
Downloaded from: http://researchonline.lshtm.ac.uk/id/eprint/2222109/

DOI: https://doi.org/10.17037/PUBS.02222109

Usage Guidelines:

Please refer to usage guidelines at https://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by-nc-nd/2.5/
Larval habitat discrimination by the African malaria vector *Anopheles gambiae sensu lato*: Observations from standardized experiments and field studies

MANUELA HERRERA-VARELA

2015

Department of Disease Control

Faculty of Infectious Diseases

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

Funded by
National Institute of Health (NIH), USA
DECLARATION OF OWN WORK

All students are required to complete the following declaration when submitting their thesis. A shortened version of the School’s definition of Plagiarism and Cheating is as follows (the full definition is given in the Research Degrees Handbook):

“Plagiarism is the act of presenting the ideas or discoveries of another as one’s own. To copy sentences, phrases or even striking expressions without acknowledgement in a manner which may deceive the reader as to the source is plagiarism. Where such copying or close paraphrase has occurred the mere mention of the source in a biography will not be deemed sufficient acknowledgement; in each instance, it must be referred specifically to its source. Verbatim quotations must be directly acknowledged, either in inverted commas or by indenting” (University of Kent).

Plagiarism may include collusion with another student, or the unacknowledged use of a fellow student’s work with or without their knowledge and consent. Similarly, the direct copying by students of their own original writings qualifies as plagiarism if the fact that the work has been or is to be presented elsewhere is not clearly stated.

Cheating is similar to plagiarism, but more serious. Cheating means submitting another student’s work, knowledge or ideas, while pretending that they are your own, for formal assessment or evaluation.

Supervisors should be consulted if there are any doubts about what is permissible.

DECLARATION BY CANDIDATE

I have read and understood the School’s definition of plagiarism and cheating given in the Research Degrees Handbook. I declare that this thesis is my own work, and that I have acknowledged all results and quotations from the published or unpublished work of other people.

I have read and understood the School’s definition and policy on the use of third parties (either paid or unpaid) who have contributed to the preparation of this thesis by providing copy editing and, or, proof reading services. I declare that no changes to the intellectual content or substance of this thesis were made as a result of this advice, and, that I have fully acknowledged all such contributions.

I have exercised reasonable care to ensure that the work is original and does not to the best of my knowledge break any UK law or infringe any third party’s copyright or other intellectual property right.

To be completed by the candidate

NAME IN FULL (Block Capitals): MANUELA HERRERA VARELA

STUDENT ID NO: LSH 272702

SIGNED: .................................................. DATE: 5th January 2014

Registry
Last updated – 04/07/13

Improving health worldwide www.lshtm.ac.uk
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 lead 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.
Acknowledgements

Four years ago I started the most important journey of my life. I left my family, friends and natal country, Colombia with the idea of becoming a doctor in Philosophy, or as my friends used to call, a world-wide mosquito catcher. After crossing two oceans, I arrived in Mbita, Kenya. This place at the shores of Lake Victoria would be the landscape of intense academic learning. More importantly it would become a place where I would meet with wonderful people that made it possible for me to write these pages.

I am most grateful to my supervisor Dr. Ulrike Fillinger. You are one of the brightest person that I know and also one of the kindest. You shared your knowledge with me without reservation and with a lot patience understood my Colombian temperament. As a female scientist, you have shown me, the perfect example towards excellence both as a scientist and as a woman. I look forward to pursuing this path.

Professor Steve W. Lindsay, thank you sincerely for giving me the opportunity to be part of OviART and for all the confidence, encouragement and challenging discussions we had throughout these years. Thank you for that unforgettable football match that we spectated – I still have the ticket as a souvenir.

To Dr. Jenny Lindh, thanks for your supervision and support in the chemical analyses from Royal Institute of Technology, KTH, Sweden. I will always admire your insatiable curiosity and enthusiasm to look for the hidden answers.

To my friends and fellow PhD students Michael Okal, Sisay Dugassa, Oscar Mbare and Lynda Eneh; immense thanks for all the time and discussions that enriched my knowledge about mosquitoes and Africa and for strengthening my way of thinking as a researcher. Mike thanks for always being there as my editor and for sharing your family, culture and ideas. I will keep our conversations as one my biggest treasures.

Thanks to my field companion and friend Paul “Puma” Ouma for his enthusiasm and tireless work while sweeping larval habitats and learning about science and real world while walking in Rusinga Island, Lwanda Nyamasare and Mbita.

I am grateful to the members of the Rusinga malaria surveillance team for their constant support and helpful insights during the field work in the Island.

I am also very grateful with the members of the OviART project, Gregory Masinde, Elizabeth Masinde, Rose Ongole, Bernard Oyembe and Joel Odero for their technical assistance and friendship. I will never forget our constant dates with 600 hungry mosquito females.

Thank you David Alila and Elisha Obudho at the ICIPE insectary for your rare rearing skills.

Maria Ulbrich and Dr. Saskia Knillman from the Helmholtz Centre of Environmental Research, thank you. Your expertise and knowledge in fresh water ecology greatly enriched my studies.

Professor Baldwyin Torto and Xavier Cheseto from the Behaviour and Chemical Ecology Department in, ICIPE, Nairobi; thank you for your advice and unwavering support.

To my students, Benjamin Francis and Martijn Disco. Thank you for your zeal in all you did. Both of you are destined for great things and I wish you well.
Life in Mbita would not be so enjoyable without sharing hundreds of stunning sunsets and tuskers baridi with all the friends that I made along the way; my fellow mbitarians, Caroline Moseti, Margaret Njoroge, Evelyn Olanga, Daniel Achinko (My friend), Caroline Kungu, Roselyn Sumba, Pauline Awuori, Fred Orwa, Chris Momanyi, Patrick Sawa and Sabina Hamisi; my wazungu Sonia Deng Chan, Cristin Zeitz, Janis Thailayil, Tobias Homan, Alexandra Hiscox, Aurelio Di Pasquale, Joelle Rosser, Will Stone, Caitlin Moe, Wietske Bouwman, Ailie Robinson, Frank Loggen, Karlijn Wouters, Marie Hermy, Elke Van Veen and Tamzin Byrne. Thank you for being my family away from home.

I cannot forget Teresa Wasonga, my house manager, for your dedication in keeping my house neat and conducive for studying.

To the Colombian Department of Science, Technology and Innovation (COLCIENCIAS) for its financial support through the Scholarship programme “Francisco Jose de Caldas”.

To my Colombian friends and family that were always present in the distance especially during the difficult times; Libertad Ochoa, Patricia Fernandez, Mauricio Rodriguez, Fabián Perez, Karina Mondragon-Shem, Natali Ortiz, Alejandro Valencia, Adriana Velez, Sara Morales, Julian Thornton, Margarita Herrera, Elpi Herrera, Gustavo Herrera, Gustavo Varela, Carlos Varela y mi segunda madre, Josefina Montoya. ¡Gracias!

A mi maravillosa hermana Claudia Marcela Herrera y a mis inigualables padres Pablo y Aracelly. Gracias por ser mis alas y mis corrientes de aire y nunca dejarme caer. Esta tesis es la prueba de un sueño vivido con amor e intensidad y está dedicada especialmente a ustedes.

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

Abstract ........................................................................................................................................ 3
Acknowledgements .................................................................................................................. 4
Table of contents .................................................................................................................... 6
List of Tables ............................................................................................................................ 9
List of Figures ............................................................................................................................ 10
Chapter 1 Introduction .............................................................................................................. 13
  1.1 Malaria ............................................................................................................................... 13
  1.2 Vector control .................................................................................................................... 15
    1.2.1. The history of vector control ................................................................................... 15
    1.2.2. Current vector control in sub-Saharan Africa relies on strategies targeting Anopheles vectors indoors .......................................................... 16
    1.2.3 The importance of the development and use outdoor vector control strategies ...... 17
    1.2.4 Renewed interest in larval source management and selection of aquatic habitats by Anopheles gambiae s.l. ......................................................... 18
  1.3 Oviposition behaviour in mosquitoes .............................................................................. 20
    1.3.1 The potential role of semiochemicals in oviposition site selection ....................... 20
    1.3.2. Microbial organisms as a source of volatile chemicals released from aquatic habitats ............................................................... 27
  1.4 Rationale ............................................................................................................................ 28
  1.5 Overall aim and hypotheses ............................................................................................. 29
Chapter 2 Habitat discrimination by gravid Anopheles gambiae sensu lato – a push-pull system ......................................................................................................................... 31
  2.1 Abstract ............................................................................................................................. 33
  2.2 Background ....................................................................................................................... 34
  2.3. Methods .......................................................................................................................... 37
    2.3.2. Mosquitoes .............................................................................................................. 37
    2.3.4. Sample size considerations .................................................................................... 43
    2.3.5 Statistical analyses .................................................................................................... 44
  2.4 Results ............................................................................................................................... 45
    2.4.1. Gravid An. gambiae s.l. females discriminate between habitats when searching for an oviposition site ....................................................... 45
    2.4.2. The female’s choice of oviposition site benefits the survival of her offspring ....... 46
    2.4.3. Gravid female An. gambiae s.s. show avoidances and preferences when selecting an oviposition site ................................................................. 46
2.4.4. Chemical cues from the infusions are responsible for the oviposition choice in cage bioassays .............................................................. 49

2.4.4. The oviposition choice of the gravid female is not influenced by her olfactory memory of her larval habitat .............................................................. 51

2.5 Discussion .................................................................................. 51

2.6 Conclusion .................................................................................. 56

Chapter 3 Do volatile chemicals of microbial origin from natural Anopheles gambiae s.s. breeding site increase oviposition responses? .............................................................. 57

3.1 Abstract .................................................................................... 59

3.2 Background ................................................................................ 60

3.3 Materials and methods .............................................................. 62

3.3.1 Study site ............................................................................... 62

3.3.2. Preparation of soil samples .................................................... 62

3.3.3. Preparation of water samples ................................................ 64

3.3.4. Two choice egg-count bioassays .......................................... 65

3.3.5. Analysis of volatile organic chemicals released from oviposition substrates ...... 67

3.3.6. Data analysis ........................................................................ 69

3.4 Results ....................................................................................... 70

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 .................................................. 70

3.4.2. Anopheles gambiae s.s. females do not show a preference for larval habitat water containing microorganisms as compared with sterile water .............................................. 73

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 ........................................ 75

3.4.4. Headspace analysis of water substrates .................................... 77

3.5 Discussion ................................................................................... 80

3.6 Conclusion .................................................................................. 83

Chapter 4 Oviposition site selection of Anopheles gambiae sensu lato in Rusinga Island, Western Kenya: A case-control approach .............................................................. 84

4.1 Abstract ..................................................................................... 86

4.2 Background ................................................................................ 87

4.3 Material and Methods ............................................................... 90

4.3.1. Study area ............................................................................ 90

4.3.2. Larval habitat mapping ........................................................ 91

4.3.3. Sweep-net method for habitat sampling ............................... 91

4.3.4. Selection of cases and controls ............................................. 92
4.3.5. Characterization of cases and control habitats ......................................................... 93
4.3.6 Bacteria community analysis ......................................................................................... 95
4.3.7. Chemical headspace analysis ....................................................................................... 97
4.3.8 Data analyses ................................................................................................................ 98
4.4 Results ............................................................................................................................... 98
  4.4.1 Differences in environmental and biological factors between control and case habitats .......................................................................................................................... 99
  4.4.2 Differences between clear and turbid case habitats ....................................................... 102
  4.4.3 Differences between clear control and clear case habitats ........................................... 105
  4.4.4 Bacteria community analyses ....................................................................................... 105
  4.4.5 Chemical headspace analyses ...................................................................................... 106
4.5 Discussion ......................................................................................................................... 111
Chapter 5 Synthesis .............................................................................................................. 117
  5.1 Key findings ..................................................................................................................... 117
    5.1.1. Gravid An. gambiae s.l. females select sites to oviposit using volatile organic compounds and turbidity .............................................................................................. 117
    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 ............... 118
    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 ........................................ 119
  5.2 Limitation of the study ..................................................................................................... 120
  5.3. Future work .................................................................................................................... 122
  5.4 General conclusions ........................................................................................................ 124
6. References ......................................................................................................................... 126
7 Appendices .......................................................................................................................... 137
Appendix A ............................................................................................................................. 137
Appendix B ............................................................................................................................. 152
Appendix C ............................................................................................................................. 160
Appendix D ............................................................................................................................. 175
List of Tables

Table 1.1 Infusions, bacteria and semiochemicals that mediate oviposition response in mosquitoes ........................................................................................................................................... 24

Table 2.1. Summary details of dual choice egg-count bioassays to evaluate oviposition choices in An. gambiae s.s. ........................................................................................................................................... 39

Table 2.2. Physical and chemical properties of pellet and soil infusions ................................................. 50

Table 3.1. Experiments carried out with sterile and non-sterile substrates in two choice cage bioassays ........................................................................................................................................... 66

Table 4.1 Differences of biological and environmental factors between control and case habitats ........................................................................................................................................... 100

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...... 103

Table 4.3. Detailed analysis of banding patterns in 16S rDNA-DGGE profiles, Diversity and Evenness indexes ........................................................................................................................................... 108

Table 4.4 Tentative compound identifications based on NIST library hits ................................. 111
List of Figures

Figure 1.1. Life cycle of malaria parasite: .................................................................14

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...18

Figure 2.1. 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. ........................................................................................................41

Figure 2.2. Natural colonization of artificial habitats. Daily average of early instar larvae in (A) Pellet infusions (B) Soil infusions.................................................................45

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.................................46

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 47

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. Multiple comparison of treatments: Treatments denoted with the same letter are not significantly different................................................................................................................48

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...............................................................................51

Figure 3.1. Water and soil collection and preparation of two choice tests ...................63

Figure 3.2. Air-entrainment system used to collect volatile compounds from oviposition substrates.............................................................................................................68

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.................................................................71
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. .................................................................73

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. .................................................................74

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. ..........................................................................................75

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. ..............................................76

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. ..............................................78

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). ..........................................................................................79

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......................................................79

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). ..........................................................................................90
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.

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

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

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.

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.

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).

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.
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).

![Life Cycle of the Malaria Parasite](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 anthropophagic 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).

![Life cycle of malaria vector mosquitoes showing different behaviours that enable them to avoid conventional control strategies and targets for novel intervention strategies](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, Menuz & 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 (Isœ, 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 Demodium (Push), and with an attractive trap plant such as Nepier 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>
<thead>
<tr>
<th>Source</th>
<th>Bacteria identified</th>
<th>Chemicals identified</th>
<th>Mosquito species</th>
<th>Response</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manure infusion</td>
<td></td>
<td></td>
<td>Ae. aegypti var. queenslandis</td>
<td>Attraction</td>
<td>(O’Gower 1963)</td>
</tr>
<tr>
<td>Log pond infusion</td>
<td></td>
<td>0-cresol alpha-Ethyl-p-methoxybenzyl alcohol Ethyl methylcarbamate Phenethyl methylcarbamate alpha-conidendrol tetraacetate N-Ethyl-o-veratrylamine 2,6 Dimethoxyphenol-ethylene oxide</td>
<td>Cx. quinquefasciatus Cx. tarsalis</td>
<td>Attraction</td>
<td>(Gjullin, Johnson &amp; Plapp 1965)</td>
</tr>
<tr>
<td>Grass infusion</td>
<td></td>
<td></td>
<td></td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Hay infusion</td>
<td>Aerobacter aerogenes</td>
<td>No identified</td>
<td>Cx. pipiens quinquefasciatus</td>
<td>Attraction</td>
<td>(Hazard, Mayer &amp; Savage 1967)</td>
</tr>
<tr>
<td>Pseudomonaceae</td>
<td>Capric acid</td>
<td>Culex restuans Culex pipiens Culex tarsalis Aedes aegypti</td>
<td></td>
<td>Attraction</td>
<td>(Maw 1970)</td>
</tr>
<tr>
<td>Breeding site water</td>
<td>Pseudomononas aeruginosa</td>
<td>Intermediate metabolites of Capric and pelargonic acids Culex pipiens fatigans Culex molestus Aedes aegypti</td>
<td></td>
<td>Attraction</td>
<td>(Ikeshoji 1966) (Ikeshoji, Saito &amp; Yano 1975)</td>
</tr>
<tr>
<td>Betula papyrifera infusion</td>
<td>P-cresol</td>
<td>Aedes triseriatus</td>
<td></td>
<td>Attraction</td>
<td>(Bentley et al. 1979)</td>
</tr>
<tr>
<td>Purina® Laboratory chow infusion</td>
<td></td>
<td>Acetic acid Propionic acid Isobutyric acid Butyric acid Isovaleric acid Caproic acid</td>
<td>Cx. quinquefasciatus Cx. tarsalis</td>
<td>Repellence</td>
<td>(Hwang, Kramer &amp; Mulla 1980)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fatty acids from C₅ to C₁₃ Nonanoic acid*</td>
<td>Cx. quinquefasciatus Cx. tarsalis Ae. Aegypti</td>
<td>Repellence</td>
<td>(Hwang et al. 1982)</td>
</tr>
<tr>
<td>Egg rafts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td></td>
<td>Erythro-6-acetoxy-5-hexadecanolide (AHD)</td>
<td>Cx. quinquefasciatus</td>
<td>Oviposition pheromone</td>
<td>(Laurence &amp; Pickett 1982)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bermuda grass infusion</td>
<td></td>
<td>phenol 4 Methylphenol 4 Ethylphenol</td>
<td>Cx. quinquefasciatus</td>
<td>Stimulation/Attraction</td>
<td>(Millar, Chaney &amp; Mulla 1992)</td>
</tr>
<tr>
<td>Source of Larval Water</td>
<td>Attraction/Repellence</td>
<td>Species Attracted</td>
<td>Chemicals Present</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------------</td>
<td>-------------------</td>
<td>-------------------</td>
<td>-----------</td>
<td></td>
</tr>
<tr>
<td>Hay infusion</td>
<td></td>
<td></td>
<td>3 Methyl indole</td>
<td>(Allan &amp; Kline 1995)</td>
<td></td>
</tr>
<tr>
<td>Field collected larval water</td>
<td></td>
<td></td>
<td>4 Methylphenol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field collected larval water</td>
<td></td>
<td></td>
<td>Indole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bermuda grass infusion</td>
<td>Attraction</td>
<td>Cx. quinquefasciatus</td>
<td>4 Methylphenol</td>
<td>(Isoe et al. 1995; Isoe &amp; Millar 1995)</td>
<td></td>
</tr>
<tr>
<td>Oak leafs infusion</td>
<td>Stimulation/Attraction</td>
<td>Ae. albopictus</td>
<td>3 Methyl indole</td>
<td>(Trexler, Apperson &amp; Schal 1998)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ae. triseriatus</td>
<td>4 Ethylphenol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ae. triseriatus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteria filtrates</td>
<td>Attraction/No effect</td>
<td>Cx. quinquefasciatus</td>
<td>Bacillus cereus</td>
<td>(Poonam, Paily &amp; Balaraman 2002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larval conditioned water</td>
<td></td>
<td>Heneicosaene</td>
<td></td>
<td>(Mendki et al. 2000)</td>
<td></td>
</tr>
<tr>
<td>Grass (Panicum maximum) infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oak leaf infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil contaminated cotton towels</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breeding site</td>
<td>Repellence</td>
<td>An. gambiae s.s.</td>
<td>4 Methylphenol</td>
<td>(Huang et al. 2006a)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 Methyl indole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grass (Panicum maximum) infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breeding site</td>
<td>Repellence</td>
<td>An. gambiae s.s.</td>
<td></td>
<td>(Huang et al. 2006a)</td>
<td></td>
</tr>
<tr>
<td>Long chain fatty acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bamboo leaf infusion</td>
<td>Attraction</td>
<td>Ae. aegypti</td>
<td></td>
<td>(Ponnusamy et al. 2008a)</td>
<td></td>
</tr>
<tr>
<td>White-oak leaf infusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 spp most of them</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 spp most of them</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Category</td>
<td>Compounds and Organisms</td>
<td>Insect Species</td>
<td>Response</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>----------------------------------------------------------------------------------------</td>
<td>-----------------</td>
<td>----------</td>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td>Midguts and breeding sites</td>
<td><em>Vibrio metschnikovii</em>, <em>Proteus</em> sp., <em>Micrococcus</em> sp., <em>Bacillus</em> sp., <em>Exiguobacterium</em> sp., <em>Comamonas</em> sp.</td>
<td><em>An. gambiae s.s.</em></td>
<td>Attraction</td>
<td>(Lindh <em>et al.</em> 2008)</td>
<td></td>
</tr>
<tr>
<td>Long chain fatty acids</td>
<td>Octadecyl propanoate, Heptadecyl butanoate, Hexadecyl pentanoate, Tetradecyl heptanoate</td>
<td><em>Anopheles stephensi</em></td>
<td>Repellence</td>
<td>(Sharma <em>et al.</em> 2009)</td>
<td></td>
</tr>
<tr>
<td>Conditioned water with <em>Notonecta maculate</em></td>
<td>n-heneicosane, n-tricosane</td>
<td><em>Culiseta longiareolata</em></td>
<td>Repellence</td>
<td>(Silberbush <em>et al.</em> 2010)</td>
<td></td>
</tr>
<tr>
<td>Synthetic analyses - Antennal transcriptome profiles</td>
<td><strong>2-propyl phenol</strong>, 4-methylcyclohexanol</td>
<td><em>An. gambiae s.s</em></td>
<td>Attraction</td>
<td>Repellence</td>
<td>(Rinker <em>et al.</em> 2013)</td>
</tr>
</tbody>
</table>

*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

This paper has been published in Malaria Journal 2014, 13:133

Manuela Herrera-Varela, Jenny Lindh, Steve W. Lindsay, Ulrike Fillinger
COVER SHEET FOR EACH 'RESEARCH PAPER' INCLUDED IN A RESEARCH THESIS

Please be aware that one cover sheet must be completed for each 'Research Paper' included in a thesis.

1. For a 'research paper' already published

   1.1. Where was the work published? Malaria Journal

   1.2. When was the work published? April 2014

     1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

     ..............................................................................................................................

   1.3. Was the work subject to academic peer review? Yes

   1.4. Have you retained the copyright for the work? Yes / No

       If yes, please attach evidence of retention.

       If no, or if the work is being included in its published format, please attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a 'research paper' prepared for publication but not yet published

   2.1. Where is the work intended to be published? .................................................................

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

   ..............................................................................................................................

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

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

   I contributed significantly 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.

NAME IN FULL (Block Capitals) MANUELA HERRERA VARELA

STUDENT ID NO: 272702

CANDIDATE’S SIGNATURE ............................................. Date 05/01/2015

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

Improving health worldwide www.lshtm.ac.uk
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; Ise et al. 1995; Ise & 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 (Gimnig et al. 2001; Fillinger et al. 2009b; Gouagna et al. 2012; Kwaka et al. 2012; Munga, Vulule & Kwaka 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; Kwaka 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; Sumba et al. 2004; Huang et al. 2005; Huang et al. 2006b; Huang et al. 2007; Lindh et al. 2008; Sumba et al. 2008; Kwaka et al. 2011). 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; Paaijman 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.***

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


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 × 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.

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), x10$^{-1}$ and x10$^{-2}$ 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.](image-url)
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.](image)

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.](image)

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%).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Lake water (%)</th>
<th>Infusion (%)</th>
<th>Odds ratio (95% CI)</th>
<th>p-value</th>
<th>Eggs per female (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake water</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>67 (61-73)</td>
</tr>
<tr>
<td>2 day pellet infusion</td>
<td></td>
<td></td>
<td>0.97 (0.50-1.90)</td>
<td>0.934</td>
<td>66 (60-72)</td>
</tr>
<tr>
<td>4 day pellet infusion</td>
<td></td>
<td></td>
<td>0.42 (0.21-0.82)</td>
<td>0.012</td>
<td>67 (62-74)</td>
</tr>
<tr>
<td>6 day pellet infusion</td>
<td></td>
<td></td>
<td>0.15 (0.06-0.32)</td>
<td>&lt;0.001</td>
<td>67 (61-73)</td>
</tr>
<tr>
<td>Lake water</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>59 (54-65)</td>
</tr>
<tr>
<td>2 day soil infusion</td>
<td></td>
<td></td>
<td>1.31 (0.86-2.01)</td>
<td>0.208</td>
<td>61 (57-67)</td>
</tr>
<tr>
<td>4 day soil infusion</td>
<td></td>
<td></td>
<td>1.55 (1.01-2.37)</td>
<td>0.046</td>
<td>63 (58-69)</td>
</tr>
<tr>
<td>6 day soil infusion</td>
<td></td>
<td></td>
<td>2.21 (1.43-3.43)</td>
<td>&lt;0.001</td>
<td>61 (56-66)</td>
</tr>
<tr>
<td>Lake water</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
<td>60 (57-63)</td>
</tr>
<tr>
<td>Autoclaved 6 day soil infusion</td>
<td></td>
<td></td>
<td>0.68 (0.47-0.98)</td>
<td>0.037</td>
<td>65 (59-70)</td>
</tr>
<tr>
<td>6 day soil infusion</td>
<td></td>
<td></td>
<td>2.24 (1.53-3.28)</td>
<td>&lt;0.001</td>
<td>66 (61-72)</td>
</tr>
<tr>
<td>Autoclaved 6 day soil infusion</td>
<td></td>
<td></td>
<td>2.60 (1.75-3.86)</td>
<td>&lt;0.001</td>
<td>66 (61-72)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tap water</th>
<th>Pellet infusions</th>
<th>Soil infusions</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2 days</td>
<td>4 days</td>
<td>6 days</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>1 (0.6-1.4)</td>
<td>22</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>Conductivity (uS/cm)</td>
<td>107 (105-110)</td>
<td>477</td>
<td>553</td>
<td>543</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/l)</td>
<td>4 (2.7-5.3)</td>
<td>0.3</td>
<td>0.7</td>
<td>0.3</td>
</tr>
<tr>
<td>pH</td>
<td>8.1 (7.9-8.1)</td>
<td>6.3</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Ammonium (mg/l)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate (mg/l)</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite (mg/l)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate (mg/l)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonate hardness (mmol/l)</td>
<td>0.1</td>
<td>3.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total hardness (mmol/l)</td>
<td>0.1</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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 repellence 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.

![Graph showing egg laying responses of An. gambiae s.s. reared in tap water or in pellet infusion to tap water and pellet infusion.]

**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
elicted. 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; Sigee 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 (Sigee 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; Sigee 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?

Manuela Herrera-Varela, Jenny Lindh, Steve W. Lindsay, Ulrike Fillinger
COVER SHEET FOR EACH ‘RESEARCH PAPER’ INCLUDED IN A RESEARCH THESIS

Please be aware that one cover sheet must be completed for each ‘Research Paper’ included in a thesis.

1. For a ‘research paper’ already published
   1.1. Where was the work published? ......................................................................................................................
   1.2. When was the work published? ...........................................................................................................................
   1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion
           ..............................................................................................................................................................................
   ..................................................................................................................................................................................
   1.3. Was the work subject to academic peer review? .................................................................................................

1.4. Have you retained the copyright for the work? Yes / No
   If yes, please attach evidence of retention.
   If no, or if the work is being included in its published format, please attach evidence of permission
   from copyright holder (publisher or other author) to include work

2. For a ‘research paper’ prepared for publication but not yet published
   2.1. Where is the work intended to be published? Parasites & Vectors .............................................................
   2.2. Please list the paper’s authors in the intended authorship order
       Manuela Herrera-Varela, Jenny Lindh, Steven Lindsay, Ulrike Fillinger
   2.3. Stage of publication – Not yet submitted / Submitted / Undergoing revision from peer reviewers’
       comments / In press

3. For multi-authored work, give full details of your role in the research included in the paper and in
   the preparation of the paper. (Attach a further sheet if necessary)
   I contributed significantly in the conception and experimental design.
   I developed all protocols and implemented the experiments, analysed the data and wrote first draft of the manuscript.

NAME IN FULL (Block Capitals) MANUELA HERRERA VARELA

STUDENT ID NO: 272702

CANDIDATE’S SIGNATURE ................................................................. Date 05/01/2015

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

Improving health worldwide www.lshtm.ac.uk
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

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Control cup</th>
<th>Test cup</th>
<th>Rounds (Replicates)</th>
<th>Number of females that laid eggs (Total of mosquitoes exposed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline/Reference</td>
<td>tap water</td>
<td>tap water</td>
<td>4</td>
<td>153 (225)</td>
</tr>
<tr>
<td><strong>Oviposition bioassays with soil from breeding site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil sterilization by autoclaving in PE bags</td>
<td>sterile soil</td>
<td>fresh soil</td>
<td>5</td>
<td>102(150)</td>
</tr>
<tr>
<td></td>
<td>sterile soil</td>
<td>distilled water</td>
<td>5</td>
<td>101(150)</td>
</tr>
<tr>
<td>Soil sterilization by autoclaving in glass</td>
<td>sterile soil</td>
<td>fresh soil</td>
<td>4</td>
<td>61(120)</td>
</tr>
<tr>
<td></td>
<td>sterile soil</td>
<td>distilled water</td>
<td>4</td>
<td>88(120)</td>
</tr>
<tr>
<td>Habitat soil vs. distilled water</td>
<td>distilled water</td>
<td>fresh soil</td>
<td>6</td>
<td>136(180)</td>
</tr>
<tr>
<td><strong>Oviposition assays with water from breeding site</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habitat water vs. micro-filtered habitat water and distilled water</td>
<td>sterile water</td>
<td>Fresh water</td>
<td>4</td>
<td>69(100)</td>
</tr>
<tr>
<td></td>
<td>distilled water</td>
<td>Fresh water</td>
<td>4</td>
<td>75(100)</td>
</tr>
<tr>
<td></td>
<td>distilled water</td>
<td>sterile water</td>
<td>4</td>
<td>70(100)</td>
</tr>
</tbody>
</table>
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 volatiles (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 65μm 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 μm 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 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).
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 (Sigee 2005). Therefore it is not possibly 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

Manuela Herrera-Varela, Maria Ulbrich, Saskia Knillmann, Steve W. Lindsay, Jenny Lindh, Ulrike Fillinger
COVERAGE SHEET FOR EACH ‘RESEARCH PAPER’ INCLUDED IN A RESEARCH THESIS

Please be aware that one cover sheet must be completed for each Research Paper included in a thesis.

1. For a ‘research paper’ already published

1.1. Where was the work published?

1.2. When was the work published?

1.2.1. If the work was published prior to registration for your research degree, give a brief rationale for its inclusion

1.3. Was the work subject to academic peer review?

1.4. Have you retained the copyright for the work? Yes / No
   If yes, please attach evidence of retention.
   If no, or if the work is being included in its published format, please attach evidence of permission from copyright holder (publisher or other author) to include work

2. For a ‘research paper’ prepared for publication but not yet published

2.1. Where is the work intended to be published?

2.2. Please list the paper’s authors in the intended authorship order
   Manuela Herrera-Varela, Maria Ulbrich, Saskia Knillmann, Steve W. Lindsay, Jenny Lindh, Ulrike Fillinger

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

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

I contributed significantly 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.

NAME IN FULL (Block Capitals) MANUELA. HERRERA-VARELA

STUDENT ID NO. 272702

CANDIDATE’S SIGNATURE

Date 05/01/2015

SUPERVISOR/SENIOR AUTHOR’S SIGNATURE (3 above)

Improving health worldwide

www.lshtm.ac.uk
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 sub-zones. 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 ≤1m² were swept three times. Two sweeps per square meter were done in habitats with an area between 1 and 20 m². When a habitat was bigger, a 20m² 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 where 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, burrow 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., Lemma 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 (PO4 3–) 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 collect 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 (PTE) 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 ($H'$):

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

$ni$ = OTU intensity (relative quantity) for ith band, $N$ = Summed intensities of all OTUs in a lane, $Pi$ = Propotion of total diversity represented by the ith species (OTUs) $Pi = \frac{ni}{N}$

Evenness index (E) was estimated for the number of bacterial species in each lane as follow
\[ E = \frac{H'}{\log S} \]

\( S = \text{Number of DGGE OTUs in each lane.} \)

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\( \mu \)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 \( \mu \)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 m²) 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).

![Graph showing frequency of occurrence of controls and cases during sampling dates and their relation with rainfall.](image)

**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>
<thead>
<tr>
<th>Factor</th>
<th>Means (95% CI)</th>
<th>Odds ratio (95% CI)</th>
<th>GLM P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Cases</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anopheles</em> late/m²</td>
<td>0.24 (0.18-0.32)</td>
<td>7.17 (5.69-9.04)</td>
<td>4.078 (1.683-9.880)</td>
</tr>
<tr>
<td>Naucoridae/m²</td>
<td>1.50 (1.12-2.02)</td>
<td>0.31 (0.25-0.39)</td>
<td>0.760 (0.300-1.926)</td>
</tr>
<tr>
<td>Coleoptera adults/m²</td>
<td>1.14 (0.85-1.53)</td>
<td>2.32 (1.84-2.92)</td>
<td>1.227 (0.951-1.584)</td>
</tr>
<tr>
<td>Fish/m²</td>
<td>1.19 (0.89-1.60)</td>
<td>0.31 (0.24-0.38)</td>
<td>0.882 (0.607-1.281)</td>
</tr>
<tr>
<td>Cyclopoida/L</td>
<td>74.8 (55.7-100)</td>
<td>189 (150-238)</td>
<td>0.998 (0.994-1.002)</td>
</tr>
<tr>
<td>Nauplius/L</td>
<td>280 (208-376)</td>
<td>620 (492-781)</td>
<td>1.000 (0.999-1001)</td>
</tr>
<tr>
<td>Moinidae/L</td>
<td>2.48 (1.85-3.34)</td>
<td>149 (118-187)</td>
<td>1.011 (0.981-1.042)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>56.2 (41.8-75.6)</td>
<td>205 (163-259)</td>
<td>1.005 (1.001-1.010)</td>
</tr>
<tr>
<td>Ammonium (mg/L)</td>
<td>0.20 (0.15-0.27)</td>
<td>0.06 (0.05-0.08)</td>
<td>0.312 (0.029-3.399)</td>
</tr>
<tr>
<td>Nitrite (mg/L)ᵇ</td>
<td>0.01 (0.01-0.02)</td>
<td>0.04 (0.03-0.04)</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵇoutput 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*.

![Box-and-Whisker plots showing the median turbidity and interquartile range for control and case habitats. Red line at 200 NTU.](image)

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, Chyoboridae 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.

<table>
<thead>
<tr>
<th>Aquatic organisms*</th>
<th>Mean (95% CI)</th>
<th>RR (95% CI), p (in reference to control &lt;200NTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anopheles early instars</td>
<td>N=40</td>
<td>N=50</td>
</tr>
<tr>
<td>Anopheles late instars</td>
<td>20.0 (13.1-30.2), p&lt;0.001</td>
<td>46.3 (27.5-78.8), p&lt;0.001</td>
</tr>
<tr>
<td>Caseinogen</td>
<td>70.70 (51.74-96.60)</td>
<td>268 (195-340)</td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>1.06 (0.70-1.60), p=0.396</td>
<td>1.06 (0.70-1.60), p=0.396</td>
</tr>
<tr>
<td>Naucoridae</td>
<td>1.27 (0.84-1.93), p=0.054</td>
<td>1.52 (0.90-2.55), p=0.117</td>
</tr>
<tr>
<td>Ostracoda</td>
<td>7.62 (5.70-10.23)</td>
<td>6.77 (4.03-11.37), p&lt;0.001</td>
</tr>
<tr>
<td>Daphniidae</td>
<td>6.15 (4.40-8.59)</td>
<td>0.13 (0.03-0.57)</td>
</tr>
<tr>
<td>Chydoridae</td>
<td>6.96 (5.17-9.36)</td>
<td></td>
</tr>
<tr>
<td>Bosminidae</td>
<td>0.08 (0.03-0.19)</td>
<td></td>
</tr>
<tr>
<td>Sibididae</td>
<td>9.60 (6.90-13.30)</td>
<td></td>
</tr>
<tr>
<td>Macrothricida</td>
<td>12.50 (9.06-17.25)</td>
<td></td>
</tr>
<tr>
<td>Haparcticoida</td>
<td>0.65 (0.40-1.07)</td>
<td></td>
</tr>
<tr>
<td>Molidae</td>
<td>0.24 (1.68-3.50)</td>
<td></td>
</tr>
<tr>
<td>Ostracoda</td>
<td>59.97 (43.70-82.30)</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin</td>
<td>70.70 (51.74-96.60)</td>
<td></td>
</tr>
<tr>
<td>Nauplius</td>
<td>243 (178-332)</td>
<td></td>
</tr>
<tr>
<td>Rotifer</td>
<td>3217 (1626-3025)</td>
<td></td>
</tr>
<tr>
<td>Fishes</td>
<td>37.66 (28.44-49.87)</td>
<td></td>
</tr>
<tr>
<td>WATER CHEMISTRY &amp; ENVIRONMENTAL FACTORS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>103.5 (23-44)</td>
<td></td>
</tr>
<tr>
<td>Phosphates (mg/L)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Conductivity (µS/sec)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Nitrates mg/L</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Dissolved oxygen (ppm)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Total hardness (mmol/L)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Carbonate hardness (mmol/L)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Habitat size (m²)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Habitat shaded (%)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Edge grass cover (%)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Surface grass cover (%)</td>
<td>554.1 (351-901)</td>
<td></td>
</tr>
<tr>
<td>Distance to next house (m)</td>
<td>25.3 (16.0-33.0)</td>
<td></td>
</tr>
<tr>
<td>Distance to next house (km)</td>
<td>148 (112-195)</td>
<td></td>
</tr>
<tr>
<td>Water depth (cm)</td>
<td>25.3 (16.0-33.0)</td>
<td></td>
</tr>
</tbody>
</table>

* 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 Macrathricidae (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

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

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

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

### 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 located 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 reaches 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 attract 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 specie Illycoris 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; Kena, 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 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 developed 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.

6. References


131


7 Appendices

Appendix A.

Herrera-Varela et al. 2014
Determining larval distribution [8-11]. Although different species are found in the same type of habitat, oviposition site selection is considered species specific [12]. Immature stages of *Anopheles gambiae* across kato (s.l.), the major malaria vector in sub-Saharan Africa, are typically described as inhabiting very small, temporary water 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 life cycle of this species [17-19]. A fact recognized over half a century ago by Holstén who reviewed the extraordinary diversity of the breeding places of *An. gambiae* s.l. [9]. Numerous studies have described how the presence of *An. gambiae* s.l. larvae [17-20] and the capacity of individual habitats for generating adult mosquitoes [6,17-24,25] 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 adult's choice.

Surprisingly, fully gravid malariavectors looking for suitable larval habitats have been greatly understudied [26]. Compared to the reach of knowledge of the physical and chemical factors used by gravid calicines for selecting an oviposition site [27-40] those potentially used by the world's most deadly malaria vector remain al- tered. For example, we have recently recognized that the distribution of larvae between seemingly suitable aquatic habitats is probably due to the choice of the gravid female [6,24,41-43] and that this choice probably im- pacts the survival of larvae in the aquatic habitat [9] in the absence of larval growth in this species [24,41-43]. This study suggests that some of these factors may be used to predict the presence of adult mosquitoes in a particular site.

**Laboratory studies** demonstrated that physical conditions of the aquatic habitat 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 ovipos- ition 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 [27]. It has been shown that gravid *An. gambiae* s.l. are sensitive to bacteria-derived odours [47,53,56] which have been associated with increased [47,55] and reduced [38] egg numbers compared to sterile media in cage bioassays. While over 20 putative oviposition semiochemicals have been suggested in the literature, based on the analyses of bacteria or habitat-derived volatile chemicals and other autocorrelation studies [53-59], there is only one report [60] of two chemicals inducing a behavioural re- sponse in gravid females (one increasing and one decreasing the oviposition response).

From 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 habitats: one made with soil from an area where natural habitats occur fre- quently, 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 pel- lets have shown to be attractive to a range of mosquito species and have been used in gravid traps [63-66]. This was aimed to explore whether *Anopheles gambiae* s.l. might also be drawn to these infusions.

Natural colonizations and larval survival was evaluated in artificial ponds filled randomly with either infusions. As a consequence of the field observations, two-choice, egg-count bioassays were used to explore patterns of oviposi- tion 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 co-ordinates 07°26'06"S, 34°12'53.3"E; altitude 1,137 m above sea level). Mbita has a typical climatic character; temperatures oscillate between 18-28°C and there is a annual rainfall of 1,106 mm (based on data from the meteorological station for 2010–2012). Two rainy sea- sons 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* [65].

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 *Ae. gambiae* s.s. (Mbita strain) supplied by Kipe'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 x 30 x 30 cm netted cages at 25-28°C and 60-75% relative humidity. To avoid mosquito desiccation, cotton towels (folded to 25-32 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 used 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 to five days after first blood meal). In the afternoon (16:30) of the day of an experiment 40–100 (depending on experiment and availability) visually presented 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 [66,69]. Whilst two meals were provided it cannot be guaranteed that two meals were taken by all females. This may 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 analysis.

Experimental procedure

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-drained field during the long dry 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 mident food, Naimbo) containing hay and grains from maize, wheat, barley, cotton, sunflower, soybean 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 kipe. Soil texture was characterized as a sandy clay loam according to the US Department of Agriculture (USDA) texture triangle [71] using the de-tangent 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 infestation. To study the oviposition response of wild mosquitoes the tubs were monitored daily between 08:00 and 16:00 by dipping five times per tub with a standard dipper (250 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 context 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 *Ae. 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 *Ae. 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 preference or avoidance? Based on the analysis of the field data a series of two-choice, egg-count bioassay were designed to investigate if the response of wild gravid females observed in the field was based on avoidance or preference of an infestation 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 aluminum 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 cleansed 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 1 l of lake water were either incubated with 10 g of pellets to prepare a pellet infusions 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 1730. The next morning between 0800 and 0900 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 3558R, WTW Germany) and a
Table 1 Summary details of dual-choice, egg-count bioassays to evaluate oviposition choices in *Anopheles gambi\ier* anna strain

<table>
<thead>
<tr>
<th>Dual-choice egg count bioassays</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Set 1: Pellet Infusions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake water</td>
<td>Lake water</td>
<td>66 (73)</td>
</tr>
<tr>
<td>Lake water</td>
<td>2-day old pellet infusion</td>
<td>64 (73)</td>
</tr>
<tr>
<td>Lake water</td>
<td>4-day old pellet infusion</td>
<td>67 (73)</td>
</tr>
<tr>
<td>Lake water</td>
<td>6-day old pellet infusion</td>
<td>68 (72)</td>
</tr>
<tr>
<td><strong>Set 2: Soil Infusions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake water</td>
<td>Lake water</td>
<td>132 (223)</td>
</tr>
<tr>
<td>Lake water</td>
<td>2-day old soil infusion</td>
<td>158 (223)</td>
</tr>
<tr>
<td>Lake water</td>
<td>4-day old soil infusion</td>
<td>159 (223)</td>
</tr>
<tr>
<td>Lake water</td>
<td>6-day old soil infusion</td>
<td>171 (223)</td>
</tr>
<tr>
<td><strong>Set 3: Water versus infusions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake water</td>
<td>Lake water</td>
<td>186 (230)</td>
</tr>
<tr>
<td>Lake water</td>
<td>4-day old soil infusion</td>
<td>156 (230)</td>
</tr>
<tr>
<td>Lake water</td>
<td>Autoclaved 6-day old soil infusion</td>
<td>121 (230)</td>
</tr>
<tr>
<td>Lake water</td>
<td>Autoclaved 6-day old soil infusion</td>
<td>101 (230)</td>
</tr>
<tr>
<td><strong>Set 4: Olfactory memory - pellet infusions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake water reared <em>A. gambi\ier</em> females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lake water</td>
<td>6-day old pellet infusion</td>
<td>31 (49)</td>
</tr>
<tr>
<td>Lake water</td>
<td>6-day old pellet infusion</td>
<td>37 (49)</td>
</tr>
</tbody>
</table>

multimeter (Multi 3401, WTW, Germany). In addition one batch of pellet, soil infusions and lake water was tested for ammonium (NH₄⁺), carbonate hardness, total hardness, nitrite (NO₂⁻), nitrate (NO₃⁻) and phosphate (PO₄³⁻) content using Aquameter*®* test kits from the compact laboratory for water testing (Aquameter*®* 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 infusions [47,78]. The experiment followed the same experimental procedures as described above. After filtering the infusion through a duktur on the day of experiment, the infusion was split in two equal volumes and half autoclaved at 121°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 cage.

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 x1 (undiluted), x10⁻¹ and x10⁻² dilutions was spread separately onto the surface of duplicate Luria broth (LB) agar plates (LB Lemo-smi-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) were 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 calico species [4,79].
To test this, approximately 2,000 An. gambiae s.s. eggs were disposed 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 Tetramin fish food twice daily following routine insectary procedures. Pupae were collected in a cup with 100 ml of rearing water and placed in 30 x 30 x 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 a single mosquito was offered a choice between six-day old pelleted 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 respondents 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 pellets infusion observed on oviposition response was much stronger than 23% in the soil infestation 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 behavioral 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 11 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 doubling 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 analyzed in R statistical software version 2.13.1 [8]. 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 infestation and lake water. Pupation time of larvae exposed to different treatments was calculated using the following formula: \((A \times 1) + (B \times 2) + (C \times 3) + (D \times 0)\). 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 model (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 analyze their impact on the outcome (proportion of eggs laid in test cups). 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 lake water and in the lake water 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 s.s. 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 34-48 hours after eggs are laid. Ponds with pelleted 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 infestation 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/liturp in natural habitats [17]. In the present study on average of two (95% CI 5–19) early instar larvae/liturp was recorded, indicating that the soil infusion ponds were a highly favourable habitat.

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

The female’s oviposition choice benefits the survival of her offspring. Anopheles gambiæ 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 age of the infusion and was on average 7.5 days (95% CI 6.6–8.3).

Gravid female Anopheles gambiæ sensu stricto show aversions 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 0.65 (95% CI 0.39-0.56) versus lake water test cup 0.55 (95% CI 0.41-0.66) 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 at 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 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 lake water than it was when both cups contained lake water (Figure 5, Set 2).

![Image of Figure 4: Proportion of gravid *Anopheles gambiae* sensu stricto laying eggs in infusions of different ages compared with control water. (A) Pellet infusion experiment (B) Soil infusion experiment.]

![Image of Figure 5: Oviposition response of caged *Anopheles gambiae* sensu stricto to pellet (Set 1) and soil (Set 2) infusions of different ages. In each panel, the number of eggs laid in each treatment is compared with the number of eggs laid in the control treatment. Multiple comparison of treatments; treatments denoted with the same letter are not significantly different.]

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Tap water</th>
<th>Infusion</th>
<th>Odds Ratio (95% CI)</th>
<th>p-value</th>
<th>Eggs per female (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake water</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>47 (61-73)</td>
</tr>
<tr>
<td>2 day pellet infusion</td>
<td>1</td>
<td></td>
<td>0.91 (0.90-0.99)</td>
<td>0.015</td>
<td>66 (66-77)</td>
</tr>
<tr>
<td>4 day pellet infusion</td>
<td>1</td>
<td></td>
<td>0.91 (0.90-0.99)</td>
<td>0.015</td>
<td>66 (66-77)</td>
</tr>
<tr>
<td>6 day pellet infusion</td>
<td>1</td>
<td></td>
<td>0.91 (0.90-0.99)</td>
<td>0.015</td>
<td>66 (66-77)</td>
</tr>
<tr>
<td>Lake water</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>59 (54.65)</td>
</tr>
<tr>
<td>2 days soil infusion</td>
<td>1</td>
<td></td>
<td>1.31 (1.06-1.61)</td>
<td>0.039</td>
<td>65 (57.47)</td>
</tr>
<tr>
<td>4 days soil infusion</td>
<td>1</td>
<td></td>
<td>1.53 (1.02-2.31)</td>
<td>0.046</td>
<td>44 (54.49)</td>
</tr>
<tr>
<td>6 days soil infusion</td>
<td>1</td>
<td></td>
<td>0.31 (0.15-0.65)</td>
<td>&lt;0.001</td>
<td>51 (51.66)</td>
</tr>
<tr>
<td>Lake water</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>58 (51.43)</td>
</tr>
<tr>
<td>Autoclaved 2 day soil inf/treatment</td>
<td>1</td>
<td></td>
<td>1.62 (1.05-2.51)</td>
<td>0.057</td>
<td>44 (36-58)</td>
</tr>
<tr>
<td>6 days soil infusion</td>
<td>1</td>
<td></td>
<td>2.34 (1.15-3.26)</td>
<td>&lt;0.001</td>
<td>44 (42-57)</td>
</tr>
<tr>
<td>Autoclaved 6 days soil infusion</td>
<td>1</td>
<td></td>
<td>1.42 (1.16-1.80)</td>
<td>&lt;0.001</td>
<td>44 (41-72)</td>
</tr>
</tbody>
</table>
On average individual females laid 61 eggs (95% CI 60–66) (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 [8] but rarely reported for *Ae. 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 *Ae. gambiae* s.s. females skip-oviposited in the four- and six-day-old pellet infusion treatments (6%, 99% 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 characterised 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 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 ammonia 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 infusions, the lake water was preferred. The oviposition preference for six-day old soil infusions 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>
<thead>
<tr>
<th>Parameter</th>
<th>Lake water</th>
<th>Pellet infusion</th>
<th>Soil infusion</th>
<th>Autoclaved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 days</td>
<td>4 days</td>
<td>6 days</td>
<td>2 days</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>1</td>
<td>22</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>Conductivity (μS/cm)</td>
<td>107</td>
<td>477</td>
<td>933</td>
<td>541</td>
</tr>
<tr>
<td>Dissolved oxygen (mg/l)</td>
<td>4</td>
<td>6</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>(2.7–5.8)</td>
<td>(4.2–7.8)</td>
<td>(0.6–1.8)</td>
<td>(0.2–2.4)</td>
</tr>
<tr>
<td>pH</td>
<td>8.1</td>
<td>6.2</td>
<td>6.7</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>(7.5–8.7)</td>
<td>(6.2–8.0)</td>
<td>(6.2–8.0)</td>
<td>(7.0–7.7)</td>
</tr>
<tr>
<td>Ammonium (mg/l)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nitrate (mg/l)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Nitrite (mg/l)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.025</td>
</tr>
<tr>
<td>Phosphate (mg/l)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Carbonate hardness (mmol/l)</td>
<td>0.1</td>
<td>3.9</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>Total hardness (mmol/l)</td>
<td>0.1</td>
<td>1.5</td>
<td>0.7</td>
<td>0.7</td>
</tr>
</tbody>
</table>
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 factory 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 1).

Discussion
The results confirm that wild and caged An. gambiae s.s. females discriminate (defined as recognition and understanding of the difference between two things [85]) 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 act over a distance (attractants and repellents [44]) or in 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 calcines 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 [81]. 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 microorganisms and chemistry 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 inundated 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 microorganisms and hence the water column will stay aerobic [85].
Anaerobic fermentation products of organic matter have been previously shown to be highly attractive to a number of gravid culicine species such as *Culex
distans* (formerly *pens*), *Culex quinquefasciatus* [33,45,50-92], *Culex pipiens* [68], *Aedes aegypti* [77,93] and *Aedes albopictus* [94,95]. These infections have been associated with a range of bacteria such as *Anabaena
erugosa*, *Pseudomonas aeruginosa*, *Brochothrix thermos* [96-98] and volatile chemicals produced by them including 4-methylpentanol, 3-methoxypropanoic acid and methyl esters [33,99,100]. 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 [48]. 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 [96,99,101]. This might explain why 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 [13,17,67]. Habitats made of pelit infusions were avoided by *Anopheles* from an early habitat age, whilst interestingly, the highest preference of the soil infusions 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 pelit 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 [194-106]. 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 pelit infusions were not rejected by *Anopheles* and even four-day old infusions still received a considerable proportion of the oviposition response 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 chor presented field experiment where good habitats were offered right next to the unfavoured one.

Importantly, *An. gambiae* s.l. appears to have an innate propensity to avoid specific chemical cues that were emitted from the pelit infusions. Rearing *An. gambiae* s. from eggs to pupae in this infusion did not alter this behaviour. Gravid females that had experienced the pelit infusions during larval development avoided the infuion 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 *Culex quinquefasciatus* where it was demonstrated that rearing the larvae in an infestation made from guinea pig faeces cancelled their innate preference for a hay infestation [4].

The cage bioassays with individual gravid females allowed a number of interesting observations that are rarely reported since the majority of studies with *Ae. gambusi* s.s. 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.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 treatment and only the distribution between cups differed when different choices were presented. This indicates that gravid females did not retain their eggs in the presence of an unfavoured substance when they were offered a suitable alternative choice. It also shows that the preferred soil infusions 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 semi-chemicals that impact on the oviposition responses of gravid *Ae. gambusi* s.s. was deduced partly by the lack of attraction of *An. gambiae* s.s. to a sterile soil infestation. However, this must be interpreted with caution since autoclaving the infusions might not only have killed the microbes but affected the chemistry of the resulting infusions, possibly altering the response of gravid mosquitoes by chemical changes rather than biological changes [77].

147
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 others rounds only a moderate preference for the soil infusion. This variation can be attributed to differences in the quality and amounts of odors 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 material over time. It has been shown previously that natural infusions can be an inconsistent source of odors 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 infusions were to be used for monitoring and 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 [34, 109].

Whilst the observed avoidance behaviour towards the ergotally rich pellet infusion was strong and in the same range as reported for other species in response to unfavourable chemical cues [107, 108, 109], the observed preference in the cages for the soil infusions was relatively weak and it is questionable whether it could compete with other suitable habitats from a larger distance. Nevertheless, chemical cues derived from over 150 replicates in two experiments likely represent a genuine effect. Further investigations are in progress to characterize the bacteria 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-electron ionization mass spectrometry.

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.s. 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.s. Soil infusions have the potential to be used for bait gravid traps for the collection of An. gambiae s.s., 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.s. 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 [64-72]. The identification of the chemicals responsible for the preference of the soil infusions might be exploited to bait gravid traps specifically for the collection of An. gambiae s.s.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JF, LL, MH-Y and SH conceived the idea for this research and developed the experimental design. MH-Y developed all procedures and implemented the experiments. JF and IP analyzed the data and wrote the first draft of the manuscript. All authors contributed to the final draft and approved the manuscript.

Acknowledgments

We thank David Nya, Bheka Dzvukamanja and Benedict Oyiente for providing mosquitoes for this study and Paul Ouma, Elizabeth Mawuli, Gregory Mwauluka and Ross Martin for many technical discussions. We also extend our gratitude to Boniface Chidzonga for statistical advice. MH-Y was supported by the Colombian Department of Science, Technology and Innovation through the scholarship programme “Mención de Excelencia”. This project received funding from the National Institute of Health NH through grant no. RO1DE021867.

Author details

1. Department of Disease Control, London School of Hygiene and Tropical Medicine, London, UK. International Centre for Insect Physiology and Ecology (IPEC), Ithabu Campus, Maha, Kenya, Royal Institute of Technology, Stockholm, Sweden.

Received: 2 February 2014 Accepted: 27 March 2014 Published: 7 April 2014

References


DOI: 10.1007/s12007-012-9633-3

Submit your next manuscript to BioMed Central and take full advantage of:
- Convenient online submission
- Thorough peer review
- No space constraints or color figure charges
- Immediate publication on acceptance
- Inclusion in PubMed, CAS, Scopus and Google Scholar
- Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit
Appendix B.

Okal et al. 2013

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

Michael N. Okal1,2, Benjamin Francis2, Manuela Herrera-Yacla2, Ulrike Fillingat3,4 and Steven W. Lindsay2,3

Abstract

Background: To date no semiochemicals affecting the pre-oviposition behaviour of the malaria vector Anopheles gambiae s.l. 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 s.s. 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 airflow olfactometer and responses measured towards collection chambers that contained cups filled with water or empty cups.

Results: Unfed females equally dispersed in the bioassay tubes to areas of high and low humidity (mean 59% 95% confidence interval (CI) 38-62%). In contrast, gravid females were 24 times (95% CI 1.3-34.2) more likely to move towards high humidity than unfed females. The results were even more pronounced in the airflow olfactometer. Gravid females were 166 times (95% CI 5.4-5208) 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
on coupled gas chromatography-electroantennogram detection [15-17]. To date, no semiochemical has been confirmed to affect the behaviour of gravid An. gambiae s.s. 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.s. 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 for Insect Physiology and Ecology, Thomas Odhiambo Campus (iipe-TOC), Mbita, on the shores of Lake Victoria, Kenya (0° 26' 86.19" S, 34° 17' 53.17E; 1,137 m above sea level). This area is characterized by an equatorial climate with a minimum temperature of 10°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 (iipe-TOC meteorological station).

Mosquitoes
Insectary-reared An. gambiae s.s. (Mbita strain) were used for all experiments. Five-day-old females were selected 30 minutes prior to the experiment from insectary colony cages where they had been kept in groups of approximately 300 males and 300 females in 30 x 30 x 30 cm netting cages and provided with 4% 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 x 30 x 30 cm netting cages and provided with 4% glucose solution ad libitum at 25-28°C and a relative humidity between 68-75%. Saturated cotton towels, 50 x 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-day-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 30 L buckets were placed on top of each other. The lower buckets 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 ran slowly through the layer. 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 attractiveness 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/closed gates between the inner and outer tubes. The two outer tubes were inserted for approximately 6 cm into small mosquito cages measuring 13 x 15 x 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 x 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, TV4000) 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
The exchange of air within the central tube and the connected cages before the gates were completely opened at 16:30 allowed the mosquitoes to move freely from the tubes into the cages. This experiment was implemented using uninfected and gravid females of the same age. At 19:00 the position of each mosquito either in the middle tube or in one of the two cages was recorded. The gates were closed at 21:30 and mosquitoes again counted in each cage or middle tube. The time period for observation was chosen based on preliminary experiments that have shown that out of 120 individual gravid females tested (5 round x 20 females) 95% (114/120) of the local insectary-reared *An. gambiae* s.s. (Mbita strain) laid all their eggs before 21:30, which is similar to the time reported for the same strain previously [19]. Experiments were done with eight mosquitoes each evening on nine occasions with uninfected females and with gravid females (total 72 per physiological stage). During the experiment with uninfected females four escaped when manipulating the gates and were excluded from the analyses. Similarly when implementing the experiments with gravid females six females were found dead in the middle tube and were excluded from the analysis, therefore a total of 68 and 56 gravid *An. gambiae* s.s. were tested. This sample size was sufficient to detect a 33% increase in the attractiveness of humid air if 6.65% 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 *Ae. gambiae* s.s. to filtered tap water (Figure 2). Each tunnel measured 40 x 100 x 30 cm and was made from polyethylene methacrylate sheets. Each tunnel was partitioned into three compartments: one large compartment for releasing the mosquitoes and two identical trapping chambers (20 x 20 x 30 cm each). Two fans (diameter 8 cm, 6 V computer casing fans (Meizu, China)) drew air through the trapping chamber into the release compartment at 0.45 m/s. Batches of 100 gravid *Ae. gambiae* s.s. females were introduced at 16:30 by inserting a 10 x 10 x 10 cm cage into the underside of the release compartment. At the same time the fans were switched on. Mosquitoes accumulated for 10 minutes and were then released by carefully opening the cage at 16:30. 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 (Peristaltic) 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 kill them of possible odour 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 *Ae. 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 formulae 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
chamber (p=0.9). The variability of the eight 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, 28 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, TV4900) 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 air-flow 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 trial, 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, quasi-binomial 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 48% at 26°C with a mean relative humidity of 54% (95% CI 52.5-55.6) in low and 97% (95% CI 95.9-99.9) 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, 62% 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
high humidity cage and the other half either remained in the middle tube (9%) or moved into the low humidity cage (4%). 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% (90% CI 90-92%), the average relative humidity in dry release compartments or dry chambers was 71% (95% CI 69-72%). The temperature did not differ between collection chambers and release compartments irrespective of the treatments and was on average 27.7°C (95% CI 27.2-27.9°C) during the 24 nights of experiments.

High responses of gravid females were recorded in the experimental treatments that presented water in either one or both collection chambers of the olfactometer (median of 69-63%, n = 103 per olfactometer/experimental unit). In contrast, when no stimulus was provided only a median of 9% of the mosquitoes responded by flying upward 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 1:1), when the other was dry (Table 1). Irrespective of whether the test cup was presented in the

---

### 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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean percentage (%) in test</th>
<th>95% CI</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>22 (16-28)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>55 (48-64)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry</td>
<td>52 (48-56)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Water</td>
<td>55 (48-64)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* *P*-value based on model parameter estimate.
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 *A. 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 area was close to 60% and above, which is similar to the relative humidity of their resting places.

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 gambiae* s.s. the hygroreceptors were located on the distal segments of the antennae bearing most of the grooved pegs [27]. Recent studies with *A. 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 present 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 to detect the humidity difference 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 air, but 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 to 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. Dagassa et al. demonstrated that when gravid *A. gambiae* s.s. females were released into a large screened semi-field system the attractiveness of a reference water source was increased by 60% when presented close to water compared with when it was presented without water [30]. In this case females traveled 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 and 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 effort 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-oviposition responses in mosquitoes (An. gambiae, An. 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 ut to an oviposition site. Water vapour has been shown to attract host seeking mosquitoes [33] and indoor resting mosquitoes [34]. For host-seeking mosquitoes water vapour can indicate a host, and for resting mosquitoes it provides an environment where the insect is less likely to desist and die, as increasing its chances of survival. Nevertheless, the results presented here clearly show a difference between the responses of non-gravid females towards water vapour suggesting that it is an important cue for gravid mosquitoes locating a potential water body, though it clearly cannot be the only one. If 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 biochemical attractants and repellents of 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 emanates from aquatic habitats making water vapour probably the major chemical signal emanating from a potential larval habitat. This study demonstrates that gravid An. gambiae ut 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 as 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 pre-oviposition response of Anopheles gambiae s.s. to filtered tap water and distilled water in two climate experiments. The document presents the background, method and results of the experiment.

Conflicts of interest
The authors have declared that they have no competing interests.

Authors’ contributions
SAM, UK, MA, D and PS were involved in the conception for the study. SAM, MA and PS developed the experimental design for the tube bioassay and implemented them with gravid females. MA designed and built the airflow apparatus. MA and PS developed the deflection detector and implemented the deflection bioassay. MA contributed the additional data comparing distilled water with tap water. MA and UP implemented the BA and UF bioassays and IF MAK and IF collected the field material. Both authors contributed to the final drafting and approval of the manuscript.

Acknowledgements
We would like to thank Steve All and Peter Ongale and Aciton Ali from the Insectary at pNTI, Mbita by providing mosquitoes for experiments in Nairobi. Paul Ouma, Rose Eni, Elizabeth J. Mclean for technical assistance. We thank Jerry Luno and from for valuable comments on the study design. This project was funded through a National Institute of Health grant no. R01 A1052177. Supporting the OAHF project/Implementation of an enhanced Malaria Control in Arusha, Likundu and Tanga, MF received travel support from the USPM, MA, and PS received travel support from the Research and Policy for Infectious Disease Dynamics (RPIDD) Program of the Science and Technology Policy, United States Department of Homeland Security’s, and the National Institute for Health Research.

Author details
Tulio Thomas, Odhiambo Campus, Mbita, Kenya, Mbita Control Department, London School of Hygiene & Tropical Medicine, London, UK.
School of Biology and Environmental Science, Durham University, Durham, UK.

Received: 10 July 2013 Accepted: 24 September 2013

Published: 11 October 2013

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

Michael N. Okal¹ ², Manuela Herrera-Varela¹ ², Paul Ouma², Baldwyn Torto², Steven W. Lindsay³, Jenny M. Lindh⁴, Ulrike Fillinger¹ ²

¹Disease Control Department, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK
²International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi, Kenya
³School of Biological and Biomedical Sciences, Science Laboratories, Durham University, Durham, DH1 3LE, UK
⁴Royal Institute of Technology, Stockholm S-100 44, Sweden

Email addresses: Michael N. Okal* - okal.mike@gmail.com, Paul Ouma - pauloumas@yahoo.co.uk, Manuela Herrera-Varela - manuelahv82@gmail.com, Baldwyn Torto - btorto@icipe.org, Jenny M. Lindh - jenlindh@kth.se, Steven W. Lindsay - s.w.linday@durham.ac.uk, Ulrike Fillinger - ufillinger@mbita.icipe.org

*Corresponding author

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-Sentinel™ 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-Sentinel™ 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 frontline 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 developed 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’ 5313” E; altitude 1,137 m above sea label). 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 m$^3$). 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 4L 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 15L of distilled water in a 20L 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 aquaous 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 45g of NaCl to 300 ml of soil infusion (150g/L) in a glass beaker and stirred to dissolve at room temperature. At this concentration small amounts of undissolved salt were observed to settle at the bottom of the beaker. Hence, for choice experiments, 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, epicak, 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 (≥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..

**Acknowledgements**

We thank Benard Oyembe, Elisha Obudho and David Alila of icipe-TOC, Mbita for maintaining colonies of mosquitoes used for these experiments and Elizabeth Masinde, Rose Atieno, Gregory Masinde and Joel Odero for technical assistance.

**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.

References


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.

Table 1: Oviposition response of gravid *Anopheles gambiae* sensu stricto to substrates in two-choice tests. Generalized linear model outputs.

<table>
<thead>
<tr>
<th>Oviposition substrates</th>
<th>Control (trap A)</th>
<th>Test (trap B)</th>
<th>Mean proportion (95% CI)</th>
<th>Odds ratio (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of gravid females trapped in test (trap B) in two choice experiments of the females trapped</td>
<td>Distilled water</td>
<td>Distilled water</td>
<td>0.50 (0.43 – 0.57)</td>
<td>1</td>
<td>0.004</td>
</tr>
<tr>
<td>led water + NaCl</td>
<td>Infusion + NaCl</td>
<td>0.77 (0.72 – 0.81)</td>
<td>3.4 (2.4 – 4.8)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Infusion</td>
<td>Infusion + NaCl</td>
<td>0.67 (0.60 – 0.69)</td>
<td>1.8 (1.3 – 2.5)</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

Response rate of released gravid females during experiments with different substrate combinations

| Response rate of released gravid females during experiments with different substrate combinations | Distilled water | Infusion | Distilled + NaCl – Infusion + NaCl | Infusion + NaCl | 0.84 (0.73 – 0.91) | 6.8 (3.1 – 15.0) | <0.001 |
|----|----------------|-------------|-----------------|---------------|----------------|----------------|----------------|---------|
| led-Distilled | 0.45 (0.33 – 0.57) | 1 | 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 |
| led-infusion | 0.84 (0.73 – 0.91) | 6.8 (3.1 – 15.0) | <0.001 |

Response of gravid females before 21:30 h of the females trapped during the night (early responders)

| Response of gravid females before 21:30 h of the females trapped during the night (early responders) | Distilled water | Infusion | Distilled + NaCl – Infusion + NaCl | Infusion + NaCl | 0.64 (0.54 – 0.73) | 1.76 (1.29 – 2.44) | 0.001 |
|----|----------------|-------------|-----------------|---------------|----------------|----------------|----------------|---------|
| led-Distilled | 0.36 (0.28 – 0.45) | 1 | 0.56 (0.46 – 0.67) | 1.59 (1.11 – 2.29) | 0.003 |
| led-infusion | 0.66 (0.58 – 0.74) | 1.92 (1.38 – 2.68) | <0.001 |
| led+NaCl – Infusion + NaCl | 0.64 (0.54 – 0.73) | 1.76 (1.29 – 2.44) | 0.001 |
Figure 1.

Figure 2.
Figure 3.
Appendix D.

Lindh et al. 2015 Article submitted to PNAS

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

Jenny Lindh1,2, Michael N. Okai1,3,1, Manuela Herrera-Varela1,3, Anna-Karin Borg-Karlson2, Baldwyn Torto3, Steven W. Lindsay3, Ulrike Fillinger1

1 Department of Chemistry, Royal Institute of Technology, SE-100 44 Stockholm, Sweden 2 Behavioral and Chemical Ecology Department, International Centre for Insect Physiology and Ecology, 00100 Nairobi, Kenya 3 Disease Control Department, London School of Hygiene & Tropical Medicine, London WC1E 7PT, UK 4 School of Biological & Biomedical Sciences, Durham University, Durham DH1 3LE, UK

Submitted to Proceedings of the National Academy of Sciences of the United States of America

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.s. 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 crocidol, occurred in >50% of natural aquatic habitats in western Kenya. When applied to water, twice as many gravid females were attracted to crocidol-treated water than to water alone in two choice cage bioassays (odds ratio (OR) 1.84; 95% confidence interval (CI) 1.16-2.81) 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 crocidol collected three times more wild malaria vectors than traps containing only water. Crocidol 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.

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 those deaths. Vector control is the most important tool to prevent transmission. However, current interventions only target vectors within houses at night. While these interventions are 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

www.pnas.org — — Volume Issue Number 1–??

PNAS | Issue Date | Volume | Issue Number | 1–??
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 compounds (51, 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 infusion). 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 make-shift hut; (B) Modified BG sentinel traps in a semi-field system; (C) Field set up of square of electrocutting nets (up) and OWART 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 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, ≥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% CI = 9.36-22.2) compared to autoclaved infusions (mean 5.7 ng, 95% CI = 4.6-6.7). In contrast, it was only detected in two out of seven lake water samples (mean in those two samples: 4.2 ng, 95% CI = 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 (11), we implemented experiments in a large (174 m²) 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 outside the trap from contacting the substrate. The experiments confirmed that cedrol was attractive...
Table 1. Probability of a mosquito female being trapped.

<table>
<thead>
<tr>
<th>Rate Ratio (95% confidence interval)</th>
<th>Anopheles arabiensis</th>
<th>Anopheles funestus</th>
<th>Anopheles costasi</th>
<th>Aedes sp.</th>
<th>Culex sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>3.3 (1.4-7.9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Test</td>
<td>2.6 (0.97-6.96)</td>
<td>0.5 (0.3-0.8)</td>
<td>0.4</td>
<td>0.8 (0.7-0.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Trap</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG</td>
<td>1</td>
<td>6.3 (1.6-25.4)</td>
<td>3.5</td>
<td>8.7 (5.0-15.1)</td>
<td></td>
</tr>
<tr>
<td>OxAIART</td>
<td>5.2 (0.9-30.9)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>E-nets</td>
<td>10.0 (0.6-18.0)</td>
<td>12.4 (2.9-52.5)</td>
<td>12.9</td>
<td>8.7 (5.0-15.1)</td>
<td></td>
</tr>
</tbody>
</table>

* no mosquitoes trapped; factor excluded from model. Treatment: control= lake water, test= lake water with 5 ppm crocidolite. Trap= E-nets of electrocuring nets (17), OxAIART= OxAIART gravid traps (18), and 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.

Mosquito species collected in gravid traps during field trial

with 69% (95% CI 66-71%) of released females collected in the treated trap (Fig. 3C). The response towards the crocidolite 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 recollected during the choice experiment when a crocidolite 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 recollected.

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 crocidolite-baited 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 pm test trap (58%, CI 53-62%).

Volatile headspace collections from both bioassay systems confirmed that crocidolite was released from the test substrates but not from the controls. Besides the crocidolite peak, no consistent difference was observed in the chromatograms from test and control treatments hence, no breakdown products of crocidolite were detected. Oviposition cups treated with 5 ppm crocidolite released 8.7 mg/min (95% CI 5.9-12.7 mg/min) and those treated with 10 ppm released 22.8 mg/min (95% CI 18.0-29.0 mg/min) during the 12 hours of experiment. The release rate from the crocidolite traps treated with 5 ppm crocidolite was on average 8.0 mg/min (95% CI 5.4-12.0 mg/min). Crocidolite 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.


dceland attracts wild malaria vectors. To test if crocidolite 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 electrocuring nets (E-nets; (17), OxAIART 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 was collected. Trap catches also included 2% of the secondary malaria vector An. coustani and 2% Aedes species. Traps baited with crocidolite were 3.3 times more trap positive (95% CI 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 electrocuring nets and the OxAIART 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 crocidolite-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 crocidolite in preference to lake water without crocidolite for laying their eggs. Furthermore, the odorous attracted colonized free-flying gravid mosquitoes in large semi-field structures and most importantly,
wild gravid mosquitoes. The attractiveness of cedrol was established in comparison to natural water from Lake Victoria which consists of the majority of the natural, highly productive anopheline habitats in the study area (20) and hence, cedrol was found to be the most stimulatory water treatment [for An. gambiae] to date in egg-count cage bioassays (21). We confirmed this using lactic acid water as a control, since it is an artificial water source that wild mosquitoes are unlikely to encounter.

Our recently developed systems of analyzing oviposition responses in comparison to a baseline that provides two equal untreated choices (15) and of measuring attraction of gravid mosquitoes to oviposition substrates with modified BG-Sentinel mosquito traps (19) allowed us to provide a detailed description of the behavior of gravid mosquitoes in response to odors. Here we showed that cedrol not only increased the proportion of gravid females that were caught in the test trap out of the total number caught, but it also increased the proportion of mosquitoes released that responded. Furthermore, cedrol induced a fast response, with two thirds of gravid mosquitoes trapped by 21:30 h. It appears that An. gambiae have evolved to use attractive chemical cues not only to select oviposition sites but also to reduce the time taken seeking suitable sites.

Our field study was implemented in an area of low vector density as confirmed by the CDC light trap and cattle-baited trap catches. Considering that only a proportion of mosquitoes that host seek obtain a sufficient amount of blood and survive long enough to become gravid, it was not unexpected that collections in gravid traps were an order of magnitude lower than catches in host-seeking traps. Despite low densities, it was three times more likely to trap An. arabiensis (the predominant species of the An. gambiae s.s. complex) than the total number of mosquitoes and cedrol-baited than when it only contained lake water. Previous observations show that the two sibling species An. arabiensis and An. gambiae s.s. share the same aquatic stages (14, 22) and therefore it is not surprising that they appear to use the same odors for orientation and selection of oviposition sites.

The collections from the gravid traps also suggested that it was worth testing the attraction of the malaria vector An. gambiae to cedrol since a slight preference for cedrol-treated traps was recorded. Finding a semiochemical or blend that could attract gravid females of the three most important vectors of malaria in Africa, An. gambiae, An. arabiensis and An. funestus would represent a tremendous breakthrough for the development of novel interventions. The fact that Anopheles mosquitoes could be caught irrespective of their low densities is promising for future development of a surveillance tool for these species using gravid traps baited with cedrol. Future work needs to be invested in testing cedrol under different eco-epidemiological conditions and in developing optimal traps that can be baited with synthetic odors for malaria vectors.

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

Cedrol-treated lake water, attracted and trapped similar proportions of gravid females in the semi-field experiments as the soil infusions from which it was identified (19). To achieve this, a nontoxic component was present in the water which is not present in the filter paper. This might be because we used a single compound rather than a blend of odors which would present the more natural situation for a mosquito. This has been extensively shown for host-seeking mosquitoes where Nedd is required for efficient attraction (24-26). Our analysis of the GC-MS data suggests another four putative semiochemicals, yet to be identified, may play a role in the attractiveness of the six day old soil infusions to gravid mosquitoes. Although it contrasts slightly, none of these could be detected in the samples from natural oviposition sites in Kenya.

The ethanol-based cedrol formulation utilized here was released in controlled raisers over the entire 12 hours trapping period each night and does therefore explain the nearly balanced response of gravid females to the traps in the semi-field experiments after 21:30 h. Less than one third of the collected mosquitoes were trapped after 21:30 h and we speculate that these were not fully gravid and therefore responded to high humidity. Cedrol is best known for its presence in the essential oil of conifery, especially in the genera Cypresus and Juniperus. However, it has been found in a large variety of plants including Sinum (27), Artemisia (28), and swing glasses of the genus Cyperus (29), which are all common in the study area. Sesquiterpenes are also known metabolites of fungi and to some extent bacteria (30, 32). We showed here that the amount of cedrol released from a soil infusio was higher than from the same infusion that had been autoclaved and previously that the odors showed no preference between infusions and controls in the field experiments.

In conclusion, our findings provide the first evidence that gravid females of the An. gambiae complex use attractive chemical cues when orienting towards a potential oviposition site and that these cues can be exploited for trapping female malaria vectors. The discovery of an odor that provides a prospect for novel entomological studies and an important step towards developing an 'attract and kill' strategy for gravid malaria vectors. This could provide a novel tool in targeting residual malaria transmission in regions where current standard indoor vector control interventions are applied at full coverage but are not enough to eliminate malaria (4, 6).

Methods

Mosquito preparation. Laboratory- and semi-field experiments were carried out with autogenically reared An. gambiae s.s. (Mbita strain) supplied by the mosquito researches at the international Centre for insect Physiology and Ecology, Nairobi. Adult females were 26±1.19 days old. 3° 15' 15'' south, 34° 14' 53.12'' east at altitude 1148 m and raised following standard operating procedures. Gravid mosquitoes were prepared by selecting 300-500 female mosquitoes, two to three days old, from their netting cages at 11:00 h and keeping them to 21:30 h. Mosquitoes were starved for seven hours before blood feeding and allowed to feed on a human arm for 15 minutes. Female mosquitoes were rejected if they required more than two minutes to engorge. Female mosquitoes were removed from the cages. Mosquitoes were then provided a 7% glucose solution ad libitum. This procedure was repeated the following day. Female mosquitoes were kept together with males for two days after the second blood meal and reared on the third day (experiment A, then four to five days after first blood meal). At 18:30 h on the day of an experiment, visually examined gravid females (enlarged, puffed abdomen) were selected from the holding cage (19).

Volatiles collection from soil infusions. Volatiles released from lake water, autoclaved and non-autoclaved six day old soil infusions were collected in parallel with bioassay cages. All the non-autoclaved infusions elicited higher response than controls. Infusion response correlated (19). Infusions were prepared by mixing 10 ml of lake water with 2 kg of soil sourced from a natural Anopheles breeding site, located within 30 km of the study site. The soil was collected and sun-dried for one day prior to preparation of the infusion. On the day of the experiment the infusions were stirred throughout the experiment and pieces of cotton cloth to remove large debris from the soil. One half of the infusion was autoclaved at 120°C for 20 minutes and left to cool to ambient temperature. Volatiles were collected on Tenax trap dynamic headspace collections from 300 ml aliquots of the three sample types in 300 ml vacuum bottles, glass 6-former traps with 2x29 octane/g: 2408 octane/g: 3219 octane/g: glass screw.
Fifty-five grams of sodium chloride (NaCl, 99.9%, Sigma-Aldrich, Steinheim, Germany) were dissolved in all aqueous samples before volatile collection to improve the release of volatile chemicals (33, 34). E-flasks were fitted with glass 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 impure imbibed empty bottles, lake water, lake water + 2 mL of soil infusions (autoclaved and auto-biofiltered). 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 sample (in duplicate) for the soil sample 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 by the sample compared to the control were also included. This peak area cut-off is derived from the peaks of samples when a volatile is present in both chromatograms. The area of each integrated peak was normalized against the area of an internal standard (n-pentadecane, 30 mg, MAC, Sigma-Aldrich, Steinheim, Germany) injected with each sample and Kovats retention indices (RI) calculated. Peaks with similar RI and mass spectra where 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 376 was confirmed using an authentic standard (1)-cadinol, 99.0% sum of isomers, GC, optical activity [α]D20 = +10.5° (Sigma, Steinheim, Germany).

Soil incubation experiment. In June 2014, water samples were collected from 116 natural water bodies (puddles, pools, ponds, ditches, scamps and pits) on the island of Wittenau (western Kyrö, 59° 30.800' N, 13° 34.130' E, altitude 518 m), during the long rainy season in April. Water samples were filtered into 250 ml E-flasks embedded in polyethylene bottle (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 sample were collected on poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) solid-phase microextraction (SPME) Fibers (50/30 mm DVB/PS-DVB, Supelco, Bellefonte, PA, USA) for 24 h. A bottle containing distilled water was also placed in the headspace above each of the water samples and served as control for background compounds. SPME fibers were analyzed immediately after volatile collection. The GC-MS file where scanned for all compounds that were detected in the headspace above the water samples and served as control for background compounds. This was repeated weekly. A soil sample was collected close to each lake during the rainy season (end of June) from the humus horizon at a depth of 0-2 cm. This sample was used for the soil incubation experiment. The humus samples were kept at 4°C until soil incubation. Soils from 16 different lake sites were sampled to obtain a wide range of edaphic conditions. Soils were placed in 9-cm Petri dishes (15 g each) covered with 3 layers of bacterial filter paper. A water column at a depth of 1 cm was added to each Petri dish and the Petri dishes were incubated aerobically at 15°C (day) and 10°C (night) for 7 days. Petri dishes were kept in a cool box at 4°C during transportation to the lab. Soil samples were homogenized with a pestle and mortar.

Statistical analyses. GC-MS data were explored using principal component analysis (PCA) with supplementary variables. Only volatile compounds present in at least four out of the seven rounds were included in the analysis. The data were centered and standardized by volatiles prior to analysis with Conoco 1.4 (33).

Dual-choice cage bioassays and attraction experiments with free-flying mosquitoes in the semi-field and full-field environments were conducted with each population of Aedes aegypti, using a quadratic distribution fitted to account for overdispersion in its statistical software version 7.13 (43). The proportion of responses (l Natural log of total responses by females) tested by the test cage group to the no-choice control was compared with the no-choice control using the binomial test (p-value) and the proportion of responses at each comparison was compared using the binomial test. Therefore, the attraction test was conducted with all female mosquitoes in one cage group (see Supplemental Information) and the proportion of responses at each comparison was compared using the binomial test. Therefore, the attraction test was conducted with all female mosquitoes in one cage group (see Supplemental Information). The correlation between the two experiments was significant and was included in the final analysis of the experiment. The data were analyzed using linear mixed-effects models, including the interaction between the parameter estimates for models with no interaction included. The analysis was conducted and standardized by volatiles prior to analysis with Conoco 1.4 (33).

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

Ethics approval for the study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol No. 36 and No. 422).

Acknowledgement. We thank Senad Gymba, Elsho Obiuwe and David Alls of Aedes-TOC, MITA for maintaining colonies of mosquitoes used for experiments and Elizabeth Masinde, Felix Ouma, Xavier Chiesa, Ross Allen, Gregory Mastlin and Joel Odum for technical assistance. This project was funded through a National Institute of Health (NHL) grant R01AI088537 supporting the OVI1 project (Department of Anopheles gambiae: Attractants, Radial Luretraps and Traps). The funders had no