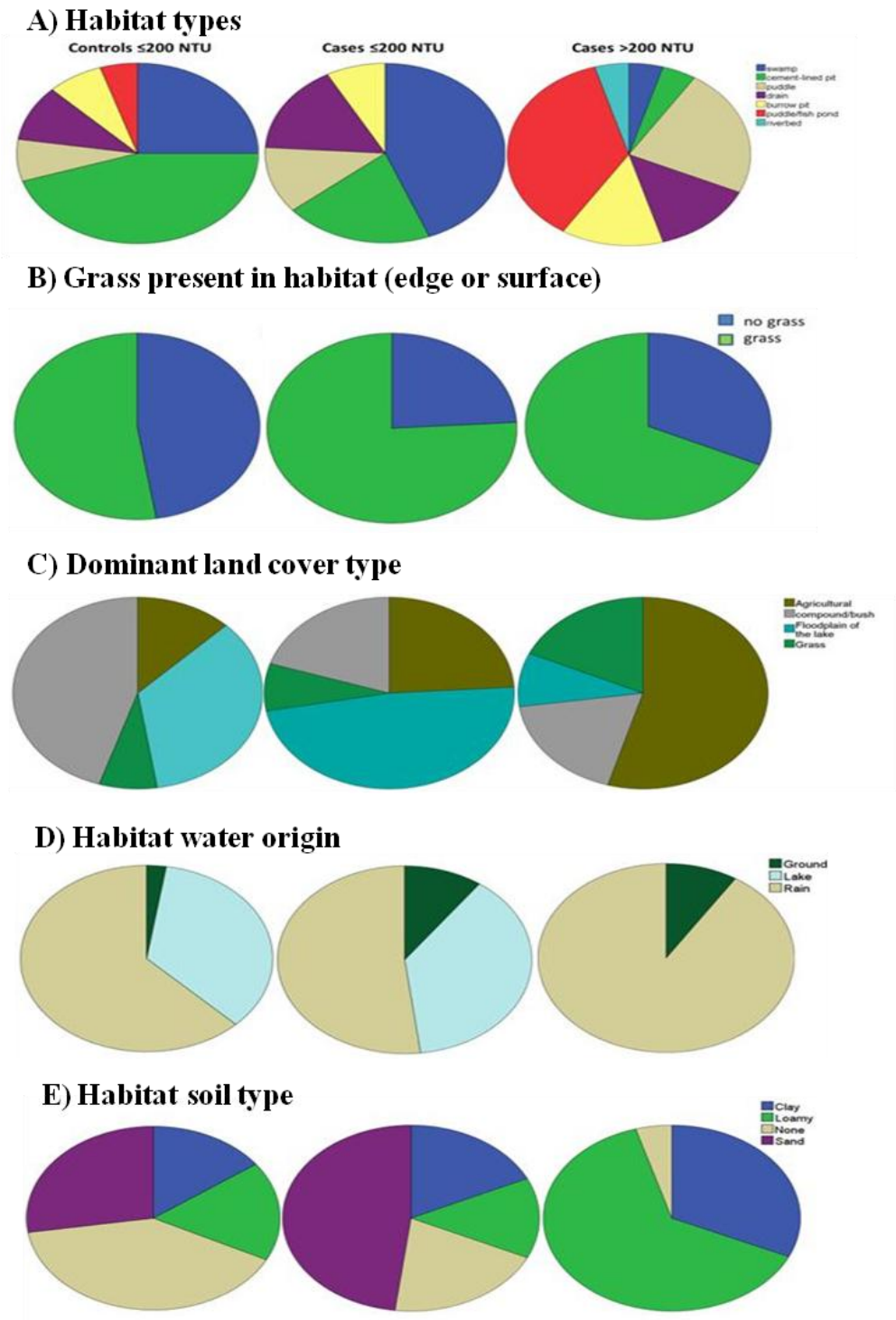


Figure 4.5. Distribution of environmental factors evaluated visually in clear control habitats (< 200 NTU), clear cases (< 200 NTU) and turbid cases (> 200 NTU). A) Habitat types, B) Grass presence in habitat including surface and edge, C) Dominant land cover type, D) Habitat water origin, E) Habitat soil type.

4.4.3 Differences between clear control and clear case habitats

Comparing the explanatory variables for clear control and clear case habitats a number of significant differences were found regarding invertebrate fauna composition, zooplankton composition and environmental factors (Table 4.2, Figure 4.5). The probability of a clear habitat becoming a case increased significantly with a unit increase of a late instar *Anopheles* larva (RR=20, 95% CI (13-30)), of cladocerans of the family Moinidae (RR=7, 95% CI (4-11)), of Chydoridae (RR=6, 95% CI (4-11)) and of Macrothricidae (RR=3, 95% CI (2-15)). Conversely, the probability of being colonized was drastically reduced with every unit increase of rotifers (RR=0.43, 95% CI (0.28-0.65)), of members of the Naucoridae family (RR=0.13, 95% CI (0.09-0.20)) and of fish (RR=0.33, 95% CI (0.22-0.50)). There were no differences between clear controls and clear cases in the density of other invertebrate predators like Odonata, Notonectidae, Corixidae, Nepidae and Coleoptera larvae; Coleoptera adults were even positively associated with cases (Table 4.2).

Grass covering some part of the water surface was significantly associated with oviposition in clear habitats. Clear cases had on average a higher percentage of the habitat covered by tufts of grass than clear controls (RR=1.55, 95% CI (1.02-2.37)). Notably, turbid case habitats did not differ in their grass coverage from controls. Grass cover in an otherwise translucent habitat might signal better protection for the offspring to the gravid female. Furthermore, for a clear habitat to be a case it had to be less than 200 meters away from the lake shore (Table 4.2) possibly indicating that the habitat is less likely to dry up than relatively small habitats further away from the lake or signalling a better water quality based on the fact that lake and ground water constantly replenishes these habitats.

4.4.4 Bacteria community analyses

DGGE analyses of 16S RNA gene PCR products were obtained for 60 water samples distributed in four habitat types: swamps, puddles, cemented lined pits and burrow pits. In each profile each band equalling an operational taxonomic unit (OTU) was assumed to represent a unique phylotype, therefore the richness of bacteria groups was reflected in the number of DGGE-DNA bands. Likewise, the intensity of a band was intended to reflect the relative abundance of that bacteria group in the sample.

Diverse DNA banding patterns were found in water samples collected from swamps (9-22 bacterial species, n=15), puddles (10-28 bacterial species, n=14), cemented lined pits (4-22 bacterial species, n=16), and burrow pits (13-22 bacterial species, n=15) (Figure 4.6.; Table 4.3)

4.4.5 Chemical headspace analyses

GC-MS analyses of headspace samples from 60 habitats lead to the detection of 556 compounds. There was a very high variability between habitats in terms of the numbers of compounds detected and amount released. The number of compounds ranged between 3-71 for a single habitat. The majority of compounds were rare with 338 out of the 556 compounds detected only once. For analysing the chemical profiles of control and case habitats, a new database was generated to include only those compounds that were detected in > 5 habitats. Consequently, the list of compounds reduced to 46.

The total relative amount of volatiles released from the headspaces of clear controls and clear cases was similar ($p=0.131$) whilst the total amount of volatiles released by turbid cases was significantly lower ($p=0.033$) compared with the amount released by clear habitats (Figure 4.7).

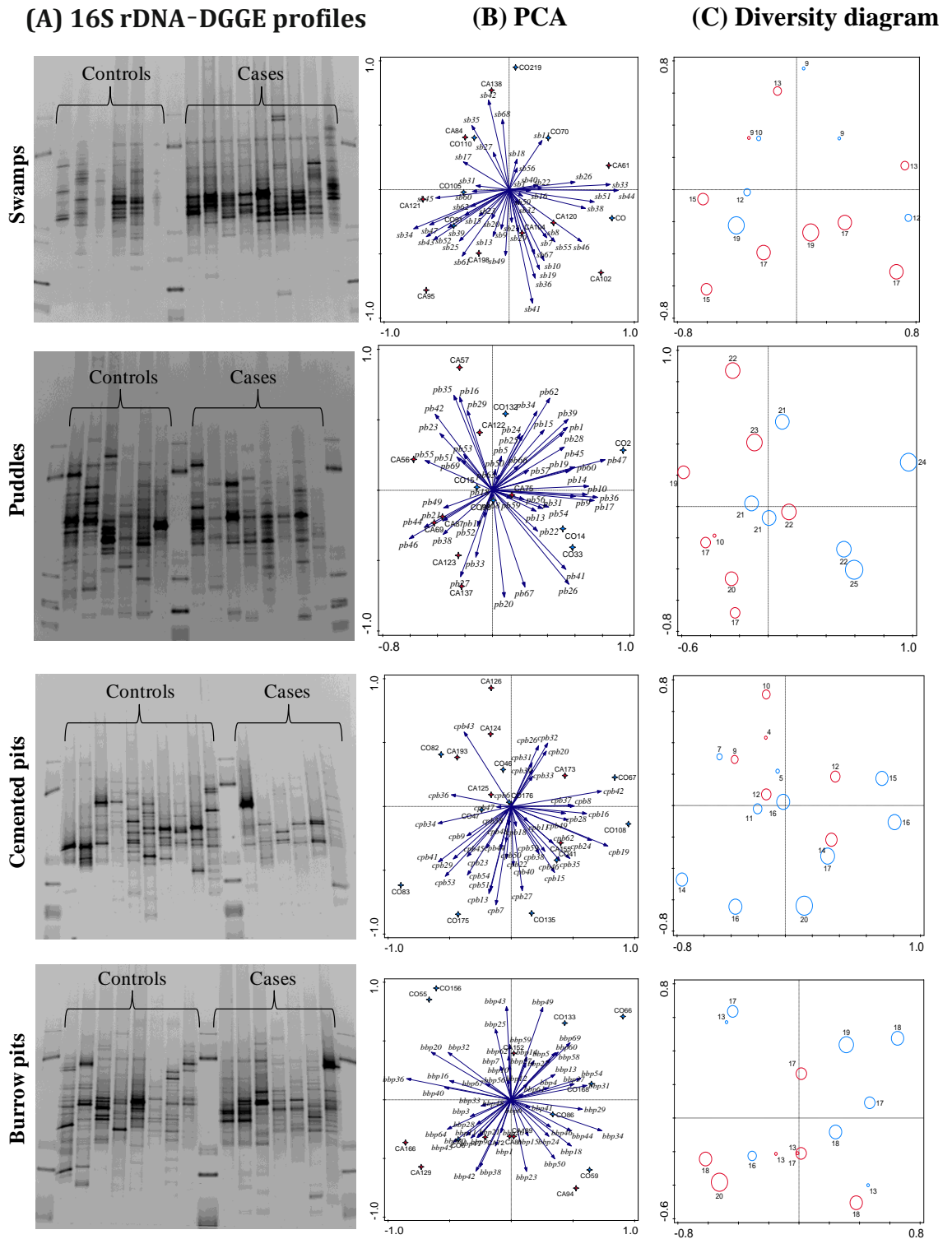


Figure 4.6. Analysis of bacteria communities from control and case habitats grouped in four different habitat types. A) 16S rDNA-DGGE profiles, every column represent an aquatic habitat and every band an operational taxonomic unit. Case and control habitats are separated by the reference (Hyperladder™ V (Bioline, London-UK)). B) Principal Component analyses plots; arrows illustrate every operational taxonomic unit detected, and the length of the arrow is associated with the amount of variation explained for that operational taxonomic unit in the

sample. C) Diversity diagrams; circle sizes and labels indicate the numbers of operational taxonomic units.

Table. 4.3. Detailed analysis of banding patterns in 16S rDNA-DGGE profiles, Diversity and Evenness indexes

Gel	Lane	No. OTUS (S)	Diversity index (H')	Evenness (H'/LogS)	Gel	Lane	No. OTUS (S)	Diversity index (H')	Evenness (H'/LogS)
Gel A	1	14	0.63	0.55	Gel C	1	18	0.80	0.64
Gel A	2	9	0.86	0.90	Gel C	2	5	0.28	0.41
Gel A	3	21	1.06	0.80	Gel C	3	13	0.69	0.62
Gel A	4	14	0.99	0.87	Gel C	4	15	0.83	0.71
Gel A	5	12	0.91	0.84	Gel C	5	8	0.80	0.88
Gel A	6	9	0.73	0.77	Gel C	6	15	0.84	0.72
Gel A	7	14	1.00	0.87	Gel C	7	19	1.07	0.84
Gel A	8	9	0.70	0.74	Gel C	8	22	1.04	0.77
Gel A	9	15	0.98	0.84	Gel C	9	20	1.00	0.77
Gel A	10	18	1.01	0.80	Gel C	10	18	0.99	0.79
Gel A	11	22	1.12	0.83	Gel C	11	4	0.50	0.83
Gel A	12	19	0.96	0.75	Gel C	12	13	0.93	0.84
Gel A	13	16	0.91	0.76	Gel C	13	10	0.67	0.67
Gel A	14	14	0.86	0.75	Gel C	14	14	0.65	0.56
Gel A	15	18	0.99	0.79	Gel C	15	12	0.76	0.70
Gel B	1	25	1.08	0.77	Gel C	16	9	0.81	0.85
Gel B	2	26	1.18	0.83	Gel D	1	16	0.08	0.07
Gel B	3	23	0.82	0.60	Gel D	2	14	0.90	0.78
Gel B	4	28	1.25	0.86	Gel D	3	13	0.92	0.83
Gel B	5	21	1.13	0.85	Gel D	4	19	1.06	0.83
Gel B	6	21	1.05	0.80	Gel D	5	18	1.02	0.82
Gel B	7	19	1.16	0.90	Gel D	6	22	1.05	0.78
Gel B	8	24	1.05	0.76	Gel D	7	17	0.97	0.79
Gel B	9	19	1.08	0.85	Gel D	8	20	1.01	0.78
Gel B	10	22	1.12	0.83	Gel D	9	13	0.92	0.83
Gel B	11	10	0.78	0.78	Gel D	10	18	1.07	0.86
Gel B	12	23	1.03	0.75	Gel D	11	19	0.93	0.73
Gel B	13	21	1.10	0.83	Gel D	12	20	1.16	0.89
Gel B	14	19	0.79	0.61	Gel D	13	19	0.91	0.71
					Gel D	14	19	1.09	0.85
					Gel D	15	14	0.93	0.81

Gel A = Swamps, Gel B= Puddles, Gel C= Cemented pits, Gel D = Burrow pits. Shaded cells correspond to habitats colonized by *An. gambiae s.l.* larvae (cases)

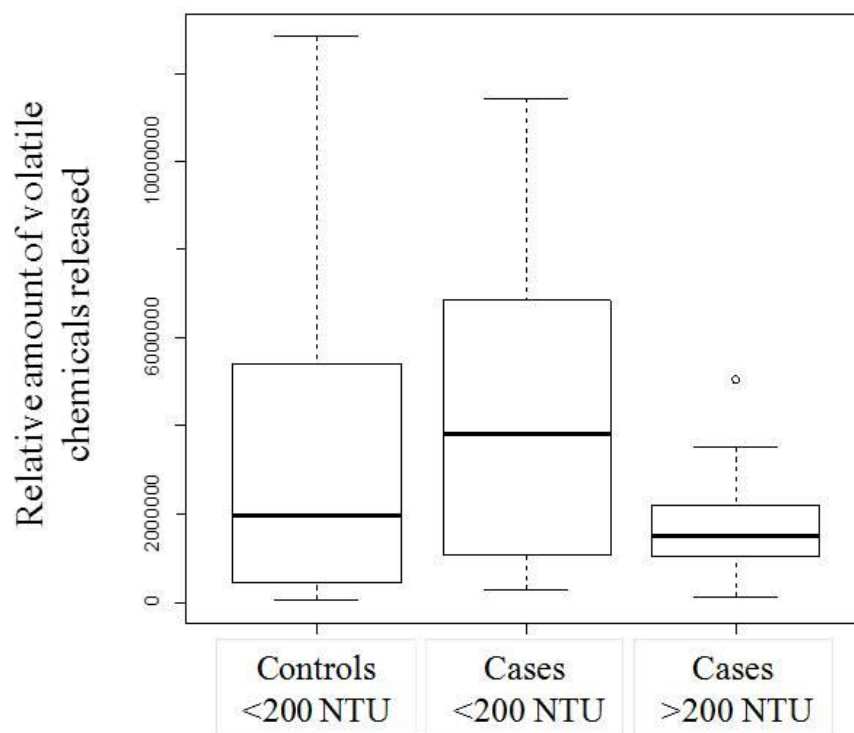


Figure 4.7. Box-and-Whisker plot showing median amount and interquartile range of the volatiles released (sum of relative amount) by clear controls (<200 NTU), clear cases (<200 NTU) and turbid cases (>200 NTU).

Principal component analysis of volatile chemicals detected in the three turbidity treatments (pooled data from all habitats in a group) revealed that most of the variation in the data (82%) was explained by the first two components which were related to the turbidity. This analysis suggests a small number of compounds as tentative markers for turbid cases (Figure 4.8). In addition, a number of compounds were common to both clear and turbid cases and few that were associated with clear cases. A tentative identification of these compounds based on the NIST library is presented in Table 4.4.

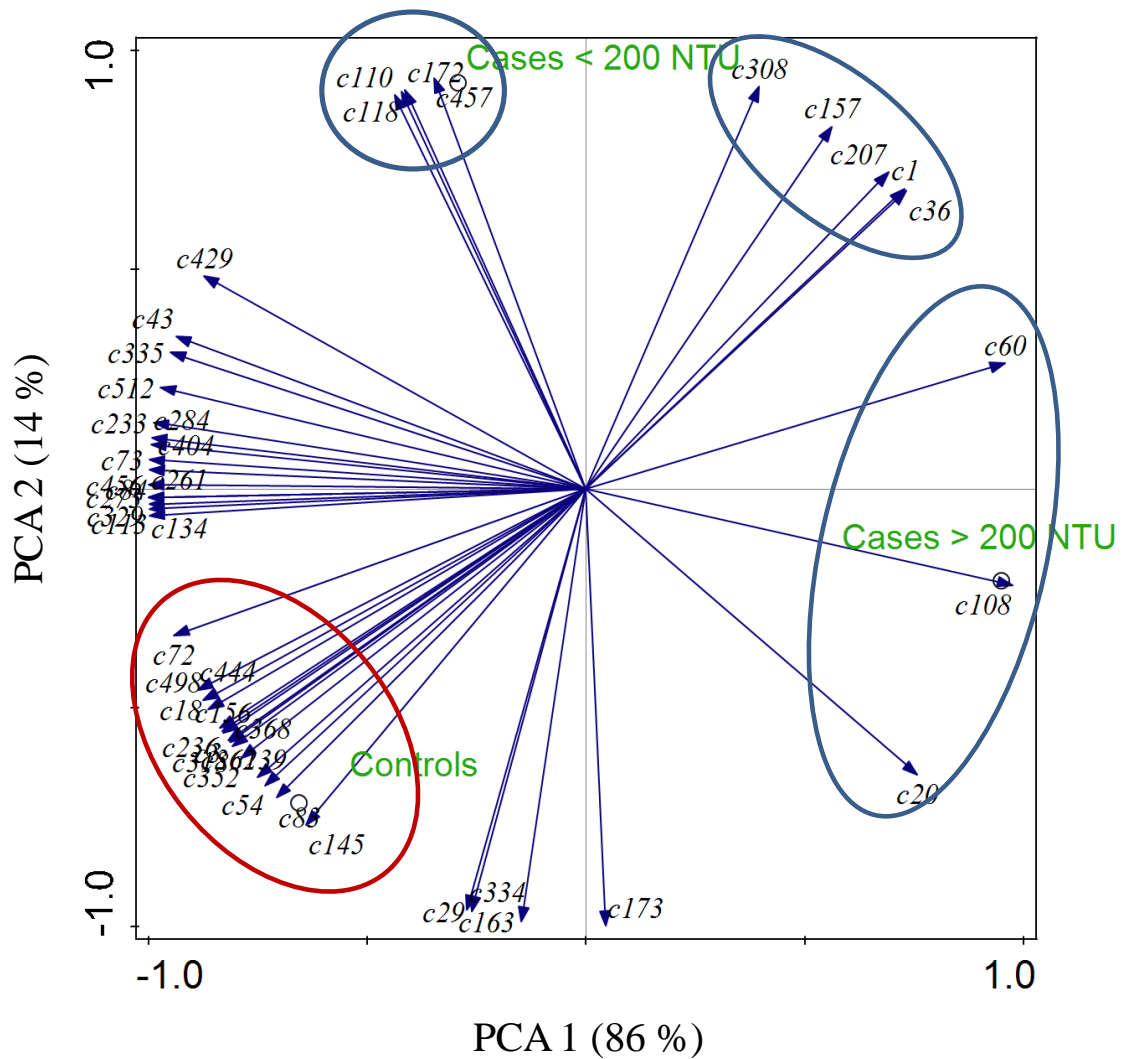


Figure 4.8. Principal Component Analysis (PCA) biplot describing the chemical profile of the three treatment groups. Blue circles highlight chemicals more strongly associated with cases whilst red circle highlights chemicals characteristic for controls.

A large number of compounds describe the control habitats in the biplot. Interestingly, when comparing the number of control habitats with the number of case habitats that released these compounds, only four were found twice as frequently in controls than in cases. All other compounds were either equally frequently detected in controls or cases or even more frequently in cases. The amount of these chemicals released from control habitats was however, on average nine times higher per habitat (1,719,890; 95% CI 1,177,211-2,262,569) than the amount released from case habitats (196,689; 95% CI 184,869-208,510).

Table 4.4 Tentative compound identifications based on NIST library hits

Habitat	Chemical ID	NIST hit name	% Quality	Functional group
Cases < 200 NTU	c110	1-Phenyl-1-butene; Benzene, 2-ethenyl-1,4-dimethyl-; Azulene, 1,2,3,3a-tetrahydro-	90	Aromatic/ Hydrocarbon
	c118	Benzene, (1-methyl-1-propenyl)-, (E)-; 1H-Indene, 2,3-dihydro-4-methyl-; Benzene, 1-ethenyl-3-ethyl-	90	Monoterpenoid, cyclic /Aldehyde
	c172	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	95	Aromatic
	c457	Pyrene, hexadecahydro-; 5(1H)-Azulenone, 2,4,6,7,8,8a-hexahydro-3,8-dimethyl-4-(1-methylethylidene)-, (8S-cis)-	70	Aliphatic
Cases > 200 NTU	c60	Cyclohexanone, 2,2,6-trimethyl-	92	Ketone, cyclic
	c108	4-Decanone;	27	Aliphatic/ Ketone
	c20	Hexanal, 2-ethyl-; 2-Heptanone, 6-methyl-	68	Aliphatic
Cases (both clear and turbid)	c157	2-Decanone	95	Ketone
	c207	Tridecane	95	Aliphatic/ Hydrocarbon
	c1	Triethylamine	76	Unidentified
	c36	2,3-Octanedione	25	Unidentified

4.5 Discussion

Our study emphasizes the complex interaction between biological, environmental and chemical factors in natural aquatic habitats along the shores of Lake Victoria, western Kenya, colonized and not colonized by early instar *Anopheles* larvae. Whilst differences between control and case habitats existed, we did not find a defined set of predictors that could explain all or at least the majority of cases. High turbidity >200 NTU was the only environmental factor that was strongly associated with cases. If a habitat was highly turbid, it was a case. Only one third of all case habitats were highly turbid. Clear cases were not as easily explained by the variables measured. Nevertheless, some risk factors were identified; clear cases were positively associated with higher grass coverage than clear controls, and were negatively associated with the abundance of creeping water bugs of the family Naucoridae and fish. These predictors though were not as strong as high turbidity. Some habitats without grass, containing Naucoridae and fish were still

colonized by early instar larvae. There was a marked difference in the zooplankton communities between clear controls, clear cases and turbid cases were always associated with a higher late instar *Anopheles* abundance.

Based on our results we have to reject the hypothesis that natural aquatic habitats without *Anopheles gambiae s.l.* larvae (controls) differ significantly from habitats that are well colonized by early instar larvae (cases) in their bacteria communities. We did not find any consistent differences between control and case habitats or between clear controls, clear cases and turbid cases. Bacteria profiles could not be associated with any environmental or biological factors. We have however, some indication that cases and control habitats differ in their chemical profile of the water headspace. We identified a small set of compounds that were strongly associated with cases, whilst a larger number was associated with controls. Compounds characteristic for controls were not more frequent in controls but the amount released was nearly an order of magnitude higher in controls as in cases. It was not possible to relate the chemical profiles of habitats to the environmental and biological parameters.

An. arabiensis was the predominant species of *An. gambiae* complex recorded in the main rainy season during this study. Although *An. gambiae s.s.* and *An. arabiensis* have been historically reported as sympatric species in Rusinga Island (Minakawa *et al.* 1999) *An. gambiae s.s.* was not detected in the present work. It is likely that similar to other places in western Kenya, decline of populations of *An. gambiae s.s.* is a consequence of intense indoor control interventions like ITNs (Bayoh *et al.* 2010). Furthermore, *An. arabiensis* and *An. gambiae s.s.* have been shown to share similar breeding habitats in the region (Minakawa *et al.* 1999; Gimnig *et al.* 2001; Minakawa *et al.* 2012) thus it is expected that both species would use similar oviposition cues to select an aquatic habitat to oviposit.

The potential role of habitat turbidity for oviposition site selection

Water turbidity results from suspended organic and inorganic particles in the water column and, it is defined “as an expression of the optical properties of a sample that causes light rays to be scattered and absorbed rather than transmitted in straight lines through the samples” (ASTM 2000). Particles such as clay and silt, finely divided organic matter, plankton and microorganisms contribute to turbidity (Paaijmans *et al.* 2008). Turbidity may affect the diversity and abundance of organisms living in aquatic ecosystems in various ways and it is not surprising that it has been positively correlated

with presence of *Anopheles* larvae in the past (Gimnig *et al.* 2001; Ye-Ebiyo *et al.* 2003; Mala & Irungu 2011).

It has been proposed that turbidity could influence immature stages of mosquitoes in three ways; First, altering their distribution given that turbidity might be used as a visual cue for gravid females to locate breeding habitats, although this assumption is based in the information available about preference of *An. gambiae s.s.* for highly contrasted substrates between clear and dark backgrounds (McCrae 1984; Huang *et al.* 2007) there is not up to date information about how *An. gambiae s.l.* detect turbidity in the natural ecosystems. It can be speculated that turbid habitats can be detected via horizontal polarized light emitted from the water source which could reach its highest point near sundown when mosquitoes might initiate flight in search for a breeding habitat (Kriska *et al.* 2009). So far the use of polarotaxis in larval habitat search in the Culicidae family has only been reported for the container-breeder mosquito *Aedes aegypti*. In its case however, polarized light was used as an attractive oviposition cue only in the absence of semiochemicals. (Bernath, Horvath & Meyer-Rochow 2012).

Second, a preference for turbid water can be explained as being advantageous for the offspring as a higher turbidity may decrease the probability of the larvae to be seen by its predators. Evidence for this approach is presented in this study, where turbid and clear habitats presented equal density of predators and yet turbid habitat presented double density of *Anopheles* larvae in comparison with clear habitats. In particular, it is argued that this mechanism could be important for visual predators like fish for which turbid water decreases its predation efficacy (Johannesen, Dunn & Morrell 2014).

Third, particles that contribute to turbidity may also interfere with larval feeding. In principle, high turbidity could prevent light from entering the water column and interfere with the growth of photosynthetic organisms i.e. algae and bacteria, affecting the availability of food for mosquito larvae; which in turn can slow down the rate of development affecting mosquito nutrient acquisition. On the other hand, proliferation of algae and bacteria can increase food availability raising the population density of mosquitoes (Ye-Ebiyo *et al.* 2003).

Strong correlation between oviposition and conspecific late instar abundance

Presence of late instar *Anopheles* larvae did not deter gravid females from laying eggs in habitats previously colonized by conspecifics as it has been suggested in other studies (Koenraadt *et al.* 2003; Sharma 2012). This observation brings out the question about

how larvae can be detected by gravid females and what are the larvae representing for gravid mosquitoes. It has been suggested that *An. gambiae* s.l. larvae could produce semiochemicals that are density-dependent. In low densities (< 5 larvae/100 ml) females could be attracted to oviposit in the same larval habitat but in high densities (>50 larvae/100 ml) gravid females are repelled (Munga *et al.* 2006; Sumba *et al.* 2008). Presence of conspecifics could also give indications about the suitability of the habitats like the presence of nutrients content, water quality and could be the reason why mosquitoes have been reported ovipositing in water from breeding sites previously colonized by *Anopheles* larvae (McCrae 1984; Sumba *et al.* 2004; Otienoburu *et al.* 2007).

The role of predators in habitat selection – oviposition versus survival and its relation to turbidity. Densities of micro invertebrates often reported as predators of mosquito larvae (Odonata, Coleoptera larvae and adults, Notonectidae) did not differ between habitats colonized and not colonized by *An. gambiae* s.l. with the exception of members of the Naucoridae family that were more abundant in control habitats. This could be an indication that gravid *An. gambiae* did not detect predators when selecting an oviposition site. Contrary previous studies have suggested that *An. gambiae* select aquatic habitats with low predation pressure (Munga *et al.* 2006; Sumba *et al.* 2008). According with our data turbid cases had lower density of predators and higher density of late instar larvae in comparison with clear cases with higher density of predators and less late instar larvae whilst their average early instar density was exactly the same. Thus, we hypothesize that predators play a role in larval survival of *An. gambiae* s.l. rather than in oviposition.

Creeping water bugs of Naucoridae family were the only micro invertebrate associated with the absence of early instar *An. gambiae* s.l. larvae. Naucoridae are recognized as voracious predators in stream tropical ecosystems, and even though mosquito larvae are counted between their preys; they can hunt almost any other organisms of minor size. In Europe the most common species *Ilyocoris cimicoides* has a feeding rate of > 20 mosquito larvae/day while their nymphs can consume as many as 35 larvae of *Aedes vexans*/day (Najera, Gonzalez-Silva & Alonso 2011). Although this insect group seems of the major importance in the regulation of other insect population in natural aquatic ecosystems little is known about how they might interact with malaria vectors larvae (Mbogho 2012). It is possible that *An. gambiae* s.l. like other species of mosquito had developed avoidance for a specific predators (Blaustein, Blaustein & Chase 2005;

Silberbush & Blaustein 2008) however, this need to be further explored in more controlled environments like microcosm or open field settings. It is clearly important to protect predators when controlling immature vector stages in the aquatic habitats since predators contribute to the control. However, the exclusive use of predators for controlling mosquito larvae is likely not successful as supported by the here presented data; even in the presence of a diverse predator community late instar anopheles larvae were abundant.

Bare habitats versus habitats with emergent vegetation/grass – the role of grass in clear habitats. Our results shown that there is a positive association between emergent vegetation and clear habitats that contained *An. gambiae s.l.* larvae, however, this association disappeared in turbid habitats that also contained early instar larvae. This evidence might be an indication that in turbid habitats the vegetation cover is an irrelevant cue for oviposition and the other way around. Likely grass and turbidity are detected by the gravid females as safety signals. Grass can confer refuge to otherwise exposed offspring in clear habitats and turbidity can reduce the probability of the larvae to be detected by visual predators like fish. This observation might explain some of the contradictory reports about *An gambiae s.l.* preferring habitats free of vegetation (Gimnig *et al.* 2001; Kenea, Balkew & Gebre-Michael 2011) (many of the turbid habitats, highly colonised are free of vegetation) and reports of positive association of tufts of grass and *Anopheles* larvae (Mwangangi *et al.* 2007; Fillinger *et al.* 2009b) which might always been observed when the habitats sampled contained a large proportion of habitats with low turbidity.

Distinct zooplankton communities in control and case habitats – the role of competitors. Controphic species of mosquito larvae particularly zooplankton was abundant in cases and control habitats and seems not to prevent *An. gambiae s.l.* colonization. Contrary, in the present study gravid females of *An. gambiae s.s.* preferred to lay eggs in aquatic habitats where high densities of members of Moinidae family were present. This is a positive association rarely reported for organisms in the same trophic level of mosquito larvae. Although there is limited information about this particular cladoceran groups in the tropics (WHO 1999) it has been suggested that zooplankton can be positively associated with mosquito larvae by consuming bacteria that are pathogenic to mosquitoes larvae or reducing predation on mosquito larvae serving as alternative prey (Blaustein & Chase 2007). In this study, competitors were not associated with negative effects in the population dynamic of early instar larvae. However, it should be

noted that the role of competition in transient aquatic habitats could be determined by other factors not studied here. It has been hypothesized that in ephemeral aquatic bodies' competition is a function of hydroperiod length suggesting that competition is less likely to be important in habitats of short hydroperiod because densities of competitors are unlikely to be high and resources are unlikely to be reduced. Whether mosquito larvae are benefited or harmed by the presence of the controphic species depends on which species is a better competitor for resources and which is more susceptible to predators (Blaustein and chase 2007).

Chemical profile analyses suggest that chemicals might be involved in habitat selection but no evidence was found to confirm or reject that these might be produced by bacteria. Diverse bacteria communities were identified in all aquatic habitat types investigated. No differences between habitats with and without early instar *Anopheles* were however detected. These findings are in agreement with a recent report of bacteria communities from domestic water storage containers with and without *Ae. aegypti* in Thailand; where diversity of bacterial communities did not differ between containers and therefore no bacteria group was associated with larvae presence (Tanner & de Savigny 2008). These results confirm our previous work suggesting that microbial communities from larval habitats are not associated directly with oviposition behaviour in *An. gambiae s.l.* In spite of the highly complex chemical profiles of natural water bodies data exploration suggests that some organic chemicals are associated with prolific oviposition sites whether turbid or not. Importantly, habitats without larvae were characterized by relatively more compounds and maybe more importantly of a higher amount of specific volatiles released from controls as compared to the amount of the same volatile released from cases. The standardized experiments and field observations confirmed that *An. gambiae s.l.* selected oviposition sites through a complex interaction of volatiles organic compounds and visual cues.

4.6 Conclusion

The relationship between environmental characteristics, biotic and abiotic factors that characterize natural oviposition sites of *Anopheles gambiae s.l.* in Rusinga Island, western Kenya are complex. Further studies are required to investigate chemical cues released from vibrant larval habitats and their interaction with potential visual cues like turbidity investigated. Turbidity and volatile chemicals might be potentially exploited to attract and kill gravid females as a novel intervention for vector surveillance and control.

Chapter 5 Synthesis

Understanding the ecology of gravid *An. gambiae s.l.* is a prerequisite for the development of interventions against malaria vectors. Factors that guide gravid females foraging for oviposition sites might be manipulated to attract and kill adult mosquitoes or used as markers to target prolific larval habitats with larvicides. This study investigated whether *An. gambiae s.l.* make informed choices when selecting oviposition sites and explored physical, chemical and biological parameters associated with these choices. Using dual-choice egg-count bioassays it was demonstrated that gravid *An. gambiae s.l.* discriminate between potential oviposition substrates using volatile chemical cues. Field studies revealed intricate mosquito colonization patterns that could be partially explained by the turbidity of water bodies. This study could not confirm that microbes in general or specific bacteria communities are associated with the selection of aquatic habitats by gravid females.

5.1 Key findings

5.1.1. Gravid *An. gambiae s.l.* females select sites to oviposit using volatile organic compounds and turbidity

This study found evidence that gravid *An. gambiae s.l.* make informed choices when selecting an egg-laying site. Firstly, the mosquitoes consistently discriminated between contrasting infusions in standardized experiments in the field and laboratory. When provided with a choice of rabbit food pellet infusions and lake water the mosquitoes avoided the infusion and laid eggs in the lake water. As the pellet infusion aged it became increasingly less likely that mosquitoes would lay eggs in it. In contrast to this, gravid mosquitoes preferred laying eggs in aging soil infusion instead of lake water. This preference for soil infusion did not depend on the visual appearance of the infusions demonstrating that volatile organic compounds dominated visual cues within that range. The volatile organic compounds that were responsible for the preference and avoidance of the organic infusions were not investigated in detail within the scope of this thesis. However, this work was continued in collaboration with colleagues in parallel (Okal *et al.* 2013) (Appendix B), Okal *et al.* 2015 under review (Appendix C), Lindh *et al.* 2015 under review (Appendix D),) and suggests that the avoided chemical cues released from the highly organic and decomposing pellet infusion were bacteria metabolites (p-cresol, skatol, indol), whilst the only attractive cue to date identified for the soil infusion in addition to water vapour (cedrol) is likely plant derived (Okal *et al.* 2014, Okal *et al.*

2015, Lindh et al 2015; Appendices C-D). Secondly, the distribution of first instar *An. gambiae s.l.* larvae in the field was non-random. Aquatic water bodies with turbidity measures greater than 200 NTU were with over 80% certainty colonized highly by early and late instar *An. gambiae s.l.* larvae. Turbid habitats interestingly released less volatile organic compounds than non-turbid habitats although a small set of chemicals associated specifically with these habitats were identified and justify further investigation. Turbidity is likely a strong visual cue at a longer range than chemical cues that increases the probability of a gravid female finding the habitat in the early evening. Visual cues aiding the orientation towards a potential habitat have been shown before in cage and semi-field settings (Huang *et al.* 2007; Dugassa *et al.* 2014). In spite of the highly complex chemical profiles of natural water bodies data exploration suggests that some organic chemicals are associated with prolific oviposition sites whether turbid or not. Importantly, habitats without larvae were characterized by relatively more compounds and maybe more importantly of a higher amount of specific volatiles released from controls as compared to the amount of the same volatile released from cases. The standardized experiments and field observations confirmed that *An. gambiae s.l.* selected oviposition sites through a complex interaction of volatiles organic compounds and visual cues.

5.1.2. The oviposition choices of gravid *An. gambiae s.l.* are not associated with the microbial community of oviposition substrates from vibrant breeding sites

In contrast to some earlier reports, it could not be confirmed that gravid malaria vectors show preferences for soil or water collected from well colonized natural larval habitats over distilled water in standardized cage tests. Removing microbes from the natural substrates did not alter this response to the two substrates. Thus this work did not add evidence to the assumption that oviposition cues used by gravid *An. gambiae s.l.* to select (prefer) a habitat over the other are majorly produced *in situ* by microbes in the habitat. Whilst it cannot be excluded that volatile chemicals were involved in the selection of sites and that they might have been produced originally by microbes, the removal of microbes did not alter the chemical profile of the substrates to affect oviposition. Natural oviposition sites exhibited a large variability in bacteria communities without discernible differences in groups of bacteria between sites with and without *An. gambiae s.l.* larvae. Likewise, there were no differences in bacteria diversity between habitat types. There was neither an association between biological/environmental factors and bacteria communities nor between the three turbidity groups (clear controls, clear cases, turbid cases) and bacteria communities.

Therefore while there is sufficient evidence that volatile organic compounds could contribute to oviposition site selection in *An. gambiae s.l.* there is no evidence that these are produced by bacteria. Other microbes like algae and fungi were not investigated during this field study and their role in natural habitat selection remains unclear, however, the sterilizing techniques used in the laboratory experiment removed all microbes from the sample and no evidence was found that could justify investigating these groups.

5.1.3. Conspecific late instar larvae, competitors and predators in natural aquatic habitats do not prevent gravid *An. gambiae s.l.* from laying eggs in these habitats

In the present study clear and turbid habitats colonized by early instar *An. gambiae s.l.* were significantly associated with high abundance of late instar *An. gambiae s.l.* High late instars in habitats selected for oviposition seems to be an indication of increased survival in these habitats probably related with other habitat characteristics like quality of the water and nutrient content that gravid females may perceive when looking for a site to lay eggs. The association between conspecific larvae and oviposition has been controversially discussed in published work. On the one hand some hypothesize that gravid females avoid oviposition in habitats with abundant late instars due to the risk of competition and the early instars being eaten by late instars (Koenraadt *et al.* 2003; Munga *et al.* 2006; Sharma 2012). On the other hand, it has been speculated that conspecific larvae produce semiochemicals (Sumba *et al.* 2008) to guide gravid females to the oviposition sites. This study clearly demonstrates that early and late instar are strongly associated in natural habitats providing evidence that gravid *Anopheles gambiae s.l.* do not avoid laying eggs when large numbers of conspecifics are present. This might warrant further investigations if late instar larvae produce semiochemicals or if the observation is associated with better survival in these habitats. Zooplankton communities regarded as competitors of mosquito larvae (Blaustein & Chase 2007) are plentiful in habitats with and without early instar *An. gambiae s.l.* larvae and there is a strong indication that certain taxonomic groups are associated with habitats where mosquito larvae are present. Gravid *An. gambiae s.l.* preferred to lay eggs in aquatic habitats with high densities of members of the Moinidae family. This is a positive association rarely reported for organisms in the same trophic level of mosquito larvae. Although there is limited information about this particular cladoceran group in the tropics it has been suggested that zooplankton can be positively associated with larvae by consuming bacteria that are pathogenic to mosquito larvae or reducing predation pressure when serving as alternative prey (Blaustein & Chase 2007). Nevertheless, none

of these hypotheses have been tested in *An. gambiae s.l.*. To my knowledge this is the first study where zooplankton from transient fresh water ecosystems was identified and related to the abundance of mosquito larvae in Kenya. The data does not suggest that zooplankton organisms compete with *Anopheles* larvae and therefore reduce larval abundance or oviposition and therefore does not present a viable option for malaria vector control as suggested by some; at least not in the naturally occurring densities.

Densities of micro-invertebrates often reported as predators of mosquitoes larvae (Odonata, Coleoptera larvae and adults, Notonectidae) did not differ between habitats colonized and not colonized by early instar *An. gambiae s.l.* indicating that gravid females did not detect and respond to predation risk when selecting a natural oviposition site. The only family of predators that might warrant further investigation are the Naucoridae. Members of this family were significantly more abundant in habitats without larvae. If this has to do with the detection and avoidance of these voracious predators by gravid females or whether the absence of Naucoridae from the cases has to do with other underlying habitat characteristics needs still to be proven - Based on the data, it is much more likely that predators played a role in larval survival than in oviposition site selection; this is supported by the relation between densities of late instar larvae and predators. Turbid cases with few predators had two fold more late instar *An. gambiae s.l.* than clear cases with a higher density of predators whilst their average early instar density was exactly the same. Predation is a vital interaction in the regulation of population dynamics and community structure in any ecosystem and mosquito larval habitats are no exception (Blaustein 1999).

5.2 Limitation of the study

This study demonstrates that volatile organic chemicals and turbidity are involved in the regulation of the oviposition site choices of *An. gambiae s.l.*, however, by no means should it be concluded that these are the only cues involved in oviposition site selection. The presented work has generated the first extensive insight into the oviposition site selection of *Anopheles gambiae s.l.* and highlighted a large number of open questions still to be investigated and also the limitations of the work that was carried out.

The findings for this study are valid for the geographical area where this research was implemented. The extent to which such findings can be generalized needs to be further explored. There is indication from a recent study that cues for oviposition in *An. gambiae s.l.* could vary between geographical locations (Ogbunugafor & Sumba 2008).

For this reason, it is important to implement oviposition studies in different environments with dissimilar topography and climate. The evidence of this study, though rigorously gathered, must be interpreted with caution when describing oviposition in geographically separated populations of *An. gambiae s.l.*

For lack of a direct way of estimating the oviposition rate of *An. gambiae s.l.* in the field, early instar larvae in natural habitats were used as a proxy for oviposition preferences and therefore aquatic water bodies were classified as case or control depending on presence or absence of these immature stages. Larvae first appear in breeding sites when eggs eclose two to three days after oviposition. Natural water bodies in the tropics are complex and dynamic environments changing quickly over time. Therefore, it cannot be excluded that the bacteria communities and chemical profiles experienced by the gravid female whilst laying eggs were not exactly the same as those collected and analysed at the day of early instar sampling.

Furthermore, the presence of larvae is also a function of other factors including the hatch rate of eggs or the relative survival of larvae. Nonetheless, until a direct method of detecting actual oviposition in natural ecosystems is found, first-instar larvae remains the most direct indicator for this behaviour. In order to analyse the chemical headspace and bacteria communities exactly on the day when oviposition occurs it would be necessary to revisit the aquatic habitat on a daily basis and collect samples and information on colonisation. This would be extremely difficult, time consuming and costly for investigating oviposition behaviour under field conditions however might be explored under more standardized conditions with artificial ponds to generate more insight on the speed and extend of change over time.

The approach taken to contrast and identify bacteria communities and volatile chemicals in natural *An. gambiae s.l.* larval habitats was very challenging with currently available tools. The complexity of the chemical profiles of natural water bodies necessitates the collection of a large numbers of samples in order to detect general patterns. Unfortunately, comparing mass spectral results from these samples can only be done manually and resulted in an extremely difficult and time consuming task; one that could be conceivably permeated with human error. However, given that this is a pioneer study in the identification of chemical headspace from natural aquatic habitats the results obtained here are valuable and can serve as a starting point for future work. The bacteria communities were analysed using DGGE, a method that could be implemented without highly specialized equipment and could therefore be done in a research field station.

Furthermore, this method allows for bands of OTUs in the gel to be cut and to be identified if any of the OTUs would have been of specific interest. However, this technique is especially sensitive to human expertise in the process of gel making and consequently the reproducibility of results is a difficult endeavour (Nocker, Burr & Camper 2007). Most importantly, comparisons of bacteria communities could only be made for habitats loaded on the same gel but not inbetween gels. It was therefore not possible to implement an analyses with all data included. However, the sub-set analyses did not provide any evidence that would suggest that an analysis of the entire data set would have resulted in any significant associations.

5.3. Future work

A large number of new research questions resulted from the here presented findings that might be worth perusing for the development of new vector control strategies targeting the gravid malaria vectors and the most preferred larval habitats.

The soil infusion tested in chapter 2 was found to be preferred by gravid females for oviposition, however, it is important to screen other soil samples to see if the observed response is a response common for all soil infusions prepared under standard conditions or, which is more likely, if there are significant differences depending on the source and characteristics of the soil.

No evidence was found that bacteria communities were associated with habitat selection in the field. When investigating the oviposition response to highly decomposed organic matter presented by rabbit food pellets it is highly likely that the chemical cues that led to the avoidance of this substrate were indeed produced by bacteria. The question arises if bacteria produced volatiles might only be involved in avoidance of habitats (which might also be supported by the field observation, that controls released the same volatile compounds as cases but frequently in a much larger amount) but not in preferential selection of habitats. Furthermore, it would be necessary to explore other microbial groups like algae and fungi, in addition to vegetation present in the aquatic habitats in order to detect the potential source of attractive semiochemicals for egg-laying.

The high probability of wild *An. gambiae s.l.* mosquitoes laying eggs in turbid habitats suggests that mosquitoes use turbidity as a visual cue. Thus there would be value in studying this in a standardize way evaluating how mosquitoes detect turbidity. There is strong evidence that water insects orientate to a given polarized light field and that one

of the highest points where polarized light can be detected is at dusk precisely the time of the day were mosquitoes are foraging for an oviposition site (Bernath, Gal & Horvath 2004; Bernath, Horvath & Meyer-Rochow 2012). Studies evaluating the responses of *An. gambiae s.l.* to polarized light emitted from turbid substrates and its relation with oviposition would aid a better understanding of the cues used by gravid females and therefore pave the way for the development of novel sampling techniques. Most importantly, it is important to study how visual and chemical cues interact under natural conditions in order to design effective attract and kill strategies.

The relationship between the absence of *Anopheles gambiae s.l.* larvae and the presence of Naucoridae in natural aquatic habitats noticed in this study requires further examination to investigate if the predators can be detected by the gravid female and deter her from egg-laying. Furthermore, the role of turbidity in predation success needs further investigation. Do predatory micro-invertebrates avoid colonizing highly turbid habitats due to reduced visibility in the water column and does this protect the vector's offspring?

Biological and environmental factors in a given habitat change over time and likely affect the oviposition behaviour in time. A longitudinal study following a small group of aquatic habitats across the rainy season on Rusinga Island could be used to confirm the observations from the case control study and further elucidate if habitat preferences change over time or are strongly associated with specific habitats throughout.

5.4 General conclusions

It has been widely acknowledged that current vector control interventions in sub-Saharan Africa are insufficient to achieve malaria elimination since residual transmission will be maintained by exophilic and insecticide resistant vectors. In order to target malaria vectors outdoors a better understanding of their outdoor behaviour is needed. This thesis presents pioneering work investigating the oviposition site selection in *Anopheles gambiae sensu lato*, the major malaria vector in sub-Saharan Africa under controlled conditions and in field settings in Western Kenya to identify if this vector species selects habitats based on specific cues and to explore if such cues can be identified and used to improve larval source management practices and to develop novel strategies for monitoring and control of gravid females.

Results from experimental laboratory and semi-field studies and from observational field studies provided evidence that a gravid *An. gambiae s.l.* female selects a suitable habitat for oviposition using chemical cues from water bodies. It was shown that natural infusions can be used to manipulate the oviposition behaviour of *An. gambiae s.l.* Soil infusions have the potential to be used to bait gravid traps for the collection of *An. gambiae s.l.* ((Herrera-Varela *et al.* 2014)/ Appendix A), although further work must be implemented to elucidate whether the observed preference was based on the specific soil type tested or whether similar responses can be achieved with any soil. In contrast, infusions routinely used in gravid traps for the monitoring of *Culex* and *Aedes* disease vectors, i.e., fermented hay infusions, rabbit food pellet (Lewis 1974; Lampman & Novak 1996; Jackson *et al.* 2005b), were strongly avoided by *An. gambiae s.l.*

Wild and caged *An. gambiae s.l.* females discriminate between potential aquatic habitats for oviposition based on habitat characteristics and experimental laboratory tests as well as the observational case-control study in the field confirmed that these choices benefit the survival of the offspring. Experience during the larval stage (rearing water characteristics) did not influence the adult's choice of oviposition substrate.

It was demonstrated that the choice of habitat is mediated by chemical cues based on both preference and avoidance. Based on this ground-breaking work, some chemical cues have been consequently been identified (Appendix C) as semiochemicals of oviposition. It was shown that the soil infusion contained at least one attractant that mediated the oviposition response of gravid females over several metres (Appendix D).

Whilst further work is required, this work lays the foundation for the development of 'push-pull' or 'attract and kill' strategies to improve malaria vector monitoring and control.

Previous reports that substrates containing microbes from vibrant larval habitats are preferred over the same substrates when sterilized were not confirmed. Elimination of live microorganisms from soil and water collected from a natural larval habitat does not necessarily influence the oviposition choices of gravid vectors. No evidence was found that volatiles produced *in situ* by these microbes are involved in the oviposition choice. Nevertheless, volatile profiles of oviposition substrates can be modified by sterilization and it was shown that substrates without any volatile headspace were avoided supporting the conclusion from the soil infusion experiments that volatile chemicals play a role in habitat selection. These findings were confirmed by the field study and based on these results it cannot be recommended to continue to invest in investigations of bacteria communities associated with preferred malaria vector habitats (but possibly with those that are avoided). Fungi and algae might be a potential source of attractive chemical cues, although the experimental work sterilizing substrates did not provide any evidence for that. Based on the results it is concluded, that rather than studying the microbial community, more time and effort should be invested in the chemical analyses of the headspaces of preferred habitats and infusions to identify individual chemical compounds and blends that could be used in traps.

The relationship between environmental characteristics, biotic and abiotic factors that characterize natural oviposition sites of *Anopheles gambiae* on the shores of Lake Victoria in western Kenya are intricate. The only factor that allowed prediction of a case with high probability was turbidity of the habitat >200 NTU. Whilst risk factors associated with selection and avoidance of habitats in the field were found, these were insufficient to guide larval source management operations targeting only habitats selected by gravid females. There might however be value in exploring experimentally or with a mathematical modelling approach what impact on malaria transmission might be achieved if only turbid habitats would be targeted for larval control since these habitats supported the highest survival of the immature stages and might therefore be the most productive sources of malaria vectors. The role of turbidity is likely visual and if further explored might provide an additional cue to be integrated in novel trapping

strategies combining chemical and visual cues to attract and kill malaria vectors in an integrated vector management approach.

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7 Appendices

Appendix A.

Herrera-Varela et al. 2014

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RESEARCH

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Habitat discrimination by gravid *Anopheles gambiae sensu lato* – a push-pull system

Manuela Herrera-Varela^{1,2}, Jenny Lindh³, Steven W Lindsay⁴ and Ulrike Fillinger^{1,2*}

Abstract

Background: The non-random distribution of anopheline larvae in natural habitats suggests that gravid females discriminate between habitats of different quality. Whilst physical and chemical cues used by *Culex* and *Aedes* vector mosquitoes for selecting an oviposition site have been extensively studied, those for *Anopheles* remain poorly explored. Here the habitat selection by *Anopheles gambiae sensu lato* (s.l.), the principal African malaria vector, was investigated when presented with a choice of two infusions made from rabbit food pellets, or soil.

Methods: Natural colonization and larval survival was evaluated in artificial ponds filled randomly with either infusion. Dual-choice, egg-count bioassays evaluated the responses of caged gravid females to (1) two- to six-day old infusions versus lake water; (2) autoclaved versus non-autoclaved soil infusions; and assessed (3) the olfactory memory of gravid females conditioned in pellet infusion as larvae.

Results: Wild *Anopheles* exclusively colonized ponds with soil infusion and avoided those with pellet infusion. When the individual infusions were tested in comparison with lake water, caged *An. gambiae sensu stricto* (s.s.) showed a dose response: females increasingly avoided the pellet infusion with increasing infusion age (six-day versus lake water: odds ratio (OR) 0.22; 95% confidence interval (CI) 0.1-0.5) and showed increasing preference to lay eggs as soil infusion age increased (six-day versus lake water: OR 2.1; 95% CI 1.4-3.3). Larvae survived in soil infusions equally well as in lake water but died in pellet infusions. *Anopheles gambiae* s.s. preferred to lay eggs in the non-autoclaved soil (OR 2.6; 95% CI 1.8-3.7) compared with autoclaved soil. There was no change in the avoidance of pellet infusion by individuals reared in the infusion compared with those reared in lake water.

Conclusion: Wild and caged *An. gambiae* s.l. females discriminate between potential aquatic habitats for oviposition. These choices benefit the survival of the offspring. Although the study was not designed to distinguish between stimuli that acted over a distance or on contact, it could be demonstrated that the choice of habitat is mediated by chemical cues based on both preference and avoidance. These cues, if identified, might be developed for 'push-pull' strategies to improve malaria vector monitoring and control.

Keywords: Oviposition site selection, *Anopheles gambiae*, Natural infusion, Olfactory memory

Background

Selection of suitable oviposition sites is a critical step in the life history of mosquitoes [1]. This is a process whereby individuals select and occupy a non-random set of aquatic habitats. Habitat selection is of major importance for the interpretation of spatial and temporal distributions of populations, and for understanding intra and

inter-specific relations that influence the abundance of individuals [2,3]. Organisms without any parental care are likely to choose habitats based on a set of innate or learned cues in order to maximize the survival and fitness of their offspring [2,4].

Mosquitoes utilize a wide range of aquatic niches for oviposition, including natural ponds, puddles, stream fringes, marshes, tree-holes and plant axils, man-made pits, drains, rice fields, and containers [5]. Field studies have shown that mosquitoes are discriminating in selecting sites for egg deposition [6,7] and that oviposition choices made by gravid females are a key factor in

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determining larval distribution [8-11]. Although different species are found in the same type of habitat, oviposition site selectivity is considerably species specific [12]. Immature stages of *Anopheles gambiae sensu lato* (s.l.), the major malaria vector in sub-Saharan Africa, are typically described as inhabiting very small, temporary sunlit pools and puddles without vegetation [13-16]. However, reviews of the literature and recent research on larval ecology have shown that this is a gross oversimplification of the wide range of habitats colonized by this species [17-19], a fact recognized over half a century ago by Holstein who reviewed the 'extraordinary diversity of the breeding places' of *An. gambiae* s.l. [19]. Numerous studies have described how the presence of *An. gambiae* s.l. larvae [17,20-23] and the capacity of individual habitats for generating adult mosquitoes [6,8,17,24,25] differs markedly over space and time, yet these surveys failed to reveal any risk factors that could consistently predict sites preferred by *An. gambiae* s.l. [17,20-23]. This might lead to the conclusion that this species randomly deposits its eggs in a large range of habitats and that the heterogeneous distribution of larvae results from the survival of larvae in the aquatic habitat [9,10] rather than the adults' choice.

Surprisingly, fully gravid malaria vectors looking for suitable larval habitats have been grossly understudied [26]. Compared to the wealth of knowledge of the physical and chemical factors used by gravid culicines for selecting an oviposition site [27-40] those potentially used by the world's most deadly malaria vector remain almost unexplored. Whereas many publications recognize that the distribution of larvae between seemingly suitable aquatic habitats is probably due to the choice of the gravid female [6,8,24,41-43] and that this choice probably impacts on the fitness of her offspring, there is little empirical evidence to support these assertions. Most recent research has evaluated the characteristics of aquatic habitats associated with the presence and absence of larvae [15,21,44-46] but the understanding of the behaviour of gravid female *An. gambiae* s.l. when searching for an oviposition site remains, at best, sketchy [8,41,47-53].

Laboratory studies demonstrated that physical conditions of the aquatic habitats influence oviposition site selection in *An. gambiae* s.l., with females preferring dark backgrounds to pale ones, muddy water to clear water and fully hydrated substrates [8,41,50,54]. Turbidity has been suggested as an important physical cue for oviposition behaviour in *An. gambiae* s.l. although the evidence for this is contradictory [55,56].

Even less is known about the chemical cues and their interaction with physical factors. Water vapour is itself an attractant to gravid mosquitoes [57]. It has been shown that gravid *An. gambiae* s.l. are sensitive to bacteria-derived odours [47,53,58] which have been

associated with increased [47,53] and reduced [58] egg numbers compared to sterile media in cage bioassays. Whilst over 20 putative oviposition semiochemicals have been suggested in the literature, based on the analyses of bacteria- or habitat-derived volatile chemicals and electro-antennogram studies [53,59], there is only one report [60] of two chemicals inducing a behavioural response in gravid females (one increasing and one decreasing the oviposition response).

Here the oviposition behaviour of *An. gambiae* s.l. was explored to test the hypotheses that a gravid *An. gambiae* s.l. female evaluates the suitability of a habitat using chemical cues from water bodies that oviposition choices made by a gravid female benefit the offspring and that this choice cannot be modified by experience in one generation.

Habitat selection by gravid *An. gambiae* s.l. was tested by presenting a choice of two infusions; one made with soil from an area where natural habitats occur frequently, and one made with rabbit food pellets. Rabbit food pellets are frequently used as diet for mosquito larvae in insectaries [61,62] and infusions made of grass, hay and other plant material, including rabbit food pellets have shown to be attractive to a range of mosquito species and have been used in gravid traps [63-66]. The aim was to explore whether *Anopheles gambiae* s.l. might also be drawn to this infusion.

Natural colonization and larval survival was evaluated in artificial ponds filled randomly with either infusion. As a consequence of the field observations, two-choice, egg-count bioassays were used to explore pattern of oviposition seen in the field. Experiments were designed to address the following questions: 1) Do gravid *An. gambiae* s.l. females discriminate between different habitats when searching for an oviposition site? 2) Does the oviposition choice benefit the survival of their offspring? 3) Are gravid females guided by preference (attractants/stimulants) or avoidance (repellents/deterrents)? 4) Are oviposition choices likely to be based on chemical cues? and, 5) Is the choice made by a gravid female influenced by her olfactory memory of her larval habitat?

Methods

Study site

Experiments were carried out at the International Centre for Insect Physiology and Ecology (icipe), Mbita, on the shores of Lake Victoria, Western Kenya (geographic coordinates 0°26'06.19" South; 34°12'53.13" East; altitude 1,137 m above sea level). Mbita has a typical tropical climate; temperatures oscillate between 18-28°C and there is annual rainfall of 1,436 mm (based on data from icipe meteorological station for 2010-2012). Two rainy seasons occur annually, the long rainy season between March and June and the short rainy season between

October and December. Malaria is endemic in the area and transmitted by three vectors, which are in order of their abundance: *Anopheles arabiensis*, *Anopheles gambiae sensu stricto* (s.s.) and *Anopheles funestus* [67].

Mosquitoes

Open-field trials were conducted with wild anopheline and culicine females that oviposited in tubs of water sunk into the ground. These were colonized within three days. Laboratory experiments were carried out with insectary-reared *An. gambiae* s.s. (Mbita strain) supplied by icipe's insectary and reared following standard operating procedures. Briefly, larvae were reared in round plastic tubs (diameter 0.6 m) filled with water from Lake Victoria and fed Tetramin® fish food twice daily. Larvae were collected randomly from several tubs on the day of experiment. Gravid mosquitoes were prepared by selecting 300 female and 300 male mosquitoes, two to three days old, from their rearing cages at 12.00 and keeping them in 30 × 30 × 30 cm netting cages at 25-28°C and 68-75% relative humidity. To avoid mosquito desiccation, cotton towels (folded to 25 × 12 cm) were saturated with lake water and 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 min at 19.00 on the same day. After feeding, mosquitoes were provided with 6% glucose solution *ad libitum*. This procedure was repeated 24 hours later. After the first blood meal unfed female mosquitoes were removed from the cages. Fed female mosquitoes were kept together with males for two days after the second blood meal before using them in an experiment (i.e. females four to five days after first blood meal). In the afternoon (16.30) of the day of an experiment 45-100 (depending on experiment and availability) visually presumed gravid females, that is with an enlarged, pale white abdomen, were selected from the holding cage. A small percentage of these mosquitoes were probably not gravid because most females needed two blood meals to reach full gravidity and some never reach full gravidity even after three feeds [68,69]. Whilst two meals were provided it cannot be guaranteed that two meals were taken by all females. This might be the reason that not all mosquitoes exposed to oviposition medium in experiments laid eggs (responded), therefore the number of responders was smaller than the number tested. Non-responders were excluded from the analyses.

Experimental procedures

Do gravid *Anopheles gambiae sensu lato* females discriminate between different habitats when searching for an oviposition site?

To explore natural colonization of habitats by wild mosquitoes, 20 artificial habitats were created by implanting

20 plastic tubs (40 cm diameter, 20 cm deep) into an open-sunlit field during the long rainy season in May 2011. The tubs were placed in four lines of five tubs each 4 m apart [70]. Two different substrates were randomly offered in the artificial habitats. Half of the tubs (ten) received 30 g of rabbit food pellets (Scooby® rabbit and rodent food, Nairobi) containing hay and grains from maize, wheat, barley, cotton, sunflower, soya bean meal, and traces of molasses, vitamins and minerals. The remaining half of the tubs (ten) received 2 kg of dry soil taken from a field at icipe. Soil texture was characterized as a silty clay loam according to the US Department of Agriculture (USDA) texture triangle [71] using the detergent method [72] to separate and quantify soil mineral particles of different size. A volume of 15 l of lake water pumped from Lake Victoria, was added to each tub and the water level was held constant by adding water to the 15 l mark daily. The two treatments are henceforth referred to as pellet and soil infusion. To study the oviposition response of wild mosquitoes the tubs were monitored daily between 08.00 and 10.00 by dipping five times per tub with a standard dipper (350 ml). Two different dippers were used for the two treatments to avoid contamination. Four dips were taken from the edge of the tubs and one from the middle. The content of each dip was emptied into a white plastic bowl and all early instars (first and second stage larvae) counted and recorded for both culicine and anopheline mosquitoes. All larvae and the water were returned to the respective tub. The tubs were followed for 16 days. Ponds were searched for pupae and collected daily to prevent any emergence of potential disease vectors. Pupae were allowed to emerge in cages in the laboratory and any anophelines emerging identified to species using morphological keys [13,73] and for specimens of the *An. gambiae* complex using the ribosomal DNA-polymerase chain reaction (PCR) method to distinguish between the two local species of the complex *An. gambiae* s.s. and *An. arabiensis* [74].

Does the oviposition choice benefit the survival of their offspring?

Larval survival was assessed by introducing individual, insectary-reared, first instar *An. gambiae* s.s. larvae in infusions collected from the tubs set up in the field. Infusion samples were taken after one, six, 11, and 16 days. One-hundred ml of infusion was collected from each of the ten tubs per treatment and pooled per treatment (soil or pellet infusion) in a plastic bottle. Lake water was used as a control. First instars were introduced in 100 ml plastic cups containing 50 ml of pellet infusion, soil infusion or lake water. Twenty larvae were exposed individually per treatment and collection day. Larvae were fed every second day with finely ground Tetramin®

Baby fish food. Food was provided with a blunt toothpick that was first wetted in lake water and then dipped quickly, not more than 1 mm deep into the ground food, and then dipped onto the surface of the test water. Larval development was monitored daily and the time of death or time to pupation and emergence recorded. This experiment was implemented under ambient conditions in a semi-field system (80 sq m) with screened walls and a glass roof [75].

Are gravid females guided by preferences or avoidance?

Based on the analysis of the field data a series of two-choice, egg-count bioassays were designed to investigate if the response of wild gravid females observed in the field was based on avoidance or preference of an infusion or both.

Gravid females were selected from insectary cages and transferred individually to 30×30×30 cm cages. In each cage two glass cups (Pyrex[®], 100 ml, 70 mm diameter), surrounded by tightly fitting aluminium cylinders, so that mosquitoes could see only the water surface, were filled with 100 ml of either the control or test medium and placed in diagonal corners of the cage. Prior to use, cups and cylinders were cleaned with detergent, then autoclaved and kept in an oven at 200°C for at least two hours. The position of oviposition cups containing the test medium was alternated between adjacent cages to control for possible position effect. The placement of the first test cup was randomly allocated for one of the four cage corners in the first cage. Subsequent test cups were rotated in the next possible corners in a clockwise direction relative to the position of the preceding cup. One control cup was added in each cage diagonal to the test cup to complete a two-choice set up. The experiments were carried out in makeshift sheds (Figure 1) that exposed the mosquitoes to ambient light, temperature and relative humidity but protected the cages from rain.

Two sets of experiments were carried out consecutively (Table 1, Set 1 and 2). In the first set oviposition choice was evaluated for two-, four- and six-day old pellet infusions compared with lake water. In the second set, the oviposition choice was evaluated for two, four and six-day old soil infusions compared with lake water. In both sets of experiments internal controls were used to validate the two-choice experiment. Here equal numbers of cages were set up where both cups in a cage contained lake water and were labelled randomly as control and test cup, assuming that gravid females lay eggs in both cups in an equal proportion.

Infusions were prepared in a similar way as for the field tests. Fifteen l of lake water were either incubated with 30 g of pellets to prepare a pellet infusion or incubated with 2 kg of soil to prepare a soil infusion. Infusions were prepared in a plastic tub (40 cm diameter 20 cm depth) six days, four days and two days before the day of experiment in order to test all ages in parallel. Tubs were covered with mosquito netting and kept in makeshift sheds at ambient conditions but protected from rain. Experiments were implemented over three to nine rounds depending on the availability of gravid females and the response rate per round (Table 1) with fresh batches of infusions and different batches of mosquitoes for every round. On the day of experiment infusions were sieved through a clean piece of cotton cloth to remove large debris remaining from the pellets or soil.

Fifteen to 25 replicate cages per treatment were set up per round. A single gravid female was introduced per cage at 17.30. 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.

Turbidity, conductivity, dissolved oxygen, and pH were measured in five cups per treatment in four different batches of pellet, soil infusions and lake water using a turbidity meter (TURB 355IR, WTW Germany) and a

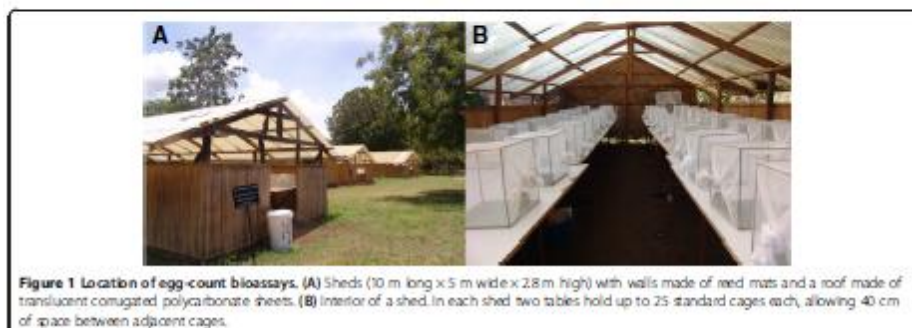


Figure 1 Location of egg-count bioassays. (A) Sheds (10 m long × 5 m wide × 2.8 m high) with walls made of reed mats and a roof made of translucent corrugated polycarbonate sheets. (B) Interior of a shed. In each shed two tables hold up to 25 standard cages each, allowing 40 cm of space between adjacent cages.

Table 1 Summary details of dual-choice, egg-count bioassays to evaluate oviposition choices in *Anopheles gambiae sensu stricto*

Dual-choice cage, egg-count bioassays	Treatments		No of rounds	Total no of females responding for all rounds (total number set up)
	Control	Test		
Set 1: Pellet infusions	Lake water	Lake water	3	66 (75)
	Lake water	2-day old pellet infusion		64 (75)
	Lake water	4-day old pellet infusion		67 (75)
	Lake water	6-day old pellet infusion		68 (75)
Set 2: Soil infusions	Lake water	Lake water	9	153 (225)
	Lake water	2-day old soil infusion		161 (225)
	Lake water	4-day old soil infusion		160 (225)
	Lake water	6-day old soil infusion		171 (225)
Set 3: Vision versus olfaction in soil infusions	Lake water	Lake water	12	186 (220)
	Lake water	6-day old soil infusion		150 (220)
	Lake water	Autoclaved 6-day old soil infusion		157 (220)
	Autoclaved 6-day old soil infusion	6-day old soil infusion		169 (220)
Set 4: Olfactory memory – pellet infusions	Lake water reared <i>An. gambiae</i> females		1	31 (43)
	Lake water	6 day old pellet infusion		
	Pellet in infusion reared <i>An. gambiae</i> females		1	37 (43)
	Lake water	6-day old pellet infusion		

multimeter (Multi 340i, WTW, Germany). In addition one batch of pellet, soil infusion and lake water was tested for ammonium (NH_4^+), carbonate hardness, total hardness, nitrate (NO_3^-), nitrite (NO_2^-), and phosphate (PO_4^{3-}) content using Aquamerck® test kits from the compact laboratory for water testing (Aquamerck® No.111151, Germany).

Are oviposition choices likely to be based on chemical cues?

Soil infusions differed strongly in colour and turbidity from lake water. To assess if the oviposition response observed was based on visual or chemical cues, a third set of dual-choice, egg-count bioassays were implemented with six-day old soil infusions (Table 1, Set 3) comparing the relative attractiveness of autoclaved and non-autoclaved infusion [47,76]. The experiment followed the same experimental procedures as described above. After filtering the infusion through a cloth on the day of experiment, the infusion was split in two equal volumes and half autoclaved at 120°C for 20 min to kill bacteria potentially involved in releasing oviposition semiochemicals [47,77] and to reduce the amount of volatile chemicals from the solution whilst maintaining the colour and turbidity of the infusion. After autoclaving, the infusion was left to cool to ambient temperature before setting up the cage bioassays. The oviposition choice of individual gravid females was evaluated for six-

day old soil infusion versus lake water, autoclaved six-day old soil infusion versus lake water and for autoclaved versus non-autoclaved six-day old infusion. An equal number of cages were set up where both cups in a cage contained lake water and were randomly labelled as control and test cup.

In order to confirm that autoclaving sterilized the infusion, samples (1 ml) of both infusions were taken during each experimental round for bacterial cultures. Samples were serially diluted (ten-fold) two times in distilled water. After dilution, 100 µl of each of the $\times 1$ (undiluted), $\times 10^{-1}$ and $\times 10^{-2}$ dilutions was spread separately onto the surface of duplicate Lysogeny Broth (LB) agar-plates (LB Lennox-Fisher Scientific) [78]. Plates were incubated overnight at 30°C and the presence of colonies recorded.

The same physical and chemical parameters were measured for the autoclaved infusion as described above for the non-autoclaved pellet and soil infusions.

Is the choice of the gravid female influenced by her olfactory memory of her larval habitat?

A fourth set of experiments (Table 1, Set 4) was designed to assess the possibility that a gravid female's choice for an oviposition site might be influenced by her olfactory memory of her larval habitat, as has been suggested for culicine species [4,79].

To test this, approximately 2,000 *An. gambiae* s.s. eggs were dispensed in 1.5 l of two-day old pellet infusion and another 2,000 eggs in lake water and reared under the same conditions to the adult stage. The infusion and lake water in the rearing pans was replaced every two days with fresh two-day old infusion or lake water until all surviving larvae pupated. Larvae were fed with Tetra-min® fish food twice daily following routine insectary procedures. Pupae were collected in a cup with 100 ml of rearing water and placed in 30×30×30 cm cages for emergence. Gravid females for cage bioassays were obtained as described above.

Dual-choice cage bioassays were carried out in parallel with gravid *An. gambiae* s.s. reared in the infusion and gravid *An. gambiae* s.s. reared in lake water. A single mosquito was offered a choice between six-day old pellet infusion or lake water. Forty-five replicates were set up in parallel for both treatment groups as described above.

Sample size considerations

The sample size (number of responders) in the four sets of cage experiments differed for a number of reasons. Due to adverse climate conditions affecting the mosquito supply during the pellet infusion bioassays, the production of colony-reared mosquitoes was low. Nevertheless, two-sample comparison of proportions power calculation showed that 66 responders in each arm in the pellet infusion bioassays (Table 1, Set 1) was sufficient to detect a 23% increase or decrease in the proportion of eggs laid in the treatment compared to the lake water only experiment with 80% power at the 5% level of significance. The effect of the pellet infusion observed on oviposition response was much stronger than 23%. In the soil infusion experiments (Table 1, Set 2 and Set 3), a minimum of 150 responders in each arm was analysed. This was sufficient to detect a 15% increase or decrease in the proportion of eggs laid in the treatment as compared to the lake water only experiment at the same power and significance level. This level of accuracy was deemed appropriate for investigating significant behavioural cues affecting the oviposition choice. The evaluation of olfactory memory required the mosquitoes to be reared in pellet infusion where larval mortality was nearly 98%. Therefore, only 45 females could be tested, out of which only 31 and 37 responded in the two arms (Table 1, Set 4). The hypothesis for this experiment was that the preference of gravid females could be changed and therefore at least double the proportion of eggs laid in pellet infusion by infusion-reared females as compared to the lake water-reared females. With 31 responders in each arm the experiment was powered (80%) to detect a change in the proportion of 35%.

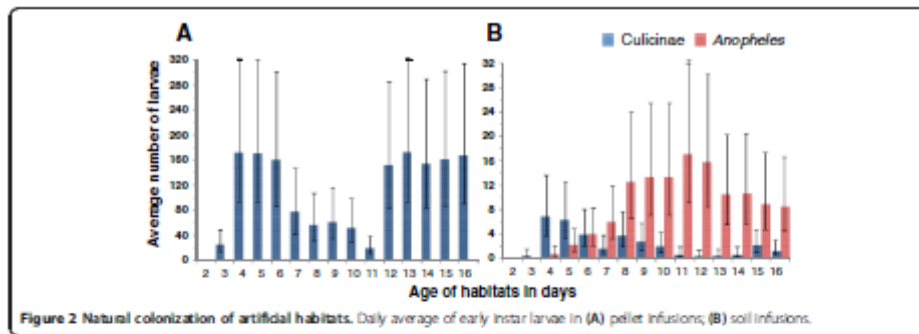
Statistical analyses

All data were analysed in R statistical software version 2.13.1 [80]. The one sample proportion test function was used to estimate the 95% confidence intervals (CI) for the proportion of larvae surviving in pellet infusion, soil infusion and lake water. Pupation time of larvae exposed to different treatments was calculated using the following formula: $(A \times 1) + (B \times 2) + (C \times 3) \dots (G \times 10) / \text{Total number of pupae collected}$, where A, B, C...G are the number of pupae collected on day 1, 2, 3 to 10. Dual-choice, egg-count bioassays were analysed using generalized linear models (glm-function) with a quasibinomial distribution fitted to account for the overdispersion. In the first three sets of experiments the proportion of eggs laid in test cups in the cages with equal treatments (lake water in both cups) were compared with the proportion of eggs laid in test cups in cages with two different treatments. It was hypothesized that gravid females presented with an identical treatment lay in both cups in an approximately equal proportion ($p = 0.5$). The statistical analysis aimed to reveal if the test treatment of interest (e.g. infusions of different age) received an increased or decreased proportion of the total number of eggs laid as compared to the lake water only treatment. Therefore, the treatment choice (e.g. lake water only cages, cages with infusion versus lake water) and the round of experiment were included as fixed factors to analyse their impact on the outcome (proportion of eggs laid in test cup). A similar analysis was used for the fourth set of experiments to compare the proportion of eggs laid in test cups (pellet infusion) by gravid females that were reared in hay infusion during their larval development, compared to the proportion of eggs laid in test cups by gravid females that were reared in lake water. The mean proportion of eggs laid in test cups in different treatments and their 95% CIs were calculated as the exponential of the parameter estimates for models with no intercept included. Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

Results

Gravid *Anopheles gambiae sensu lato* females discriminate between habitats when searching for an oviposition site

Mosquitoes oviposited in the artificial ponds shortly after they had been set up since early instar larvae were found from day 3 and larvae hatch approximately 24–48 hours after eggs are laid. Ponds with pellet infusion were colonized exclusively and in high densities by culicine mosquitoes. Not a single *Anopheles* larva was detected over the 16-day observation period. In sharp contrast, early *Anopheles* instar were consistently found from day 3 to day 16 in the soil infusion ponds (Figure 2). Based on the pattern of larval abundance,



peak oviposition occurred six to ten days after setting up the ponds. *Anopheles* nearly always occurred in higher densities than culicines. *Anopheles* larval densities are naturally relatively low in the study area, with approximately one to three larvae/dip in natural habitats [17]. In the present study an average of ten (95% CI 5–18) early instar larvae/dip was recorded, indicating that the soil infusion ponds were a highly favourable habitat.

All pupae collected from the artificial habitats belonged to the *An. gambiae* complex. PCR-based species analysis revealed that nearly all the wild *An. gambiae s.l.* were *An. arabiensis* (98%, 49/50).

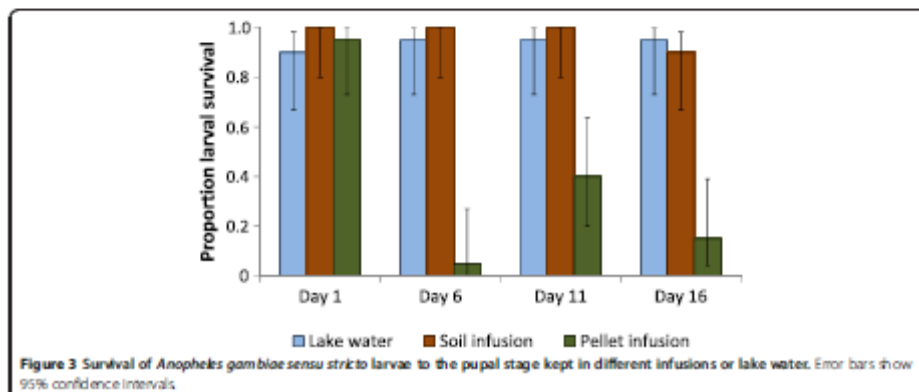
The female's oviposition choice benefits the survival of her offspring

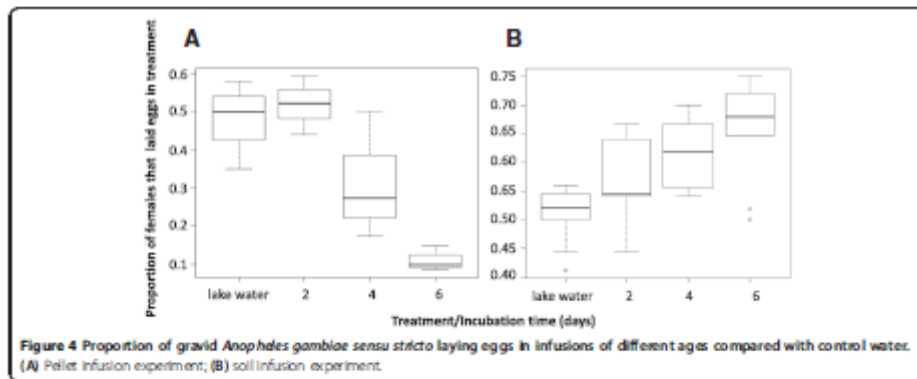
Anopheles gambiae s.s. larvae survived equally well in soil infusion and lake water irrespective of the age of the infusion. In contrast, larvae placed in pellet infusion only survived in the one-day old infusion in similar numbers

but survival was reduced by over 60% ($p < 0.001$) in pellet infusions six days and older compared to lake water or soil infusions of the same age (Figure 3). Mean pupation time for survivors did not significantly differ between treatments or ages of the infusion and was on average 7.5 days (95% CI 6.6–8.3).

Gravid female *Anopheles gambiae sensu stricto* show avoidances and preferences when selecting an oviposition site

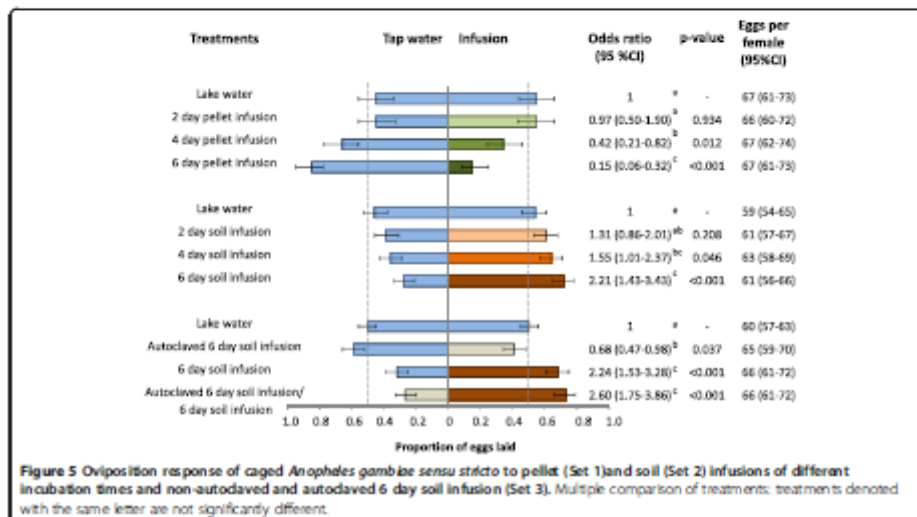
Figure 4 shows the median response rate of the gravid females to the test cup in pellet infusion and soil infusion experiments. An approximately equal proportion of females laid eggs in test and control cups when an equal choice of lake water was provided. Fewer females laid their eggs in pellet infusion as it aged, whilst for soil infusion the opposite was the case with an increasing proportion of females laying eggs in soil infusion as it aged.





Similar results were seen for the proportions of eggs laid although the distribution of eggs between the two equal choices of lake water was slightly skewed (Figure 5, Set 1, lake water control cup 0.45 (95% CI 0.34-0.56) versus lake water test cup 0.55 (95% CI 0.44-0.66)) though not significantly different from 0.5. The distribution of eggs between lake water and two-day old pellet infusion did not significantly differ from the distribution between the two cups with lake water only. However, pellet water became unattractive from day 4 (Figure 5, Set 1). It was 6.7 times less likely for an egg to be laid in the test cup in the treatments that contained six-day old

pellet infusion versus lake water than it was when both cups contained lake water. During the experiment with soil infusions a similarly skewed distribution in the proportion of eggs laid in the two cups with lake water was observed due to chance alone. (Figure 5, Set 2). In contrast to the pellet infusion, larger proportions of eggs were laid in the test cup in the treatments that contained six-day old soil infusions compared with lake water than it was when both cups contained lake water (Figure 5, Set 2).



On average individual females laid 63 eggs (95% CI 60–65) (Figure 5) irrespective of the experiment and treatment. Notably, 18% (95% CI 11–26%) of gravid females laid eggs in both cups provided in a cage, a behaviour known as skip oviposition in other mosquito species [81] but rarely reported for *An. gambiae* s.s. [82]. The average number of eggs laid by skip-ovipositing females and by females that chose only a single cup was similar. Whilst the percentage of skip-ovipositing females was similar in all treatments with two equal lake water choices and in all soil infusion treatments, this behaviour was affected by the pellet infusion. Only a few *An. gambiae* s.s. females skip-oviposited in the four- and six-day old pellet infusion treatments (6%, 95% CI 3–9%).

Pellet and soil infusions differed in key physical and chemical parameters. All pellet infusions had a strong smell to the human nose, were more or less transparent and had a slightly green colour but differed little in appearance compared with lake water in the oviposition cups. Correspondingly, turbidity levels were low. In contrast, soil infusions did not have any smell to the human nose, were light brown in colour and turbid, providing a strong visual contrast to the lake water. Pellet infusions were also characterized by relatively high conductivity, low pH and oxygen deprivation. In contrast the soil infusions' conductivity was approximately half that of pellet infusions, was saturated with dissolved oxygen and had a higher pH (Table 2). The variability of these measures between infusions of different incubation times within a treatment group was relatively low and does not appear to explain the differences in the behavioural responses. The only factor that changed over time was turbidity in

the soil infusion and notably the most preferred six-day old infusion was less turbid than the others.

The increased carbon and total hardness of the pellet infusion corresponded with the increased conductivity levels and the high ammonium and phosphate levels compared with the soil infusion (Table 2).

Chemical cues from the infusions are responsible for the oviposition choice in cage bioassays

Since soil infusions differed in appearance from the lake water, an additional set of experiments was carried out to evaluate whether the attractiveness of this infusion was due to visual cues. Cage experiments with two equal choices of lake water confirmed an equal distribution of eggs between control and test cup. Notably, when gravid females had a choice between lake water and autoclaved soil infusion, the lake water was preferred. The oviposition preference for six-day old soil infusion compared with lake water was also confirmed in this set of experiments with nearly identical odds ratios as before of 2.2. The preference for the six-day old infusion was corroborated when given a choice between autoclaved and non-autoclaved infusions of similar colour and turbidity (Figure 5, Set 3).

Results from this set of experiments suggest that chemical cues are involved in the oviposition responses observed. If the preference for the six-day old soil infusion over lake water was based on turbidity and/or colour of the infusion alone, a similar response in the choice tests with autoclaved versus non-autoclaved infusion should have been seen as in the choice tests with lake water versus autoclaved infusion. Due to the slight

Table 2 Physical and chemical properties of pellet and soil infusions

Parameter	Oviposition substrates in choice experiments							
	Lake water	Pellet infusions			Soil infusions			
		2 days	4 days	6 days	2 days	4 days	6 days	Auto-claved
Turbidity (NTU)	1 (0.6–1.4)	22 (20–23)	17 (14–19)	25 (20–29)	222 (197–248)	97 (92–102)	73 (61–84)	137 (108–166)
Conductivity (µS/cm)	107 (105–110)	477 (462–491)	553 (547–559)	543 (532–555)	173 (171–176)	207 (208–212)	237 (233–242)	266 (258–274)
Dissolved oxygen (mg/l)	4 (2.7–5.3)	0.3 (0.21–1.4)	0.7 (0.9–1.4)	0.3 (0.2–0.4)	5.3 (5.0–5.6)	6 (5.7–6.3)	6.3 (6.0–6.6)	4.8 (4.6–5.1)
pH	8.1 (7.9–8.1)	6.3 (6.2–6.3)	6.7 (6.5–6.9)	7.4 (7.3–7.5)	7.7 (7.6–7.7)	7.9 (7.8–7.9)	8 (7.9–8.0)	8.9 (8.8–8.9)
Ammonium (mg/l)	0			5			0	0
Nitrate (mg/l)	10			10			10	10
Nitrite (mg/l)	0			0			0.025	0.025
Phosphate (mg/l)	0			3			1.5	1.5
Carbonate hardness (mmol/l)	0.1			3.9			2.7	2.5
Total hardness (mmol/l)	0.1			1.5			0.7	0.7

avoidance of the autoclaved infusion the odds of finding an egg in the non-autoclaved six-day old infusion should have been approximately 1.3 (decreased choice for autoclaved infusion by 30% or increased choice of fresh infusion by 30%). Nevertheless, the remaining odds of 2.2 can only be explained by chemical cues being either involved in attracting the female from a short distance or stimulating the female to lay eggs on contact with water. Physical and chemical water parameters were similar for autoclaved and non-autoclaved soil infusions. Bacteria cultures from autoclaved infusions confirmed that samples did not contain any bacteria that could grow on LB plates as opposed to the non-autoclaved infusion where colonies of at least three different morphologies were observed.

The oviposition choice of the gravid female is not influenced by her olfactory memory of her larval habitat

Rearing *An. gambiae* s.s. in two- to four-day old pellet infusion did not alter their oviposition response towards the infusion ($p = 0.392$). Gravid females reared in lake water and gravid females reared in pellet infusion show an equally strong avoidance of the six-day old pellet infusion provided in choice experiments (Figure 6, Set 4).

Discussion

The results confirm that wild and caged *An. gambiae* s.l. females discriminate (defined as recognition and understanding of the difference between two things [83]) between potential aquatic habitats for oviposition and make clear choices when presented with contrasting oviposition media. These choices benefit the survival of the offspring. Although the experimental design does not allow a conclusion whether the stimuli acted over a distance (attractants and repellents [84]) or on contact with the oviposition medium (stimulants and deterrents [85]), it could be demonstrated that the choice of breeding site is guided by both avoidance and preference. However, the exclusive way in which the artificial ponds were chosen in the field, where they were set up relatively

close to each other, suggest that these characteristics were detected by both culicines and anophelines from a distance rather than on contact.

Muddy water has previously been suggested to increase oviposition response of gravid *An. gambiae* s.s. in cages when offered together with clear water [41], however in this study cage experiments could not confirm this observation. The difference in turbidity between lake water and infusions cannot explain the avoidance and preference observed at short range in the cages. Two-day and six-day old pellet infusions did not differ in their turbidity but significantly differed in the oviposition response they received. Similarly, all soil infusions should have elicited equally strong responses from gravid females if turbidity was an important oviposition cue at short range. On the contrary, the six-day old soil infusion remained equally preferred for oviposition when tested against a turbid and autoclaved infusion than when tested against clear lake water. The results suggest that chemical and not visual cues were responsible for the responses observed in the cage experiments. The previously published preference of muddy water [41] may have been based on chemical cues associated with the muddy water which was taken from a natural habitat. However the possibility that visual cues played a role in the selection of oviposition sites by wild mosquitoes in field experiments especially when searching for water bodies from a distance cannot be entirely excluded.

It is likely that the chemical cues used by mosquitoes to avoid pellet infusions and to prefer soil infusions were at least partly of microbial origin, which is supported by the lack of attraction to the autoclaved soil infusion compared to clear lake water. It is most likely that the observed oviposition choices as well as the larval survival were rooted in the water quality of the habitat and consequently the associated micro-organisms and chemicals in the water. The physical and chemical water parameters measured for the two infusions suggest that they represented aquatic habitats in different stages of decay. Contrary to the expectation at the onset of the experiment, pellet infusions created with increasing age a habitat type in a severe state of decomposition. High ammonia and phosphate levels are characteristic of recently undated organic material [86]. The odour of the pellet infusion is associated with fermentation of organic material by facultative and anaerobic bacteria. This leads to depletion of the oxygen supply and a decrease in pH as a result of accumulation of organic acids in the water [87-89]. The soil infusion had the characteristics of a less nutrient-rich habitat containing relatively little organic matter. This limits the removal of oxygen by aerobic heterotrophic micro-organisms and hence the water column will stay aerobic [89].

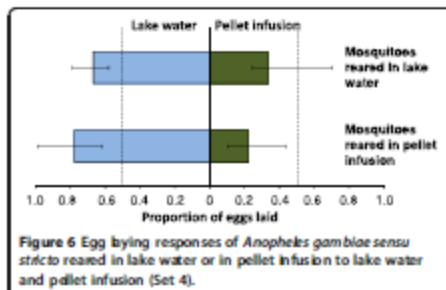


Figure 6 Egg laying responses of *Anopheles gambiae sensu stricto* reared in lake water or in pellet infusion to lake water and pellet infusion (Set 4).

Anaerobic fermentation products of organic matter have been previously shown to be highly attractive to a number of gravid culicine species such as *Culex stigmatosoma* (formerly *peus*) [87], *Culex quinquefasciatus* [33,65,90-92], *Culex pipiens* [64], *Aedes aegypti* [77,93] and *Aedes albopictus* [94,95]. These infusions have been associated with a range of bacteria such as *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* [96-98] and volatile chemicals produced by them including 4-methylphenol, 3-methylindole, carboxylic acids and methyl esters [33,39,99,100]. It is likely that similar factors were responsible for the strong repellent/deterrent effect on gravid *An. gambiae s.l.* females.

Most stagnant water bodies will show increasing signs of decomposition over time but the speed and extent of this will depend largely on habitat quality [101]. Therefore, it is argued that habitat age or permanence alone is not a good predictor for the oviposition response of *An. gambiae s.l.* as has been suggested [45]. For example, the content and input of organic matter, source of water and frequency of fresh water inflow will affect the composition of the biotic community and chemical and physical characteristics of an aquatic habitat [86,89,101]. This might explain why in some environments semi-permanent and permanent habitats are just as well colonized as temporary habitats traditionally thought to be the preferred *An. gambiae s.l.* habitats [13,17,67]. Habitats made of pellet infusion were avoided by *Anopheles* from an early habitat age, whilst interestingly, the highest preference of the soil infusion was recorded on day 6 in the laboratory and between day 6 and 10 in the field after the habitats were well established, contradicting the idea that *An. gambiae s.l.* is a pioneer species colonizing temporary habitats immediately after their occurrence [13].

Typically, it is reported that *An. gambiae s.l.*, although largely a generalist, is not found in heavily polluted waters [19,102]. Hancock [103] further observed that *An. gambiae s.l.* avoided water with a low pH when it was also accompanied with high organic matter content. Addition of freshly cut vegetation (i.e. grass cuttings) to aquatic habitats has also been shown to prevent the larval development of *An. gambiae s.l.* [16]. The results from the experiments with pellet infusions support these observations. On the other hand, there have been recent reports of *An. gambiae s.l.* colonizing polluted habitats especially in urban areas [104-106]. Clearly, the degree of avoidance or acceptance of a polluted habitat by *An. gambiae s.l.* depends on the extent and nature of pollution [19]. Results show that two-day old pellet infusions were not rejected by *Anopheles* and even four-day old infusions still received a considerable proportion of the oviposition responses despite their adverse water characteristics. This supports the idea that *An. gambiae s.l.* has

a very high tolerance level of what they accept as oviposition sites, especially in the absence of better alternatives in close vicinity as is often the case in urban environments and in contrast to the here presented field experiment where good habitats were offered right next to the unfavoured ones.

Importantly, *An. gambiae s.l.* appears to have an innate propensity to avoid specific chemical cues that were emitted from the pellet infusion. Rearing *An. gambiae s.l.* from egg to pupae in this infusion did not alter this behaviour. Gravid females that had experienced the pellet infusion during larval development avoided the infusion for oviposition as much as the females that had no prior experience of it. This suggests that the environment in which *An. gambiae s.l.* develop as larvae does not determine the preferred oviposition site when they return to lay eggs. This is in contrast to published work on *Cx. quinquefasciatus* where it was demonstrated that rearing the larvae in an infusion made from guinea-pig faeces cancelled their innate preference for a hay infusion [4].

The cage bioassays with individual gravid females allowed a number of interesting observations that are rarely reported since the majority of studies with *An. gambiae s.l.* are done with groups of mosquitoes where the actual number of females laying per cage is unknown [41]. The occurrence of skip-oviposition in gravid *An. gambiae s.l.* and how this is affected by chemical cues was demonstrated. Furthermore, the design revealed that the mean number of eggs laid per female in a cage was similar irrespective of the experiment and treatment; only the distribution between cups differed when two different choices were presented. This indicates that gravid females did not retain their eggs in the presence of an unfavoured substrate when they were offered a suitable alternative choice. It also shows that the preferred soil infusion did not stimulate individual females to lay more eggs than they would do in lake water. Testing individual females also excludes potential aggregation effects. Whilst from the field experiments it might have been possible that gravid females selected habitats that already received eggs from conspecific females, cage bioassays with individual females showed the same avoidance and preference behaviour as observed in the field, confirming that conspecifics alone cannot explain the observed choice.

The potential involvement of microbial activity in breaking down organic matter and producing semiochemicals that impact on the oviposition responses of gravid *An. gambiae s.l.* was deduced partly by the lack of attraction of *An. gambiae s.l.* to a sterile soil infusion. However, this must be interpreted with caution since autoclaving the infusion might not only have killed the microbes but affected the chemistry of the resulting infusion, possibly altering the response of gravid mosquitoes by chemical changes rather than biological changes [77].

Batch-to-batch variations were recorded in the response of gravid mosquitoes to the infusions, resulting, for example, in some rounds showing a high preference and other rounds only a moderate preference for the soil infusion. This variation can be attributed to differences in the quality and amounts of odorants released from the infusions and stochastic events. Fresh infusions were prepared for every test round with different batches of pellets and soil. Especially, for the soil it is highly likely that there were differences in the soil condition as well as differences in the species composition of the microbial community associated with the natural materials over time. It has been shown previously that natural infusions can be an inconsistent source of odorants for oviposition site-seeking mosquitoes and therefore every batch needs to be verified to be behaviourally active before it can be used for subsequent experiments [77]. Ideally, if info-chemicals were to be used for monitoring and/or controlling gravid malaria vectors, specific chemically defined oviposition cues would be preferred over natural infusions to ensure a consistent response in gravid females either pushing them away from human population [107,108] or pulling them towards a gravid trap [38,109].

Whilst the observed avoidance behaviour towards the organically rich pellet infusion was strong and in the same range as reported for other species in response to unfavourable chemical cues [107,108,110], the observed preference in the cages for the soil infusion was relatively weak and it is questionable whether it could compete with other suitable habitats from a larger distance. Nevertheless, consistent response derived from over 150 replicates in two experiments likely represents a genuine effect. Further investigations are in progress to characterize the bacteria communities associated with the infusions and the volatile chemicals emitted from the infusions and detected by gravid *An. gambiae* s.s. using gas-chromatography coupled to mass-spectrometry and coupled gas chromatography-electroantennogram detection.

It must be cautioned that not all soils and all rabbit food pellets will lead to the same physical and chemical parameters as the infusions presented here. Therefore the two infusions of this study only serve as specific examples for two highly contrasting media. Further work is needed to screen other soil samples to see if the observed response is a response common for all soil infusions prepared under standard conditions and if the same bacteria and chemical profiles can be detected, or, which is more likely, that there are significant differences depending on the source of the soil.

Conclusion

This work illustrates that a gravid *An. gambiae* s.l. female selects a suitable habitat for oviposition using chemical cues from water bodies. It furthermore emphasizes that

natural infusions can be used to manipulate the oviposition behaviour of *An. gambiae* s.l. Soil infusions have the potential to be used to bait gravid traps for the collection of *An. gambiae* s.l., although further work must be implemented to elucidate whether the observed preference was based on the specific soil type tested or whether similar responses can be achieved with any soil. The low *An. gambiae* s.l. catching efficacy reported for gravid traps operationally used for *Culex* and *Aedes* monitoring might partly be explained by the infusions routinely used in these traps, i.e. fermented hay infusions, rabbit food pellet and cow manure infusions [64-112]. The identification of the chemicals responsible for the preference of the soil infusion might be exploited to bait gravid traps specifically for the collection of *An. gambiae* s.l.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UF, J., MH-V and SWL conceived the idea for this research and developed the experimental design. MHV developed all protocols and implemented the experiments. MH-V and UF analysed the data and wrote the first draft of the manuscript. All authors contributed to the final draft, read and approved the manuscript.

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

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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 orientated movements towards the source.

Methods: Three experiments were carried out with insectary-reared *An. gambiae s.s.* One with unfed females and two with gravid females during their peak oviposition time in the early evening. 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 24 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.

Keywords: *Anopheles gambiae*, Oviposition, Water vapour, Gravid mosquitoes

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 [1,2], yet this is a gross oversimplification of the types of habitat actually colonized by these mosquitoes [3]. 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 [4-7]. 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 [5-7]. Nearly every type of water accumulation, apart from excessively polluted smelly water, may contain anopheline larvae [3-5,8-11]. 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 [12], 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 [1,13,14]. Although some putative semiochemicals have been suggested based

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on coupled gas chromatography-electroantennogram detection [15-17], to date, no semiochemical has been confirmed to affect the behaviour of gravid *An. gambiae* s.l. 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 [14]. 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.

Methods

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).

Mosquitoes

Insectary-reared *An. gambiae* s.s. (Mbita strain) were used for all experiments. Five-days-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 30 × 30 × 30 cm netting cages and provided with 6% glucose solution *ad libitum*. These females never had a bloodmeal 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 30 × 30 × 30 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, 50 × 25 cm in area, were folded and placed over the cages to avoid mosquito desiccation. Mosquitoes were starved from 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.

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 purification. 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 Additional file 1. Gravid females did not have a significant preference for either filtered tap water or distilled water.

WHO-tube bioassays

Choice tests were carried out in the laboratory under ambient conditions. Natural moonlight came from a window located 2 m from the set-up. For each choice test, three WHO bioassay tubes, each 12 cm long [18] 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 15 × 15 × 15 cm. Cages were wrapped in commercially available kitchen cling-film (Figure 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 15 × 15 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 around 18.00 with the gates opened by 2 mm (not too wide to let the mosquito through) allowing some

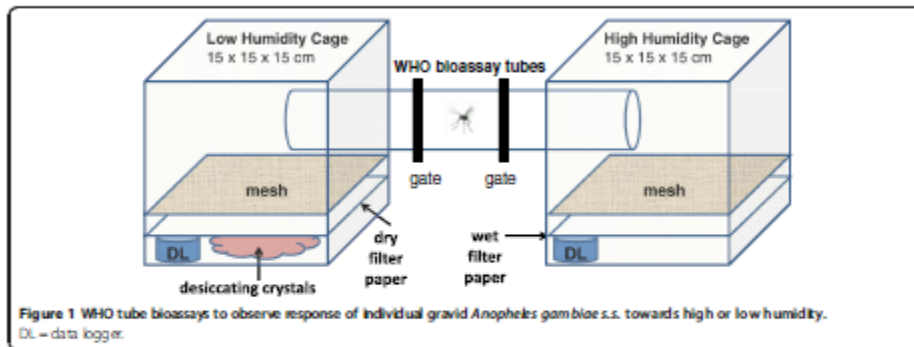


Figure 1 WHO tube bioassays to observe response of individual gravid *Anopheles gambiae* s.s. towards high or low humidity. DL = data logger.

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 \times 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 [19]. 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 [20]).

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 2). Each tunnel measured 40 \times 100 \times 30 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 (20 \times 20 \times 30 cm each). Two fans (diameter 8 cm, 6 V 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 10 \times 10 \times 10 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 odorant 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 [21] 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

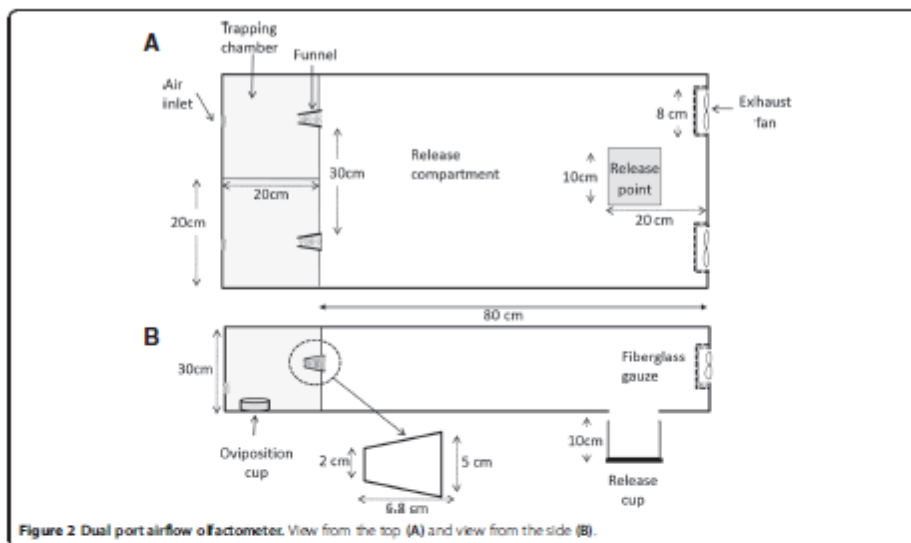


Figure 2 Dual port airflow olfactometer. View from the top (A) and view from the side (B).

chamber ($p1 = 0.5$). 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 ($p1$ and $p2$) can detect an increase or decrease in the catch rate of 20% ($p2 = 0.7$) 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.

Statistical analysis

Data were analysed using generalized 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 over-dispersed, 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 [22].

Results

WHO-tube bioassays

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 3). Average temperatures during the experiments ranged between 27 and 28°C. Conditions were similar in both experiments with unfed and gravid females.

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 had moved only in small and similar proportions to the low and high humidity cages (Figure 4).

Unfed females showed no preference for any of the two conditions provided (Table 1, Figure 4). When gates were closed at 21.30 half of the unfed females had moved in the

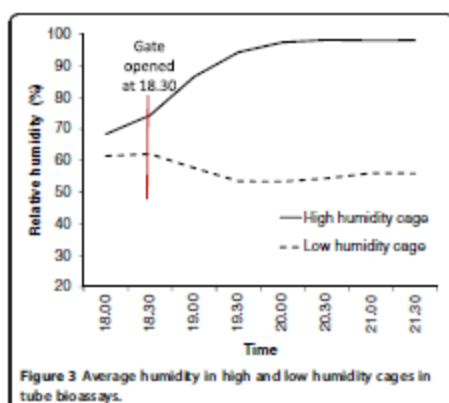


Figure 3 Average humidity in high and low humidity cages in tube bioassays.

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 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.

Airflow olfactometer bioassays

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%).

Table 1 The mean percentage of gravid *Anopheles gambiae* s.s. attracted to the test cage in the WHO-tube bioassays and to the test compartment in the airflow olfactometer bioassays

Experimental treatment	Mean percentage (%) in test (95% CI)*	Odds ratio (95% CI)	p
Response towards high humidity cage (test) in WHO-tube bioassays at 21.30			
Non-fed females	50 (38-62)	1	
Gravid females	71 (59-81)	2.4 (1.3-4.7)	0.018
Airflow olfactometer bioassays with gravid <i>An. gambiae</i> s.s. in three experimental treatments			
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.

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 up-wind in any of the two chambers whilst the rest remained in the release compartment (Figure 5).

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 1) irrespective of whether the test cup was presented in the

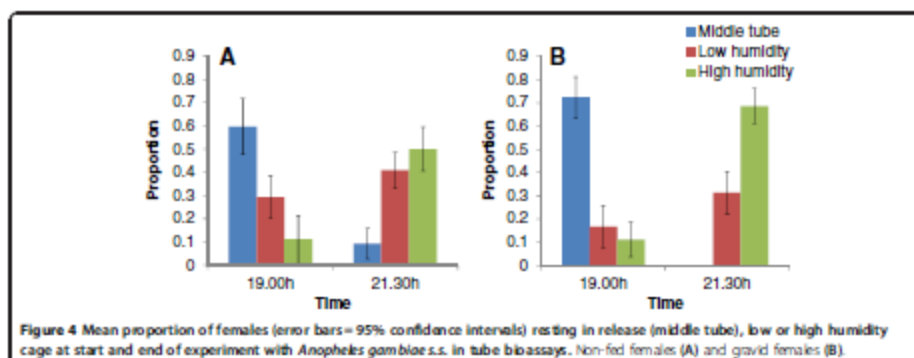


Figure 4 Mean proportion of females (error bars = 95% confidence intervals) resting in release (middle tube), low or high humidity cage at start and end of experiment with *Anopheles gambiae* s.s. in tube bioassays. Non-fed females (A) and gravid females (B).

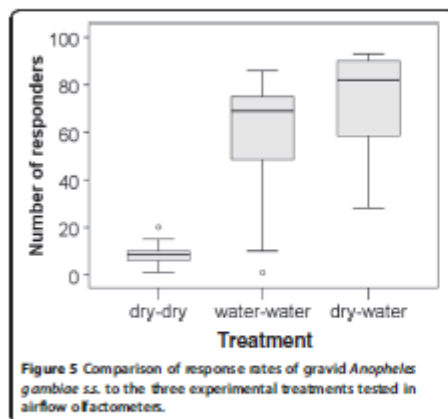


Figure 5 Comparison of response rates of gravid *Anopheles gambiae* s.s. to the three experimental treatments tested in airflow olfactometers.

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).

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 [23,24]. 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 [25]. Early studies indicated that in *Aedes aegypti* humidity receptors were present on the antennae of females [26]. In *Anopheles atroparvus* the hygroreceptors were located on the distal segments of the antennae bearing most of the grooved pegs [27]. Recent 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 [28]. Whilst it has been shown that humidity is

important for the survival of mosquitoes [29], a clear difference in the behaviour of unfed and gravid females was demonstrated in the presented WHO tube experiments. The strong responses observed in gravid mosquitoes towards moving to areas of very high humidity is likely to increase the reproductive success of females, since they are more likely to find an aquatic habitat that might serve as a potential oviposition site, and would therefore 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 for detecting the humidity differences and direction before moving, 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 [30]. 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 [31].

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 (Additional file 1).

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*)' [32]. 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 [33]. 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 [13] and indoor-resting mosquitoes [34]. 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 [35] and with semiochemicals attracting and repelling gravid *An. gambiae* mosquitoes over short distances [14,15,36,37].

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.

Additional file

Additional file 1: Cage bioassays comparing the oviposition response of *Anopheles gambiae* s.s. to filtered tap water and distilled water in two choice experiments. The document presents the background, method and results of the experiment.

Competing interests

The authors have declared that they have no competing interests.

Authors' contributions

SWL, UF, MD, MH, conceived the idea for this research. SWL, BF and MH developed the experimental design for the tube bioassays and implemented them with gravid females. MD implemented them with non-fed females. MD designed and built the airflow olfactometers. MD and UF developed the experimental design and implemented the olfactometer bioassays. MH contributed the additional data comparing distilled water with tap water. MD and UF analysed the data and BF, SWL and UF collated the first manuscript draft. All authors contributed to the final draft, read and approved the manuscript.

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Analyzing chemical attraction of gravid *Anopheles gambiae sensu stricto* with modified BG-Sentinel traps

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Abstract

Background: Insight into the oviposition behaviour of malaria vectors in Africa could lead to strategies that target gravid mosquitoes for monitoring and control. However, cues that guide gravid females to these sites are not well understood. This study aimed to develop a bioassay system that can be used to analyse chemical attraction of gravid *Anopheles gambiae sensu stricto*.

Methods: BG-SentinelTM mosquito traps that utilize convection currents to release odorants were modified to contain aqueous substrates. Two-choice tests were implemented within an 80 m² screened semi-field system where 200 gravid females were released per experimental night. Choices tested were (1) distilled versus distilled water (baseline) and (2) distilled water versus soil infusion. To study the effect of salting-out of volatile chemicals from the aqueous solutions, we also implemented tests with 150g/l NaCl added.

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-SentinelTM mosquito traps turned it into a powerful bioassay tool for evaluating the orientation of gravid mosquitoes to putative oviposition substrates using olfaction. We provide evidence that gravid *An. gambiae s.s.* are attracted to and can be baited with odorants and water vapour over several meters.

Introduction

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 [1]. Effective interventions need to be scaled up [2] and new approaches added to the armamentarium for controlling the disease and its vectors [3,4]. 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 led to a major reduction of 29% in malaria cases worldwide [2] justifying efforts to scale up LLINs and IRS. However, because of the growing problem of insecticide resistance [5,6,7], changing vector behaviour [4,8,9,10] and increasing importance of outdoor-biting vector populations [4] the effectiveness of these may be compromised and additional strategies required.

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 considerable progress towards identification of odorants from skin emanations of humans and other primary blood meal hosts [11,12] and host plants [13]. These volatiles have been incorporated into baits and tested in traps [13,14]. 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 [15,16,17,18,19] 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 [20,21]. 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 [22]. 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 them either into traps or to insecticides [23].

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 [24,25] and the presence or absence of bacteria [15,26,27]. A recent study demonstrated that at short-range gravid *An. gambiae s.s.* can avoid or select substrates using olfactory cues [28]. In another comparable laboratory study one synthetic odorant, 2-propylphenol was shown to increase the egg-laying rate of *An. gambiae s.s.* in cage tests [29]. However, to the best of our knowledge there is no published evidence to date that gravid females of the *An. gambiae* complex orient towards a suitable aquatic habitat over a distance of several metres using attractant chemical cues.

The aim of the present study was to develop a simple bioassay for measuring olfactory orientation of gravid *An. gambiae s.s.* in the semi-field and evaluated the response of gravid mosquitoes to soil infusions previously described [28] to increase the egg-laying rate of these species in small experimental cages.

Materials and methods

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.

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 through visual inspection on the third day. Females were presumed gravid when they had an opaque and pale distended abdomen.

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 1). A netting ceiling was stretched across the cage 2.4 m above the ground (176.3 m³). The floor was covered with sand to a depth of 50 cm. The rectangular floor plan 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 1). Sites along the shorter walls that received approximately the same proportion of mosquitoes in preliminary tests (data not shown) were paired up (site 2 + 3 and site 1 + 4; Figure 1). The pair and the position of the control (trap A) and test treatment (trap B) 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 [25] 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 [30] 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.

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

We chose to modify and test the commercially available BG-Sentinel™ mosquito trap (Biogents, Regensburg, Germany). 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 [31,32]. 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’ [33]. 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. [25]. 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 2).

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 distilled water (Buyimpex Agencies LTD, Kenya), with the position of the traps allocated randomly.

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 [28]. 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 [26,34]. Here, the same soil was used to prepare infusions in the same way as before [28] 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. [28] 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.

Testing of the impact of salting-out of volatile chemicals from infusions on gravid female attraction to traps

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 [35,36,37,38,39,40,41]. For instance, Mozuraitis et al. [42] 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. Here we aimed at testing whether we could increase the attractiveness of the soil infusion by adding sodium chloride (NaCl). Following published data on salt concentrations [42], we implemented a preliminary experiment where we slowly added 45 g of NaCl to 300 ml of soil infusion (150 g/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, we added 150 g NaCl 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.

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 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 [43].

Ethics statement

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

Results

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, which validates the experimental design. Only about one third of all mosquitoes were trapped before 21:30 h (Figure 3, Table 1).

Soil infusions contain odorants 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. Moreover, adding NaCl increased the attractiveness of the infusion; females were 3.5 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 more likely to be collected in the trap containing the infusion with salt (Figure 3, Table 1).

The presence of attractive odorants 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 for a mosquito to respond and be collected in either trap than when only distilled water was presented in both traps (Figure 3, Table 1).

Odorant cues from soil infusions prompt early oviposition site seeking in *Anopheles gambiae s.s.*

The presence of soil-infusion odorants doubled the proportion of mosquitoes that responded before 21.30 h (Figure 3, Table 1).

Discussion

Minor modifications of the commercially available BG-Sentinel mosquito trap turned this trap into a powerful 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 ($\geq 20\%$) in the proportion of gravid mosquitoes attracted to one of two competing substrates (odorant 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 the first evidence that gravid females of the major malaria vector *An. gambiae s.s.* can use attractive odorant 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 [15,16,26,28]. 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 [44,45], or discount

stimulation. We show that odorants from the soil infusions reported by Herrera-Varela et al. [28] attract gravid *An. gambiae s.s.*. Furthermore, our results show that oviposition attraction to odorant 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 this suggests 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 [25]. Previous studies were done in small, closed laboratory systems, free of external odorants with standardized water vapour and artificial moisture gradients [24,25]. With the bioassay, where both traps contained distilled water only, we provide 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 and moisture most likely indicate the presence of water bodies while chemical odorants enable mosquitoes to assess the suitability of this potential niche.

Based on our findings we hypothesise that the soil infusions we tested contained at least one odorant that prompted habitat searching in gravid *An. gambiae s.s.*. The odorant 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 [46]. In nature such an odorant or collection of odorants 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. We show 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 can not 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 [35,36,37,38,39,40,41,42]. 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 [39,47] which most frequently is positive (salting-out) but can also be negative (salting-in) [48,49]. 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 on 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 [50,51] proving its versatility and effectiveness. This study now shows that with only small modifications it has potential for collecting gravid mosquitoes too. If it can be exploited in ecological studies or in vector control programmes needs to be evaluated in the field.

Conclusion

In summary we (1) describe an efficient bioassay tool and potential new odour-baited trap for gravid females of the *An. gambiae* species complex; (2) provide evidence for the strong involvement of olfaction in the location and selection of potential breeding sites by *An. gambiae s.s.*; and (3) describe 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.*

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Author contributions

MNO, UF, MH, JML, BT and SWL conceived the idea for this research and jointly developed the experimental designs. MNO developed all protocols and implemented the experiments

together with PO. MNO and UF analysed the data and drafted the manuscript. SWL, BT, JML, MH and PO contributed to the final draft, read and approved the manuscript.

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Figure legends

Figure 1: Semi-field system (A) and schematic diagram of trap 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.

Figure 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.

Figure 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 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.

Table1: Oviposition response of gravid *Anopheles gambiae sensu stricto* to substrates in two-choice tests. Generalized linear model outputs.

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

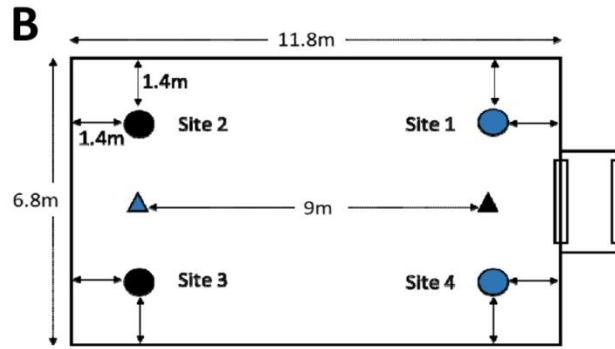


Figure 1.

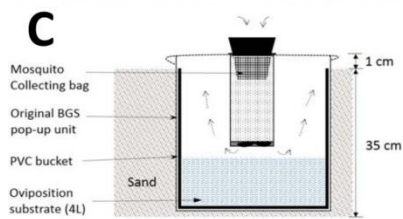
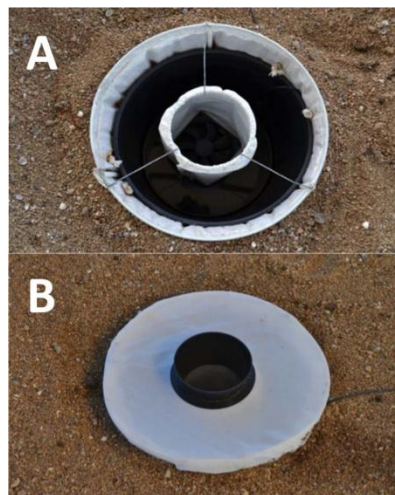


Figure 2.

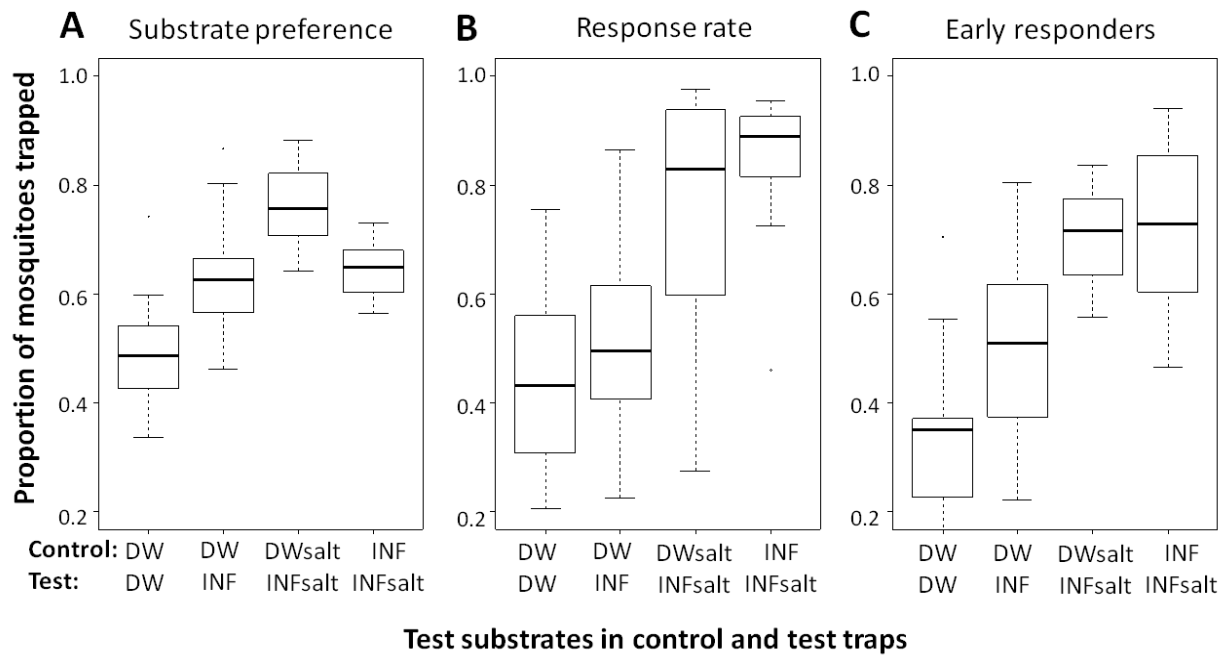


Figure 3.

Discovery of an odor bait for gravid malaria vector mosquitoes of the *Anopheles gambiae* species complex

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New strategies are needed to manage malaria vector mosquitoes that resist insecticides and bite outdoors. Here we explore the first steps to develop an 'attract and kill' strategy targeting gravid females by identifying and evaluating oviposition attractants for *An. gambiae* s.l. Previously, we found that lake water infused for six days with soil from a natural oviposition site in western Kenya doubled the chances of eggs being laid by gravid *An. gambiae* s.s. females compared to lake water alone. In this study we combine gas-chromatography coupled to mass-spectrometry (GC-MS) and cage and field bioassays to identify an oviposition attractant from this soil infusion. Analysis of the GC-MS data revealed a group of putative semiochemicals of which one, identified as cedrol, occurred in >50% of natural aquatic habitats in western Kenya. When applied to water, 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 free-flying experiments conducted in large-screened cages (OR 1.92; 95% CI 1.63-2.27). When tested in the field, traps baited with cedrol collected three times more wild malaria vectors than traps containing only water. Cedrol is the first oviposition bait identified for gravid *An. gambiae* s.l. This finding paves the way for developing new 'attract and kill strategies' for malaria control.

malaria | *Anopheles gambiae* s.l. | attract and kill | oviposition attractant | cedrol

Significance Statement

In Africa a child dies every minute from malaria. Mosquitoes of the *Anopheles gambiae* species complex are among the most efficient vectors of malaria on the planet and are responsible for most of these deaths. Vector control is the most important tool to prevent transmission. However, current interventions only target vectors within houses at night. While these interventions remain effective, a significant gap remains in addressing the residual transmission that occurs outside the protection of indoor-based interventions. It has been recently highlighted that additional tools that target outdoor mosquito populations are urgently needed. Our findings pave the way for the development of novel 'attract and kill' strategies targeting gravid females outdoors.

Introduction

Mosquitoes of the *Anopheles gambiae* species complex (*Anopheles gambiae sensu lato* (s.l.)) including *Anopheles 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 (1). The most effective way to prevent malaria is vector control. The interventions used today to reduce vector numbers primarily target host-seeking mosquitoes indoors (2, 3). 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 (4). The development of an efficient attract and kill strategy for oviposition site seeking females could

be a one of the novel vector control tools that are urgently called for (5, 6).

To date, there has been little research investigating how *An. gambiae* s.l. females find and choose oviposition sites. It is known that water vapor helps to guide them (7, 8), however, in nature many aquatic sites remain uncolonized suggesting that some are more attractive to gravid females than others (9-11). Although a number of chemicals have been proposed as oviposition semiochemicals for *An. gambiae* s.s. (12-14), 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.

Recently, we found that lake water infused for six days with soil from a natural oviposition site in western Kenya doubled the chances of eggs being laid by gravid females in two choice egg count cage bioassays compared to lake water alone. This preference was lost when the infusion was autoclaved (15) suggesting that volatile chemicals, rather than visual cues attracted mosquitoes to breeding sites. Here we analyzed the volatiles released from autoclaved and unmodified soil infusions and the lake water utilized as control in the bioassays. Multivariate analysis revealed a number of putative semiochemicals of which one was selected for evaluation in (1) egg-count cage bioassays to confirm preferential egg-laying, (2) large semi-field systems with free-flying females to test for attraction over large distances and (3) under natural field conditions. Through these experiments we describe the discovery of the first oviposition bait for gravid *An. gambiae* s.l.

Significance

In Africa a child dies every minute from malaria. Mosquitoes of the *Anopheles gambiae* species complex are among the most efficient vectors of malaria on the planet and are responsible for most of these deaths. Vector control is the most important tool to prevent transmission. However, current interventions only target vectors within houses at night. While these interventions remain effective, a significant gap remains in addressing the residual transmission that occurs outside the protection of indoor-based interventions. It has been recently highlighted that additional tools that target outdoor mosquito populations are urgently needed. Our findings pave the way for the development of novel attract and kill strategies targeting gravid females outdoors.

Reserved for Publication Footnotes

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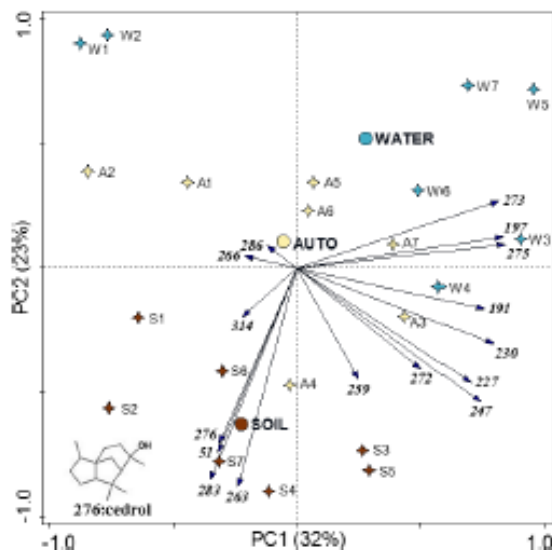


Fig. 1. Biplot of the GC-MS data from lake water, non-autoclaved and autoclaved soil infusions. The three sample types form distinct groups, mainly separated by the second principal component. Four compound IDs (S1, 263, 276 and 283) group closely with the unmodified soil samples. Data from seven rounds of each sample type were centered 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.

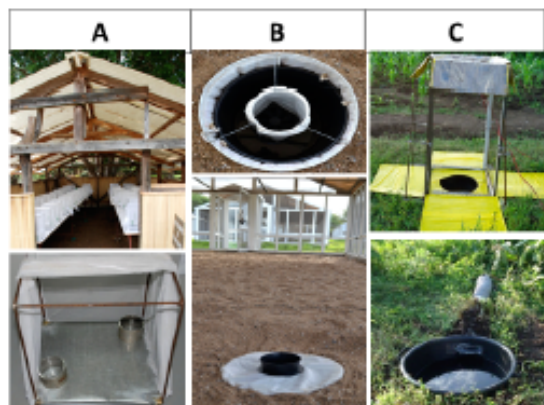


Fig. 2. Experimental set up. (A) Cage bioassays with individual gravid females under ambient conditions in makeshift huts; (B) Modified BG sentinel traps in a semi-field system; (C) Field set up of square of electrocuting nets (up) and OVIART gravid trap (down).

Results

Identification of putative oviposition semiochemicals. Volatile chemicals released from autoclaved and unmodified soil infusions as well as the control lake water were sampled in parallel to behavioral assays and analyzed by gas-chromatography coupled with mass-spectrometry (GC-MS, Fig 1, SFig. 1). Exploration

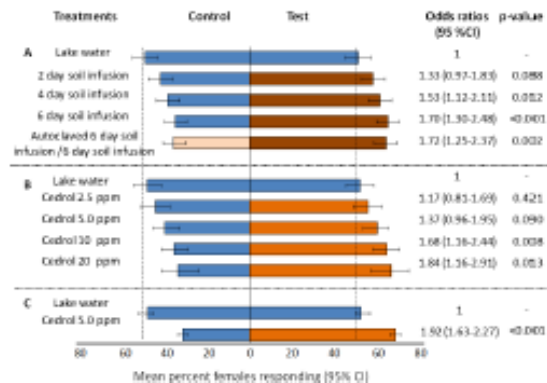


Fig. 3. Mean percent of gravid *Anopheles gambiae* responding to control and test treatments in choice experiments. (A) Cage bioassays with soil infusions of increasing incubation time and comparison of autoclaved versus non-autoclaved infusion. The data from Herrera-Varela et al. 2014 (15) have been re-analyzed for this figure to show the percent of females responding. These data present the background for the current study. Headspace collections for identification of volatile chemicals were implemented for autoclaved and non-autoclaved six-day old soil infusions in parallel to these behavioral 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.

of the GC-MS data using principal component analysis (PCA) indicated similarities within the replicates of the sample types but different chemical profiles between the treatments (Fig. 1). Four compounds (IDs 51, 263, 276 and 283) grouped closely with the unmodified soil samples in the PCA. GC-MS data containing volatiles collected above samples from natural aquatic habitats situated along the shores of Lake Victoria in western Kenya were screened for these four compounds; ID 276 was detected in 62 of the 116 samples whereas none of the other three compounds (IDs 51, 263, 283) were found. This compound (ID 276) was identified as cedrol by comparison of mass spectral data to the NIST08 library and an authentic standard (+-cedrol, $\geq 99.0\%$ sum of enantiomers, Sigma). Cedrol was present in all the soil infusion samples investigated ($n=14$ for unmodified and autoclaved combined) and the amount was three times as high in unmodified soil infusions (mean 15.8 ng, 95% CIs = 9.36-22.2) compared to autoclaved infusions (mean 5.7 ng, 95% CIs = 4.6-6.7). In contrast, it was only detected in two out of seven lake water samples (mean in those two samples: 4.2 ng, 95% CIs = 3.8-4.5).

Cedrol attracts laboratory-reared gravid *Anopheles gambiae* 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 responded to cedrol (Fig. 2 and 3). The cage bioassays demonstrated a dose-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 results for the soil infusions of increasing incubation time when compared to lake water (Fig. 3A and 3B).

Since these egg-count cage bioassays cannot distinguish between contact stimulants and long-range attractants (16) we implemented experiments in a large (174 m³) semi-field system using modified BG-Sentinel traps (Fig. 2B). These traps allowed us to assess the relative attractiveness of volatiles released from the trap, without the influence of visual cues or contact stimulants; it prevented mosquitoes from seeing or accessing the test substrate. The experiments confirmed that cedrol was attractive

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Table 1. Probability of a mosquito female being trapped.

	Rate Ratio (95% confidence interval)				
	<i>Anopheles arabiensis</i>	<i>Anopheles funestus</i> s.s.	<i>Anopheles coustani</i>	<i>Aedes</i> sp.	<i>Culex</i> sp.
Treatment					
Control 1	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)
Trap					
BG	1	1	1	1	1
OviART	5.2 (0.9-30.9)	6.3 (1.6-25.4)	- ^a	- ^a	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)

^a no mosquitoes trapped; factor excluded from model. Treatment: control= lake water, test= lake water with 5 ppm cedrol. Traps: E-nets= squares of electrocuting nets (17), OviART=OviART gravid traps (18), BG= modified BG-Sentinel mosquito traps (19).

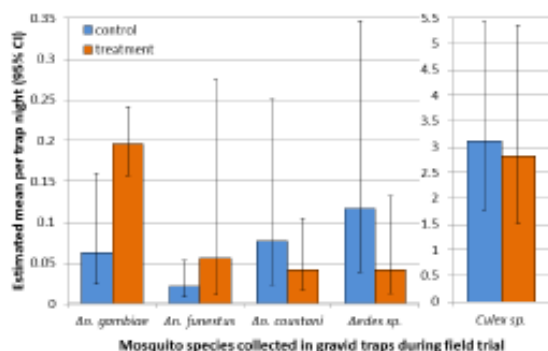


Fig. 4. Estimated mean number of female mosquitoes per trap night collected during the field trial. Error bars represent 95% confidence intervals.

with 69% (95% CI 66-71%) of released females collected in the treated trap (Fig. 3C). 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 lake water only in which only 34% (95% CI 29-38%) of all released females were recaptured.

The peak oviposition time of our caged *An. gambiae* is between 19:00 and 21:30 h (8). 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 h 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/min (95% CIs 5.9-12.7 ng/min) and those treated with 10 ppm released 22.8 ng/min (95% CIs 18.0-29.0 ng/min) during the 12 hours of experiment. The release rate from the convection traps treated with 5 ppm cedrol was on average 8.0 ng/min (95% CIs 5.4-12.0 ng/min). 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 h) and the rest of the night.

Cedrol attracts wild malaria vectors. To test if cedrol also attracts gravid malaria vectors under natural field conditions we carried out a study at the end of the long rainy season in June 2014 in villages close to the shores of Lake Victoria in western Kenya. We used three types of collecting devices: squares of electrocuting nets (E-nets; (17), OviART gravid traps (18) and modified BG-Sentinel mosquito traps (19). A total of 933 female mosquitoes were collected in 288 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 sensu stricto* were collected. Trap catches also included 2% of the secondary malaria vector *An. 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 1, Fig. 4). However, the three traps 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 (Table 1). Whilst the overall number of *An. arabiensis* females collected per trap night was very low, collections of host-seeking females indoors with Centre of Disease Control (CDC) light traps and outdoors with cattle baited traps (CBT) at the same time confirmed that the overall population density of vectors was low during the study period. In CDC traps a mean of 0.73 (95% CI 0.28-1.90) and in CBT 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.

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 1). On the contrary, *An. coustani*, *Aedes* species and the abundant *Culex* species preferred the untreated traps (Table 1).

Discussion

Our 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 in preference to lake water without cedrol for laying their eggs. Furthermore, the odorant attracted colonized free-flying gravid mosquitoes in large semi-field structures and most importantly,

409 wild gravid mosquitoes. The attractiveness of cedrol was est-
410 tablished in comparison to natural water from Lake Victoria
411 which constitutes the majority of the natural, highly productive
412 anopheline habitats in the study area (20) and which previously
413 was found 'to be the most stimulatory water treatment [for *An.*
414 *gambiae*] uncovered to date' in egg-count cage bioassays (21). We
415 considered this comparison more realistic than one using distilled
416 water as a comparator, since it is an artificial water source that
417 wild mosquitoes are unlikely to encounter.

418 Our recently developed systems of analyzing oviposition res-
419 sponses in comparison to a baseline that provides two equal,
420 untreated choices (15) and of measuring attraction of gravid
421 mosquitoes to oviposition substrates with modified BG-Sentinel
422 mosquito traps (19) allowed us to provide a detailed description
423 of the behavior of gravid mosquitoes in response to odorants.
424 Here we showed that cedrol not only increased the proportion
425 of gravid females that were caught in the test trap out of the total
426 number caught, but it also increased the proportion of mosquitoes
427 released that responded. Furthermore, cedrol induced a fast re-
428 sponse, with two third of gravid mosquitoes trapped by 21:30 h. It
429 appears that *An. gambiae* have evolved to use attractive chemical
430 cues not only to select oviposition sites but also to reduce the time
431 taken seeking suitable sites.

432 Our field study was implemented in an area of low vector
433 density as confirmed by the CDC light trap and cattle baited trap
434 catches. Considering that only a proportion of mosquitoes that
435 host seek obtain a sufficient amount of blood and survive long
436 enough to become gravid, it was not unexpected that collections
437 in gravid traps were an order of magnitude lower than catches in
438 gravid traps. Despite low densities, it was three times more
439 likely to trap *An. arabiensis* (the predominant species of the *An.*
440 *gambiae* species complex in the study area) when the trap was
441 cedrol-baited than when it only contained lake water. Previous
442 observations show that the two sibling species *An. arabiensis*
443 and *An. gambiae* s.s. share the same aquatic habitats (11, 22)
444 and therefore it is not surprising that they appear to use the
445 same odorants for orientation and selection of oviposition sites.
446 The collections from the gravid traps also suggested that it is
447 worth testing the attraction of the malaria vector *An. funestus*
448 to cedrol since a slight preference for cedrol-treated traps was
449 recorded. Finding a semiochemical or blend that could attract
450 gravid females of the three most important vectors of malaria
451 in Africa, *An. gambiae*, *An. arabiensis* and *An. funestus* would
452 represent a tremendous breakthrough for the development of
453 novel interventions. The fact that Anopheline mosquitoes could
454 be caught irrespective of their low densities is promising for future
455 development of a surveillance tool for these species using gravid
456 traps baited with cedrol. Future work needs to be invested in
457 testing cedrol under different eco-epidemiological conditions and
458 in developing optimal traps that can be baited with synthetic
459 odorants for routine use.

460 Our results show that the modified BG-Sentinel mosquito
461 traps work extremely well under semi-field conditions but were
462 less effective in the field. We hypothesize that visual cues interact
463 with olfactory signals (23), explaining the better performance
464 of traps with open water surfaces in our study. Further under-
465 standing of the interaction between visual and chemical cues and
466 more effective traps will increase the possibility to efficiently lure
467 vectors into the traps when competing with natural habitats.

468 Cedrol-treated lake water, attracted and trapped similar pro-
469 portions of gravid females in the semi-field experiments as the
470 soil infusions from which it was identified (19). To achieve this,
471 a release rate of cedrol which was much higher than from the
472 natural source was required. This might be because we used a
473 single compound rather than a blend of odorants which would
474 present the more natural situation for a mosquito. This has been
475 extensively shown for host-seeking mosquitoes, where blends are

477 required for efficient attraction (24-26). Our analysis of the GC-
478 MS data suggests another four putative semiochemicals, yet to be
479 identified, may play a role in the attractiveness of the six day old
480 soil infusion to gravid mosquitoes. Although in contrast to cedrol,
481 none of these could be detected in the samples from natural
482 oviposition sites in Kenya.

483 The ethanol-based cedrol formulation utilized here was re-
484 leased in consistent rates over the entire 12 hours trapping period
485 each night and does therefore not explain the nearly balanced re-
486 sponse of gravid females to the traps in the semi-field experiment
487 after 21.30 h. Less than one third of the collected mosquitoes were
488 trapped after 21.30 h and we speculate that these were not fully
489 gravid and therefore responded to high humidity.

490 Cedrol is best known for its presence in the essential oil
491 of conifers, especially in the genera *Cupressus* and *Juniperus*.
492 However, it has been found in a large variety of plants includ-
493 ing *Sorghum* (27), *Artemisia* (28), and swamp grasses of the
494 genus *Cyperus* (29), which are all common in the study area.
495 Sesquiterpenes are also known metabolites of fungi and to some
496 extent bacteria (30-32). We showed here that the amount of
497 cedrol released from a soil infusion was higher than from the
498 same infusion that had been autoclaved and previously that the
499 oviposition preference increased with increasing incubation time
500 of the infusion (15). This suggests that the release of cedrol is
501 associated with microbial activity possibly by metabolism of plant
502 products. Finding the source of cedrol might further elucidate
503 why *An. gambiae* and *An. arabiensis* prefer to lay eggs in habitats
504 containing it and might help predict habitat selection and guide
505 malaria vector control interventions.

506 In conclusion, our findings provide the first evidence that
507 gravid females of the *An. gambiae* complex use attractive chemical
508 cues when orienting towards a potential oviposition sites and
509 that these cues can be exploited for trapping female malaria
510 vectors. The discovery of an odor bait provides prospects for novel
511 ecological studies and is an important step towards developing an
512 'attract and kill' strategy for gravid malaria vectors. This could
513 provide a novel tool in targeting residual malaria transmission in
514 areas where current gold-standard indoor vector control interven-
515 tions are applied at full coverage but are not enough to eliminate
516 malaria (4, 6).

517 Methods

518 **Mosquito preparation.** Laboratory and semi-field experiments were carried
519 out with insectary-reared *An. gambiae* s.s. (Mbita strain) supplied by
520 the mosquito insectaries at the International Centre of Insect Physiology and
521 Ecology-Thomas Odhiambo Campus (Icipe-TOC), Mbita, western Kenya (0°
522 26' 06.19" South; 34° 12' 53.12" East; altitude 1149 m), and reared following
523 standard operating procedures. Gravid mosquitoes were prepared by select-
524 ing 300 female and 300 male mosquitoes, two to three days old, from their
525 rearing cages at 12.00 h and keeping them in 30x30x30 cm netting cages
526 at 25-28°C and 68-75% relative humidity. To avoid mosquito desiccation,
527 folded cotton towels, saturated with tap water were placed over the cages.
528 Mosquitoes were starved of sugar for seven hours before blood feeding and
529 allowed to feed on a human arm for 15 minutes at 19.00 h. Afterwards unfed
530 female mosquitoes were removed from the cages. Mosquitoes were then
531 provided with 6% glucose solution *ad libitum*. This procedure was repeated
532 the following day. Fed female mosquitoes were kept together with males
533 for two days after the second blood meal and used on the third day for
534 experiments (i.e. four to five days after first blood meal). At 16.30 h on the
535 day of an experiment visually presumed gravid females (enlarged, pale white
536 abdomen) were selected from the holding cage (15).

537 **Volatiles collections from soil infusions.** Volatiles released from lake wa-
538 ter, autoclaved and non-autoclaved six day old soil infusions were collected
539 in parallel with behavioral cage bioassays. All the non-autoclaved infusions
540 elicited higher oviposition responses compared to lake water (15). Infusions
541 were prepared by mixing 15 L of lake water with 2 kg of soil sourced from
542 a natural *Anopheles* breeding site, located within Icipe-TOC. The soil was
543 collected and sun-dried for one day prior to preparation of the infusion.
544 On the day of the experiment the infusions were sieved through clean
545 pieces of cotton cloth to remove large debris from the soil. One half of the
546 infusion was autoclaved at 120°C for 20 minutes and left to cool to ambient
547 temperatures. Volatiles were collected on Tenax traps by dynamic headspace
548 collections from 300 ml aliquots of the three sample types in 500 ml conical
549 borosilicate glass Erlenmeyer flasks with 24/29 sockets (Quickfit® glassware).
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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 (33, 34). E-flasks were fitted with gas wash bottle heads and charcoal-filtered air was pumped at 100 ml/min through the inlet and drawn out at the same speed through the Tenax trap over 20 h 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 empty bottles, lake water and duplicates of soil infusions (autoclaved and non-autoclaved). This was repeated over seven rounds.

Analysis of soil infusion volatiles. The Tenax traps were thermally desorbed. 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 an external standard (heptylacetate, 35 ng, SAFC, Sigma-Aldrich, Steinheim, Germany) injected with each sample and Kovats retention indices (RI) calculated. Peaks with similar RI and mass spectra were given the same compound identification number (ID). Mass spectral data was compared to a MS library (NIST 2008) for tentative identification. The identity of ID 276 was confirmed using an authentic standard. (+)-cedrol, 99.0% sum of enantiomers, GC, optical activity $[\alpha]_D^{20} +10.5 \pm 1^\circ$ (Sigma-Aldrich, Steinheim, Germany).

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 1377 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) fibers (65µm 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-fibers were analyzed immediately after volatile collection. The GC-MS files were screened for the main ions of the four compounds closely associated with the unmodified soil infusion samples in the 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 150) at the retention time that matched cedrol were scored as positive for the compound.

Dual-choice cage experiments to study substrate preferences. Experiments were done in previously described (15) make-shift sheds at Icipe-TOC (Fig. 2A). 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 had two glass cups (Pyrex®, 100 ml, 70 mm diameter) 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. A single gravid female was introduced per cage at 18:00 h. The next morning between 8:00 - 9:00 h 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.

Free-flying gravid mosquito attraction experiments to study odor discrimination. Free-flight 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 meters (35)) were done in a screened semi-field structure (10.8 m long x 6.7 m wide x 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 (Fig. 2B, (19)). 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. Approximately 200 gravid mosquitoes were released per round over 12 rounds. Mosquitoes were introduced at 18:00 h, about 5 minutes after the BG-Sentinel traps were

started. Gravid mosquitoes that oriented towards either trap were sucked into a catch bag in the trap. Catch bags were changed at 21:30 h and collected the next day between 8:00 - 9:00 h. 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). Field assessment of trapping efficiency of wild mosquitoes with odor-baited gravid traps. Field work was implemented during the end of the long rainy season in June 2014 approximately 14 km south of Icipe-TOC in Kaugage location. Collecting gravid malaria vectors has never been done routinely and gravid traps have only been developed recently (18, 19, 36). Therefore, we used three types of traps (Fig. 2B-C): E-nets, OviArt gravid traps and modified BG-Sentinel mosquito traps. The operating procedures for these devices have been published in detail elsewhere (17-19). Three study sites in close vicinity to residential houses and within 200 m from the lake shores were selected. The sites were separated by between 70 and 500 m. In each site four locations were chosen 10 m apart from each other and 5 m from the nearest house. One out of the three sites was randomly selected to receive two E-nets and two OviArt gravid traps whilst the remaining two sites received BG-Sentinel traps in all four 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 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 h. Mosquitoes were collected from the traps in the morning at 6:00 h. 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 were in each site once (three rounds of eight days). In parallel, host-seeking vectors were collected weekly 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 with CDC light traps (37) and outdoors with cattle baited traps (38). Mosquitoes were morphologically identified to genus level and *Anopheles* mosquitoes to species level (39, 40). Molecular tools were used to identify members of the sibling species of the *Anopheles gambiae* complex and the *Anopheles funestus* complex following published procedures (41, 42).

Statistical analyses. GC-MS data were explored using principal component analysis (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 centered and standardized by volatiles prior to analysis with Conoco 5 (43).

Dual choice cage bioassays and attraction experiments with free-flying mosquitoes in the semi-field were analyzed using generalized linear models with a quasibinomial distribution fitted to account for overdispersion in R statistical software version 2.13 (44). The proportion of responses (eggs laid by female) received by the test cups or test traps in the cages or semi-field systems, respectively, with equal treatments (lake water in both cups/traps) were compared with the proportion of responses received by the test cups/traps in cages/semi-field systems with two different treatments. 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. Therefore, the treatment (e.g. lake water only cages, cages with cedrol versus lake water) and the round of experiment were included as fixed factors to analyze 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 analyzed using generalized estimating equations. Prior to the final analysis we tested if there were significant between-group variations in trap location and study area. Only study area varied significantly and was included in the final analyses 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% CIs were estimated as the exponentials of the parameter estimates for models with no intercept included.

Supplemental information. Supplemental experimental procedures can be found with this article online.

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

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role in study design, data collection and interpretation, or the decision to submit the work for publication.

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