Development of a gravid mosquito trap for surveillance of the malaria vector *Anopheles gambiae s.l.* Giles (Diptera: Culicidae)

BY

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A THESIS SUBMITTED TO THE UNIVERSITY OF NAIROBI SCHOOL OF BIOLOGICAL SCIENCES IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN MEDICAL ENTOMOLOGY

February, 2014

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I would like to dedicate this dissertation to my beloved family Lemane Getaneh, Natole Sisay, Moera Sisay and my brother Taye Dugassa and his family. Thank you all for your support and sacrifice to help my dream come true.

ACKNOWLEDGEMENTS

I would like to express my profound gratitude and deep regards to my supervisors Professor Florence Oyieke, Professor Richard Mukabana and Dr Ulrike Fillinger for their exemplary guidance, monitoring and constant encouragement throughout the course of this thesis. The blessing, help and guidance given by them from time to time shall carry me a long way in the journey of my future career. I would like to express a deep sense of gratitude to Dr Jenny Lindh (Royal Institute of Technology, Stockholm, Sweden), Professor Steve Lindsay (School of Biological and Biomedical Sciences, Durham University, Durham, UK) and Professor Steve Torr (Natural Resources Institute, University of Greenwich, UK) for their valuable advice, guidance and information which helped me in completing various stages of this thesis.

I am obliged to the staff members of International Centre of Insect Physiology and Ecology Thomas Odhiambo Campus (*icipe* TOC), for the valuable information provided by them in their respective fields. Specially, I am thankful to Benard Oyiembe for the technical assistance and other members of the research team, Arthur Sune, Joel Odera, Gregory Masinde, Rose Emelda, Elizabeth Masinde, Paul Ouma, Oscar Mbare, Mike Okal and Manuela Hererra for all the constructive inputs.

My sincere gratitude goes to German Academic Exchange Service (DAAD) for paying my monthly stipend, university fee, Medical insurance and provision of four months extended support in addition to the three years program. I am grateful to National Institute of Health (NIH) for funding the project, International Centre of Insect Physiology and Ecology (*icipe*) for hosting me and allowing me to use resources, University of Nairobi (UoN) for offering the training opportunity and London School of Hygiene and Tropical Medicine (LSHTM) for administration of the project fund.

Finally, I thank my parents, family, relatives, friends and all people who provided me constant encouragement without which this assignment would not be possible.

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ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CI	Confidence interval
DNA	Deoxyribonucleic acid
E-net	electric net
EtBr	ethidium bromide
GNP	gross national product
HLC	Human-landing catch
ICIPE, TOC	International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
LLINs	Long Lasting Insecticidal Nets
MM-X	Mosquito Magnet® - X
NMCP	National Malaria Control Programme
PCR	Polymerase Chain Reaction
RBM	Roll Back Malaria
s. l.	*sensu lato*
s. s.	*sensu stricto*
WHO	World Health Organization

ABSTRACT

This study was intended to develop a trap for surveillance of gravid malaria vectors, *Anopheles gambiae s.l.*, in outdoor venue.

First, a suite of tools were developed for studying the oviposition behaviour of gravid *An. gambiae s.l.* A square of four electric nets with yellow sticky film-mounted boards to collect electrocuted mosquitoes was developed as a tool to quantify gravid mosquitoes approaching an oviposition site. On average 33% (95% CI 28-38%) of mosquitoes released were recollected with the e-nets. The electric net (e-net) setup was evaluated for any influence on the response of gravid mosquitoes to a pond it surrounds compared to distribution of mosquitoes in two similar ponds; it collected a higher proportion of mosquitoes (OR 1.7, 95%CI 1.1 - 2.7; p<0.017). Following this, yellow boards placed around a pond were also tested and it attracted more mosquitoes as well (60.6% 95%CI 47.9 – 72.0). The combination of the yellow boards, the black pond at the centre and the surrounding floor might have formed a preferred contrast by the mosquitoes. The yellow film might not attract by itself as the mosquitoes hardly land on it compared with the transparent and shiny black surfaces (OR 41.6, 95% CI 19.8 – 87.3, p < 0.001 and OR 28.8, 95% CI 14.5 – 56.8, p < 0.001, respectively). Detergent and spray glue applied to water, insect glue applied on transparency and wire screen placed above the surface of the water (test for landing on the surface) and spray glue, yellow sticky film and transparent double-sided sticky film applied on the edge of artificial habitats were used to assess the mosquitoes' landing behaviour during oviposition. Over 80% of collected females were found on the water surface (Mean 103, 95% CI 93-115) as compared to the edge of the artificial pond (Mean 24, 95% CI 20-28).

The catching efficiency of commercially available gravid culicine traps were evaluated and the square of electric net was used to investigate the factors that are responsible for a reduced acceptability of gravid malaria vectors to approach these traps. Only less than 30% of released mosquitoes were recollected per night by Box, CDC and Frommer gravid traps (59.3, 95% CI 50.3–70.0). The number of mosquitoes approaching the Box trap was significantly reduced when the trap was

positioned over a water-filled basin compared to an open pond (OR 0.7 95% CI 0.6–0.7; $p < 0.001$). Based on this result a new prototype trap (OviART gravid trap) that provides open landing space was developed for the collection of gravid malaria vectors. The catch was significantly increased with the OviART gravid trap both in semi-field and field systems (OR 1.6, 95%CI 1.2–2.2; $p = 0.001$; OR 3.3, 95%CI 1.5, 7.1 respectively) compared with the Box gravid trap.

In conclusion, a square of four e-nets with yellow sticky collection devices can be used for quantifying the numbers of mosquitoes approaching a small oviposition site. *An. gambiae s.l.* primarily land on the water surface for oviposition, a behaviour exploited for the development of an OviART gravid trap. The OviART gravid trap can be used as outdoor sampling tool for surveillance of malaria vectors.

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. General Introduction

Malaria is a communicable disease caused by *Plasmodium* species and is transmitted by female *Anopheles* mosquitoes from infected to a healthy person. Malaria has declined in recent years due to tremendous efforts made to alleviate the disease. However, it still remains one of the major global health and economic threats. The current advocated malaria vector control interventions of insecticide treated nets/long-lasting insecticidal nets (ITNs/LLINs) and indoor residual spraying (IRS) have resulted in substantial reductions in malaria transmission in many countries over the last thirteen years (WHO 2010b; O'Meara *et al.,* 2010; White *et al.,* 2011; Breeveld *et al.,* 2012; Hiwat *et al.,* 2012; Meyrowitsch *et al.,* 2012). The impact of these tools on mortality and morbidity reduction has been remarkable. According to the WHO report 2011 (WHO 2011c), the mortality due to malaria has decreased by over 25% globally and by 33% in the WHO African Region since 2000. Specific reports from different countries including Eritrea, Rwanda, Zanzibar, Tanzania, Pemba, Kenya, and Zambia showed reduction in malaria cases and deaths (O'Meara *et al.,* 2008; O'Meara *et al.,* 2010; WHO 2009b; Chizema-Kawesha *et al.,* 2010).

Nevertheless, there is much work remaining to achieve the global malaria elimination and eradication goals (RBM 2008). Fifty-eight countries of the ninety nine countries with consistent malaria transmission provided complete and consistent data on malaria cases from 2000 to 2011. However, it was indicated that surveillance systems were weak in forty one countries which account for 85% of the estimated malaria cases. Accordingly, fifty countries (including nine countries in the African Region) are on the right track to reduce their malaria cases by 75% by 2015 (WHO 2012b). Nevertheless, these countries account only 3% (7 million) of the total estimated worldwide malaria cases. The global goals include: 1) complete coverage of populations at risk with locally suitable intervention measures for prevention and management of cases by 2010, 2) 50% decrease in malaria cases in

2010 and 75% in 2015 from the 2000 levels, 3) global malaria death reduction by 50% in 2010 from 2000 levels and to near zero preventable deaths in 2015, 4) elimination of malaria from eight to ten countries by 2015 and then in all countries at pre-elimination phase and finally eradication of malaria globally through progressive elimination in countries (WHO 2009b; RBM 2008; WHO 2005).

Nevertheless, malaria is still the major economic and public health problem in tropical countries particularly Africa. The number of global malaria cases is estimated to be 216 million and the number of deaths to be 655, 000 in 2009 (WHO 2011c). It also affects individuals' income which affects a gross national economy of a country in general. According to Mia and colleagues (Mia *et al.,* 2012), the household level costs of malaria includes the direct (costs of prevention and treatment), indirect (productivity loss due to sickness and death) and intangible (pain, suffering and loss of leisure time due to illness) costs. It was reported that there had been 1.3% less economic growth per person per year in countries with high malaria incidences than countries without malaria from 1965 to 1990 (Gallup and Sachs 2001; WHO 2004). It was also indicated that a malaria reduction by 10% in countries with high malaria prevalence had led to 0.3% higher economic growth (Gallup and Sachs 2001). The household level losses have long-term impacts on gross national product (GNP) (Mia *et al.,* 2012; Asenso-Okyere *et al.,* 2010). This is an indication that malaria problem alleviation would significantly contribute to the socioeconomic development of malaria endemic countries. Teklehaimanot and Mejia, (Teklehaimanot and Mejia 2008) suggest that malaria control should be considered as a strategy of poverty reduction.

Methods of vector population control are strategic approaches for prevention and control of malaria and other vector-transmitted diseases (WHO 1982; Greenwood 2008; Enayati *et al.,* 2009; Mendis *et al.,* 2009). Most scholars agree that vector control has a major role to achieve the intended goal of malaria reduction or elimination (Greenwood 2008; Enayati *et al.,* 2009; Mendis *et al.,* 2009; Barclay *et al.,* 2012). Various vector control techniques such as the use of chemicals on long lasting insecticidal nets (LLINs), for indoor residual spraying (IRS) and for larviciding, environmental management like larval source reduction and use of space and topical repellents have been devised against malaria vectors. Each of the control tools is associated with advantages and disadvantages. Since a single vector control method is unlikely to be sufficient or effective under all ecological settings in Africa (Ferguson *et al.,* 2010), the current emphasis is integration of various complementary methods known as integrated vector management (IVM). IVM is defined as a rational decision-making process for the optimal use of resources for vector control (WHO 2009a, 2012c; Killeen *et al.,* 2000; Beier *et al.,* 2008). Monitoring vector population and disease risk through inspection of the pathogens in the vectors are essential components of the IVM.

Monitoring tools play crucial roles in malaria vector control activities. They provide essential bases for devising vector management methods. These include studying species composition and longevity of mosquitoes, studying resting and/or ovipositing behaviours of mosquitoes in relation to the indoor and outdoor venues, analysing the sources from which blood meals were taken, inspecting the presence of parasites in vectors to assess potential disease risk, examining alleles responsible for insecticide resistance in mosquito vectors and evaluation of the impact of mosquito control measures (Nelson 1994; Facchinelli *et al.,* 2007; Facchinelli *et al.,* 2008; WHO 2009a; Marini *et al.,* 2010; Dekoninck *et al.,* 2010; Githeko *et al.,* 1994).

So far various tools have been developed and utilized for sampling mosquito vectors and the parasite they transmit. These include for Disease Control and Prevention (CDC) light traps, Mosquito Magnet ® X (MM-X) trap, Biogents Sentinel trap (BG Sentinel trap), Biogents mosquitito trap (BG mosquitito trap), human-landing catch (HLC), pyrethrum spray collection (PSC), aspirator collection from resting sites, resting boxes, pit shelters and gravid traps (Oyewole *et al.,* 2007; Mahande *et al.,* 2007; Odiere 2007). Most of the widely used collection tools target host-seeking mosquitoes indoors and they are effective for sampling those mosquitoes (Oyewole *et al.,* 2007; Mahande *et al.,* 2007; Odiere 2007). However, collecting mosquitoes that do not enter houses for feeding or resting has been difficult. Outdoor sampling is more challenging than indoor sampling since the outdoor vector population is more dispersed over the landscape and the interacting factors in the natural field system are complex.

This study, the development of a trap for surveillance of gravid *An. gambiae*, was one component of a large project with the aim to study the oviposition behaviour of *An. gambiae* and develop novel tools for surveillance and control of this malaria vector. Gravid traps could be used outdoors to monitor availability and density of gravid females in a given area.

1.2. Literature review

1.2.1 The major vectors of malaria in the Afro-tropical region

The malaria-vector system is very complex. Over 500 species of *Anopheles* mosquitoes exist worldwide, of which only 70 are so far recognized as potential vectors of malaria (Hay *et al.*, 2010). They are distributed worldwide with the exception of Antarctica and they exhibit a capacity to adapt to increasing ecological and environmental changes rapidly (Chaves *et al.*, 2010). These adaptations are also accompanied with an increasing number of species complexes, thus indicating a high level of genetic diversity and flexibility (Molyneux 1998). The members of *An. gambiae* complex and *An. funestus s.l.* are the important vectors (primary vectors) of malaria in sub-Sahara Africa (Gillies and Coetzee 1987; Logue 2013). Two species of the *An. gambiae* complex: *An. gambiae s.s.* and *An. arabiensis*, are both most broadly distributed and the most efficient vectors of malaria in the region (Coetzee *et al.*, 2000; Fontenille and Lochouarn 1999). Other malaria vector species of *Anopheles* in other parts of the world include *An. darlingi* and *An. albitarsis* in South America, *An. dirus* and *An. minimus* in Southeast Asia (SEA) and *An. punctulatus* in Southwest Pacific (SWPacific) (Logue 2013).

The proportion of the two important members of *An. gambiae s.l.*, *An. gambiae s.s.* and *An. arabiensis,* varies from place to place. According to Lindsay *et al.*, the range and relative abundance of *An. gambiae s.s.* and *An. arabiensis* are strongly influenced by climatological factors, especially total annual rain falls (Lindsay 1998). *An. gambiae s.s.* tends to dominate in arid savannahs where as *An. arabiensis* is the dominant species in humid forest zones. Nevertheless, the two species occur in sypmpatry in most African countries (Coetzee *et al.,* 2000). It is recognized that *An. gambiae s.s.* are in most areas highly anthropophilic (feeds on humans) whereas *An. arabiensis* is frequently more opportunistic (Sindato *et al.,* 2011). *An. gambiae s.s.* is known to enter houses for feeding on people (endophagic) and resting indoors (endophilic) (Gillies and DeMeillon 1968).

An. funestus complex has at least nine sibling species. Of these, *An. funestus s.l.* is a highly anthropophilic and a primary malaria vector. These mosquitoes are important vectors particularly at the end of rainy seasons (Gillies and DeMeillon 1968; Kelly-Hope 2009; Mendis *et al*., 2000). Other members of the complex such as *Anopheles parensis* Gillies, *Anopheles vaneedeni* Gillies and Coetzee, *Anopheles aruni* Sobti, *Anopheles confusus* Evans and Leesoni, *Anopheles fuscivenosus* Leesoni, *Anopheles leesoni* Evans, and *Anopheles brucei* Service mainly feed on animals and are not malaria vectors except *Anopheles rivulorum* Leesoni which is a secondary vector in Tanzania (Muturi *et al.,* 2009). *An. pharoensis, An. coustani, An. bwambae, An. merus, An. melas, An. rivulorum* and *An. nili* are also important species in malaria transmission in localized areas; they are secondary vectors in many places (Coetzee *et al.,* 2000). *An. pharoensis* bites animals or man both indoors and outdoors, but rests predominantly outdoors (Muriu *et al.*, 2008). The report by Muriu *et al.* indicated that they fed on animals more than humans outdoors(Muriu *et al.,* 2008). Although *An. coustani* can bite humans outdoor (Mwangangi *et al.,* 2013), they mainly feed on cattle outdoors (Mendis *et al.,* 2000). This behaviour makes them relatively less important malaria vectors.

1.2.2 Current status of malaria vector control

Long Lasting Insecticidal Nets (LLINs) and Indoor residual spraying (IRS) are the currently prioritized intervention measures for malaria reduction and interruption of transmission through vector control in Africa (Okumu and Moore 2011; Bhatt *et al.,* 2012; West *et al.,* 2012; Okell *et al.,* 2012). Both depend on the use of chemicals to kill and/or repel the vectors. IRS is a vector control method recommended by WHO for malaria control in 73 countries of which 36 were in the African Region in 2010 (WHO 2006a, 2011b). The use of DDT has declined due to its detrimental impacts on human and environmental health (WHO 2006a) and it was even banned on Stockholm Convention. However, it is still one of the 12 insecticides recently recommended for IRS (WHO 2011b). There are reasons why the use of DDT is continued. It has the longest residual efficacy (6 – 12 months based on the substrate and dosage) and fast knockdown effect when sprayed on walls and ceilings as compared to the alternative chemicals (WHO 2011b, c). Its alternative chemicals (pyrethroids; organophosphates and carbamates) have shorter residual efficacy (3 – 6 months) and might require more than two spray cycles per year (WHO 2011b). In addition, DDT's spatial repellence and irritant effect on malaria vectors reduces man-vector contact (Miller *et al.,* 1991; Pates and Curtis 2005). The mosquitoes that are not directly killed by DDT are repelled and forced to feed and rest outdoors. According to WHO (WHO 2011b), this contributes to effective disease transmission control. Therefore, IRS including DDT is recommended to be used as a component of the IVM strategy (WHO 2011b). LLINs are not only used for personal protection but also to kill the vectors that come in contact with the nets. In addition, the pyrethroids in which the nets are impregnated also have excite-repellent effect on the mosquitoes and hence reducing man-vector contact (Miller *et al.,* 1991; Pates and Curtis 2005; Zoulani *et al.,* 1994).

The decreasing trend of malaria cases and deaths is undoubted. However, the underlying reasons for this decrease and the long-term sustainability of the current malaria intervention require further investigations. Most scholars agree that much of the reduction is due to large scale investments in intervention programmes especially aimed at achieving high coverage of bed nets, IRS-campaigns and the use of effective antimalarial drugs (WHO 2011c). It was reported that IRS protected a total of 185 million people globally in 2010 which makes 6% of the global population who were at risk (WHO 2011c). In the African region, the number of people protected by IRS increased from 10 million in 2005 to 78 million in 2010 which corresponds to protection of about 11% of the population at risk in the continent (WHO 2011c). It was also reported that ITNs saved over 908,000 lives between 2000 and 2010. However, some scholars emphasized that the current malaria reduction is not only due to the current vector control measures and the effective treatment approaches but also due to other factors such as climate changes resulting in changes in rainfall, humidity and temperature that have impacts on malaria vectors (Meyrowitsch *et al.,* 2012). The significance of these climatic factors on the distribution and rate of development of both malaria parasites and vectors was also indicated by others (Steresman 2010; Rogers and Randolph 2006; Parham and Michael 2010). Nevertheless, there is substantial debate about the exact role that climate plays in driving malaria situations (Hay *et al.,* 2002; Hay *et al.,* 2005; Lindsay and Martens 1998; Pascual *et al.,* 2006; Pascual *et al.,* 2008; Chaves *et al.*, 2008; Patz *et al.*, 2002; Zhou *et al.*, 2004; Parham and Michael 2010).

Factors other than intervention techniques and climate changes such as urbanization, changes in agricultural practices and land use, and economic development which help improved housing construction might have affected mosquito vectors resulting in reduction of malaria transmission (O'Meara *et al.*, 2008). It was also indicated by WHO (WHO 2011c) that the number of deaths from all causes among children under 5 have been reduced due to socio-economic improvements in many countries in sub-Saharan Africa. Climatic change leads more likely to expansion of malaria vectors to new areas. However, it may lead to contraction of the expansion in other places (WHO 2011a). Malaria vectors and the parasites have optimum temperature and relative humidity (e.g. temperature 26–32°C and relative humidity 60%) for effective survival, dispersal and reproduction (Becker 2008). If an area’s temperature is too high and/or relative humidity is too low, the vectors and the parasites cannot survive (Becker 2008). On the other hand, with growth in economy land use also improves which would result in reduction of breeding habitats. Improved house construction with proper screens and plastering of the walls results in reduced entry of malaria vectors into houses.

The figures of malaria cases and deaths are still unacceptably high especially since it is a preventable and treatable disease. Moreover, the long-term sustainability of the current malaria reduction is uncertain mainly due to the rising vectors' resistance, the changing biting and resting behaviour of the vectors and uncertainty of future condition of the other contributing factors for the decline (Bayoh *et al.*, 2010; WHO 2011c; Reddy *et al.*, 2011; Sindato *et al.*, 2011; Yohannes and Boelee 2011). It was pointed out by Ferguson *et al.*, (Ferguson *et al.*, 2010) that the currently prioritized control measures cannot break the transmission cycle of *Plasmodium falciparum* in the areas with high entomological inoculation rate (EIR), the most direct measure of human exposure. EIR is the number of infectious mosquito bites a person is exposed to in a certain time period, typically a year. Movement of a single infected person into an area with such a high biting rate would result in the disease outbreak even if local parasite population were eliminated by mass drug administration (Smith 1955; Ferguson *et al.*, 2010). Hence, an integrated vector management is given a due attention to achieve national and global goals of malaria prevention and control (WHO 2006b; Mutero *et al.*, 2012; Enayati *et al.*, 2009; Mendis *et al.*, 2009; WHO 2012c). IVM requires monitoring vector population, disease risk and an impact of a control program (Beier *et al.*, 2008; WHO 2009a, 2012c). It is, therefore, necessary to understand vector ecology and biology to yield several new and complementary strategies of integrated vector control measures (Ferguson *et al.*, 2010).

1.2.3 Challenges facing malaria vector control

1.2.3.1 Insecticide resistance

Resistance is the decreased susceptibility of a vector population to a pesticide that was previously effective at controlling the vector. The success of malaria vector control is being threatened in sub-Saharan Africa due to resistance development by the vectors (Ramphul *et al.*, 2009). Resistance could be either physiological in which mosquitoes develop a mechanism of overcoming the toxicity of chemicals or behavioural where vectors avoid on contaminated places.

1.2.3.1.1 Physiological resistance

Vectors' resistance development to various insecticides has been reported from many countries (Kawada *et al.*, 2011; Abilio *et al.,* 2011; Hunt *et al.,* 2011). Studies were conducted in 87 countries about the prevalence of insecticide resistance in malaria vectors. Accordingly, resistance had been detected in 45 countries to at least one insecticide used for malaria vector control (WHO 2011b). Most reports indicated the existence of pyrethroid resistances in 27 countries of sub-Saharan Africa. Resistance to DDT is also common worldwide (14 countries), and there are also some cases of resistance to organophosphates (5 countries) and carbamates (8 countries) (WHO 2011b). Resistance could reduce the effectiveness of vector control in a given locality and could have serious consequences on vector intervention measures (WHO 2011b).

The current malaria control methods are highly dependent on a single class of insecticides, the pyrethroids (WHO 2011c, 2012a). This is the only insecticide class used for LLINs which also accounts about 77% of IRS coverage. This is due to its less persistence and less environmental risk than DDT which has been used in IRS for long period. Such extreme use of a single set of chemical insecticides results in a high risk of resistance development by vectors (WHO 2012a). Moreover, cross-resistance may occur to DDT and pyrethroids due to their similar mode of action regardless of their structural differences (WHO 2012a, 2010a).

1.2.3.1.2 Behavioural resistance

Malaria vectors vary in respect to their biting and resting behaviours. Some of the vectors feed and rest indoors (endophilic/exophilic) and other feed outdoors and rest outdoors (exophagic/exophilic). Currently there is behavioural shift from endophagy/endophilly to exophagy/exophilly in response to continued use of chemicals inside houses through LLINs and IRS. This is a behavioural resistance against these effective tools. The current malaria vector intervention measures and monitoring tools mainly target indoor host-seeking mosquito population. The major challenge here is that the exophagic (those that feed outside houses), exophilic (those that rest outside houses) and egg-laying mosquitoes are not directly and effectively affected by those methods. A study by Oyewole and others (Oyewole *et al.,* 2007) indicated that biting by *An. arabiensis* and *An. moucheti* occurred more outdoors while *An. gambiae* and *An. funestus* were found to bite indoors. *An. arabiensis* tend to adapt to endophagic/endophilic patterns in areas where hosts are domestic and indoor and adopts exophagic patterns where hosts are mainly outdoors (Tirados *et al.,* 2006; Mahande *et al.,* 2007). However, they are generally more exophagic and exophilic than the *An. gambiae s.s.* (Mahande *et al.*, 2007; Tirados *et al.*, 2006). They become completely exophilic in areas with high coverage of indoor spraying (Mendis *et al.,* 2000; Ameneshewa and Service 1996).

More interestingly, various current reports show that *An. gambiae,* the primary vectors of malaria, are shifting their biting and resting behaviours from indoor to outdoor in response to high utilization of ITNs and IRS (Bayoh *et al.,* 2010; Sindato *et al.,* 2011; Reddy *et al.*, 2011; Yohannes and Boelee 2011). Though *An. gambiae s.s.* predominantly endophilic, exophily has been reported in northern Tanzania (Mahande *et al.,* 2007). This risks the sustainability of the current malaria vector control methods. Both endophilic and exophilic mosquitoes eventually fly outside houses in search for oviposition sites where they are less affected by the indoor malaria vector control methods. Tools that target mosquitoes at oviposition site are important.

1.2.3.2 Species composition shift

Reports show that proportion of *An. gambiae s.l.* species composition is being shifted to the more flexible exophagic/exophilic *An. arabiensis* (Tirados *et al.,* 2006; Russell *et al.,* 2011; Derua *et al.,* 2012). There has been a declining trend of *An. gambiae s.s.* proportion of adult females from indoor collections in western Kenya, one of the malaria endemic areas. These mosquitoes had been dominant in indoor collection from 1970 to 1998 (85%). However, their proportion started decreasing relative to *An. arabiensis* since 1999 (Bayoh *et al.,* 2010). According to this study, they even became rare in indoor collection with increased bed net ownership. A study in Punta Europa region of Equatorial Guinea indicated that *An. gambiae s.s.* and *An. melas* bite mainly outdoor throughout night including the periods when people are not protected by ITNs, early evening and morning hours (Reddy *et al.,* 2011). ITNs were found to have repellence or irritancy effect on mosquitoes and hence divert them to bite in the early evening before people retire to bed (Zoulani *et al.,* 1994). They reported that *An. arabiensis* bite early in the evening with biting peak at 19.00 hours and 20.00 hours in three villages in northern Ethiopia. They found that over 70% of biting activity occurred before 22.00 hours, when people go for sleeping. It is generally evident that the biting and resting behaviours of *An. gambiae s.l.* are changing over time mainly due to the current intervention measures. In addition, new malaria vector species that bites humans outside houses in the early evening when people are not protected by the current control measures is recently investigated (Stevenson *et al.,* 2012). This report indicated that these vectors are different from the malaria vectors previously described in the area.

1.2.4 Oviposition behaviour of mosquitoes

Oviposition is the act of laying eggs by female oviparous arthropods. Mosquitoes must first find an aquatic habitat which is suitable for their offspring before laying eggs. Many mosquito species select aquatic habitats primarily based on chemical substances found and emanated from the habitats (Bentley and Day 1989; Ponnusamy *et al.,* 2010b; Burkett-Cadena and Mullen 2007; Braks *et al.,* 2007). Some mosquitoes such as *An. gambiae s.l.* are relatively specific in oviposition site and host selection while others such as *Culex nigripalpus* exhibit opportunistic behaviour with respect to both behaviours (Bentley and Day 1989). *Culex nigripalpus* mosquitoes lay their eggs in almost any aquatic habitat ranging from salt and marsh water to artificial containers such as discarded jars, cans, and tires.

An. gambiae mosquitoes make choices of oviposition site before they lay eggs (Huang *et al.*, 2005; Munga *et al.,* 2005; Sumba *et al.,* 2008). Munga *et al.,* (Munga *et al.,* 2005) evaluated oviposition preference of *An. gambiae* to water from farmlands, forests, natural wetlands and rain water. They found that the mosquitoes preferred rainwater in both dry and wet seasons (41.1% and 44.9% of the total eggs laid respectively) over waters from farmland (32.2% and 21.7%), natural wetland (15.7% and 17.1%) and forest (10.7% and 16.3%). This suggests that water with few impurities is selected by *An. gambiae* for oviposition. Their study also indicated that the average number of eggs per experiment laid by females was significantly higher during the wet season (112.8 $\pm$ 8.4 eggs, mean $\pm$ SE; average temperature = 21.1^{0}C and RH = 76.8%) than during the dry season (77.2 $\pm$ 5.6; average temperature = 23.4^{0}C and RH = 63.8%). According to Huang *et al.*, (Huang *et al.*, 2005), *An. gambiae* laid the highest number of eggs (50% of the total eggs) on sand that was provided brown background and topped with standing water as compared to dry sand, sand and soil with 2.5% - 15% water contents and water alone without brown background. Therefore, those mosquitoes are sensitive to moisture content differences and background colour of aquatic habitats. Concerning oviposition site selection based on visual cue, Huang *et al.* reported that black substrates were preferred to white substrates by these mosquitoes (Huang *et al.*, 2007). It was indicated that black substrate against white floor was the most preferred site as compared to black – black, white – white, and white – black dish vs. floor combinations. Reports indicate that visual contrasts highly influence the search for aquatic habitats (McCrae 1984; Huang *et al.*, 2007).

Fresh, small, sun-lit, and spatially spread temporary ponds (with low emergent plant coverage) are frequently cited to be the reproductive habitats for *An. gambia*e mosquitoes (Minakawa *et al.*, 1999; Mutuku *et al.*, 2006; Sumba *et al.*, 2008). A study by Huang and others suggested that they oviposit into a soil which is not covered by vegetation (Huang *et al.*, 2006a). However, they lay in aquatic habitats with different level of grass covers if they do not find open alternative pond. Nevertheless, caution should be taken not to limit the breeding habitats of these mosquitoes to exposed temporary habitats alone. Fillinger and others showed in their study that habitats with patches of emergent grass had a higher density of *An. gambiae* larvae than those without (Fillinger *et al.*, 2004). This was also confirmed by Minakawa *et al*. (Minakawa *et al.*, 2005). Historic studies suggest that these mosquitoes exhibit quite some flexibility in selecting an aquatic habitat and shown to adapt to a new habitat in a relatively few years under urban setting (Fillinger *et al.,* 2004; Huang *et al.*, 2006b; Chinery 1984). Munga *et al.* comparatively studied oviposition preferences of *An. gambiae* to rainwater conditioned with different numbers (none, five or 50 in 200ml) of conspecific larvae and rainwater with different densities of larvae (none, five, 40, 70 and 100 in 200ml) (Munga *et al.*, 2006). They reported that fewer eggs were laid in rainwater conditioned with larvae than in unconditioned rainwater. In the presence of different densities of larvae, they found that more eggs were laid in rainwater that had the least or no larvae. A study by Sumba *et al.* suggested that these mosquitoes prefer puddles which have no larvae and if they do not find such an alternative, they can lay eggs in pools with low density of larvae or even in habitats with high larval density (Sumba *et al.*, 2008). Although some studies have been carried out, there is generally a large knowledge gap concerning the oviposition behaviour of *An. gambiae* compared to that of culicines.

1.2.4.1 Oviposition cues

Gravid female mosquitoes make a choice of habitats prior to depositing their eggs. This choice is made based on cues or information they obtain from a given aquatic habitat. These include physical (e.g., reflectance, colour, temperature, humidity, pH, presence of vegetation) and chemical (volatiles from aquatic habitats like oviposition attractants, arrestants and stimulants) cues (Bentley and Day 1989; Kline 2007; Ponnusamy *et al.*, 2010b). Oviposition site semiochemicals are among the important components of a number of the external influences such as habitat size; substrate, microbial fauna, predators, vegetation, and land cover types that together may bring about the choice of aquatic habitat by female mosquitoes (Bentley and Day 1989; Sumba *et al.,* 2004; Munga *et al.,* 2005). The role of chemical cues in the attraction of mosquitoes has been investigated for culicine mosquitoes. Though few studies are available on the role of physical cues of a habitat that determines preference of *An. gambiae* (Kennedy 1942; McCrae 1984), attractant chemicals or infusions are not identified for these mosquitoes. Knowledge of how infusions and chemical substances are used for attraction of culicine mosquitoes may be used as bases to study oviposition behaviour of malaria vectors.

1.2.4.1.1 Infusions

An artificial infusion is a mixture of tap water and an organic material such as grass, hay, oak leaves, acacia leaves, manure, rabbit food pellets, algae that is fermented to allow bacterial growth (Reiter 1983; Barbosa *et al.,* 2007; Burkett-Cadena and Mullen 2007). They are prepared to provide oviposition medium that simulates natural breeding habitat condition for egg laying mosquitoes. However, their preparation is not standardised i.e. various organic matters are mixed with distilled water in varying proportions. Artificial infusions, such as hay or sod, have been shown to elicit oviposition by *Culex* mosquitoes (Reiter 1983) studied infusion-baited gravid traps, typically intended to attract *Culex* spp., for collection of *Ochlerotatus japonicus* and reported that the traps were found to effectively collect the adult mosquitoes in the state of New Jersey. According to Hazard *et al*., of the gravid *Cx. pipiens* and *Cx .quinquefasciatus* attracted to a glass trap inserted in the end of an olfactometer, 66% favoured the odours of hay infusion over distilled water, and 78% responded to the odour of the bacteria (Hazard *et al.,* 1967). In their comparative study of selected bacterial species as ovipositional attractants for *Aedes aegypti*, Asselschwert and Rockett (Hasselschwert and Rockett 1988) reported that bacteria act as ovipositional attractants for *A. aegypti*. These investigations further indicated that *Ae. aegypti* displays an actual preference for various bacterial species. Accordingly, *Bacillus cereus* and *Pseudomonas aeruginosa* were noted as being effective attractants among the compared bacterial species.

1.2.4.1.2 Semiochemicals

Semiochemicals are chemical signals produced by an organism that provoke changes in the behaviour or physiology of another organism of the same or different species (Kline 2007). The semiochemicals that act between members of the same species are known as ***pheromones***. Pheromonal systems are usually the most highly developed semiochemical systems because the species directly benefits from any improvement. Semiochemicals that act between different species are known as ***kairomones*** if the response in the recipient is favourable to the recipient and ***allomones*** if the response in the recipient benefits the producer of the signal (Kline 2007). The semiochemicals that are released from aquatic habitats of mosquitoes are referred to as ***oviposition semiochemicals*.**

Oviposition semiochemicals can be used with gravid traps or ovitraps to attract egg-laying mosquitoes. The use of oviposition attractants/stimulants to increase the collection efficacy of these traps has considerable potential in monitoring and control activities (Reiter 1983; Allan and Kline 1995; Reiter 1986a; Otieno and Onyango 1988). Attraction (i.e. orientation towards the source) and stimulation (i.e. eliciting oviposition) were not differentiated in many studies as distinction of the two acts is difficult and complex. Ikosheji   demonstrated the presence of two types of chemicals in-surface water, an ovipositing attractant and an ovipositing stimulant (Ikeshoji 1966). In the establishment of the presence of the stimulant factor, he forced gravid females to touch the testing water with tarsi and proboscis in which stimulation of oviposition occurred three times faster on breeding-site water than on tap water.

1.2.4.1.2.1 Attractants or stimulants

Attractant semiochemicals are chemical cues that direct gravid female mosquitoes to an aquatic habitat. Several studies indicate that attractants are produced by microorganisms (Hazard *et al.,* 1967; Rockett 1987; Beehler *et al.,* 1994; Millar *et al.,* 1992; Isoe *et al.,* 1995; Ponnusamy *et al.,* 2010a). For instance, fungus of the *Trichoderma* genus and the entomopathogenic bacteria *Bacillus thuringiensis* var. *israelensis* (*Bti*) and *Bacillus sphaericus* may produce attractants for some mosquito species (Poonam *et al.,* 2002; Stoops 2005; Geetha *et al.,* 2003; Hazard *et al.,* 1967; Rockett 1987; Hasselschwert and Rockett 1988). Attractants for culicine mosquitoes that have been described include 3-methylindole, indole, 4-methylphenol, 4-ethylphenol, phenol and methyl butyrate (Beehler *et al.,* 1992; Barbosa *et al.,* 2007;

Bentley and Day 1989). Culicine mosquitoes release pheromones that adhere to the tip of their eggs (Laurence *et al.,* 1985; Braks *et al.,* 2007). Pheromones laid with eggs also attract gravid females to a habitat. Erythro-6-acetoxy-5-hexadecanolide, n-heneicosane and synthetic racemic pheromone (SRP) are among the attractive pheromones identified and tested for culicine and aedine mosquitoes (Hwang *et al.,* 1987; Mboera *et al.,* 1999; Seenivasagan *et al.,* 2009). However, chemical attractants have not yet been identified and standardised for anopheline mosquitoes. Stimulant semiochemicals are chemicals that elicit oviposition behaviour in mosquitoes at the aquatic habitat (Bentley and Day 1989; Isoe *et al.,* 1995). Stimulant chemicals are non-volatile chemicals and mosquitoes need to come in contact to perceive them (Isoe *et al.,* 1995; Ikeshoji 1966).

1.2.4.1.2.2 Repellents or deterrents

Repellents drive mosquitoes away from a source. Chemicals from natural enemies and plants may have repellent activity against egg-laying mosquitoes. *An. gambiae s.s* was deterred from a target conditioned with predators and competitors (Munga *et al.,* 2006). The work of Kweka *et al.* indicated that essential oil extracts from *Ocimum suave* and *O. kilimandscharicum* deter oviposition by *An. gambiae s.l.* (Kweka *et al.,* 2010). Attraction and repellence of some chemicals depend on concentration of the chemical i.e. a given chemical could be attractive, neutral or repellent at different concentrations. For instance, Isoe *et al.,* reported that a steam distillate at a concentration equivalent to crude grass infusion repelled *Culex tarsalis* mosquitoes while a concentration equivalent to 10% grass infusion attracted the mosquitoes and a concentration equivalent to 1% grass infusion did not mediate any effect (Isoe *et al.,* 1995).

1.2.5 Mosquito collection methods

Mosquito collection is important for vector population surveillance, evaluate potential disease outbreak , study biting and resting behaviours of the vectors, determine species abundance and distribution and evaluate the effectiveness of certain control methods implemented (Nelson 1994; Amusan *et al.,* 2005; Facchinelli *et al.,* 2007; Facchinelli *et al.,* 2008; WHO 2009a; Marini *et al.,* 2010; Dekoninck *et al.,* 2010). Furthermore, effective tools and mechanisms could be used for mass trapping and suppression of the population density of the vectors.

The choice of mosquito sampling technique is determined by the purpose of the collection, an entomological parameter intended to be studied, species and stage of the mosquitoes to be sampled (Nelson 1994; Mathenge *et al.,* 2005). Nevertheless, such reasonable choices are hindered due to main limitations associated with the use of most of the mosquito sampling methods (Service 1977). There is a need of using different complementary methods when surveillance of mosquitoes of various species and physiological status is required. All sampling methods have advantages and disadvantages, which should be considered. The available tools include Centres for Disease Control and Prevention (CDC) light traps, biting collection or human-landing catch (HLC), pyrethrum spray collection (PSC), mechanical collection using aspirators (mouth aspirators and backpack aspirators), pit shelters, clay pots, empty drums and gravid traps (Oyewole *et al.*, 2007; Mahande *et al.*, 2007; Odiere 2007).

The sampling tools are classified in to two broad categories, attractant and non-attractant (Nelson 1994). In attractant sampling tools or methods certain stimuli such as light, CO_2, human or animal bait, shelter, synthetic attractant chemical blends and oviposition sites are used (Nelson 1994; Scott *et al.,* 2001; Smallegange *et al.,* 2010; Verhulst *et al.,* 2011; Mukabana *et al.,* 2012a). Active searches for resting mosquitoes by using various types of aspirators (Sindato *et al.,* 2011) and passive collection methods relying upon accidental entry to a trap (Townes, 1963 – Malaise trap) are examples of non-attractant sampling methods (Nelson 1994). Furthermore, the commonly used collection tools could be categorised into indoor and outdoor mosquito collection techniques depending on their utilisation. The outdoor mosquito populations are less targeted in the widely used sampling tools. Various attempts have also been made to collect malaria vectors outside houses. However, it has been challenging to have effective tools unlike the indoor venue.

1.2.5.1 Human landing catches (HLC)

Human landing catches are carried out by human volunteers who serve both as baits and trappers (Andrianaivolambo *et al.,* 2010). The mosquitoes land on the volunteers for feeding and are collected using aspirators. Human landing catch technique is advantageous in providing a reliable method for estimating the human biting rates (human-vector contact). Furthermore, it does not require special equipment and elementary training is enough for the collectors (WHO 1975; Lines *et al.,* 1991; Nelson 1994; Mboera 2005; Govella *et al.*, 2010). HLC has been the standard sampling tool for mosquitoes (WHO 1975), however alternative methods are needed that avoid using humans as subject in collection of disease vectors (Kweka and Mahande 2009; Kline 2002). Other disadvantage of this tool is the variation in attractiveness of humans (the catch size may be biased) and the labour intensity (Nelson 1994; Dia *et al.,* 2005; Kweka *et al.,* 2009).

1.2.5.2 Pyrethrum spray collection (PSC)

The PSC is done by covering the floor and furniture in bedrooms with white sheets. The room is then sprayed with pyrethrum where the knocked-down mosquitoes are collected from the white sheets after 10 minutes (WHO 1975; Premasiri *et al.,* 2005). This sampling technique has also its own limitations: 1.) only endophilic mosquito species are collected; 2.) it is laborious in that it requires carrying insecticide, spray equipment, and drop cloths from house to house and hence less suitable for large-scale sampling (Harbison *et al.,* 2006; van den Bijllaardta *et al.,* 2009); 3.) like with the light traps the catches indoors means interference with peoples private life (Odiere 2007).

1.2.5.3 Aspirator collection from resting places

Various aspirators like mouth aspirators, battery operated aspirators and back pack aspirators are used to collect mosquitoes from resting places (Vazquez-Prokopec *et* *al.,* 2009; Sindato *et al.,* 2011). Like for PSC this method is labour intensive and less suitable for large-scale sampling (van den Bijllaardta *et al.,* 2009; Harbison *et al.,* 2006) furthermore the collection indoors might interfere with peoples private life (van den Bijllaardta *et al.,* 2009).

1.2.5.4 Pit shelter, resting boxes and clay pot collection methods

The outdoor mosquito population remains unaffected with the use of indoor collection tools. Given the behavioural avoidance of malaria vectors on contaminated surfaces indoors, effective vector sampling is becoming more and more challenged. There have been studies on developing sampling tools that can be deployed outdoors. Tools such as pit shelters, clay pots, resting boxes, empty drums and gravid culicine traps have been used as outdoor collection techniques (Muirhead-Thomson 1958; van den Bijllaardta *et al.,* 2009; Kweka *et al.,* 2009). These tools have been used to sample malaria vectors; however they have not been well standardized or widely used. A gravid trap has been developed for sampling egg-laying anophelines. In pit shelter technique, a pit usually 1.5 m × 1.2 m × 1 m is dug near residential houses under shades of trees or bushes where outdoor resting mosquitoes are collected with aspirators early in the mornings (Muirhead-Thomson 1958; Bhatt *et al.,* 1989). This method may be dangerous for children and livestock wandering in a village as the pits can gather water.

Resting boxes are rectangular wooden boxes measuring approximately 30.5 m × 30.5 m × 30.5 m with an open end. Boxes with black cloths lining the inside part is used for sampling outdoor resting mosquitoes in the morning just like pit shelters (Kweka *et al.,* 2009; Sikulu *et al.,* 2009). A disadvantage is that they may provide a resting place for other animals like lizards, spiders, and scorpions, some of which are potential mosquito predators.

The traditional water storage clay pots are used to sample exophilic mosquitoes, they are placed in shaded sites near houses (Odiere 2007; van den Bijllaardta *et al.,* 2009). Clay pot sampling is cheap, does not require power supply (battery or electricity) and is less labour intensive than many other tools. Even though, clay pot method was shown to be relatively effective for sampling outdoor mosquitoes, it has also its own limitations. These outdoor sampling tools are generally associated with so many drawbacks. They may provide a resting place for other animals like lizards, spiders, and scorpions, some of which are potential mosquito predators (Odiere 2007; Kweka *et al.*, 2009). The resting mosquitoes might fly away due to disturbances by other animals and may also escape while aspiration is going on.

1.2.5.5 Mosquito traps

Adult mosquito traps work using light or various chemical odours to attract and catch host-seeking mosquitoes. Light and/ or chemicals such as CO_2, L-lactic acid and 1-octen-3-ol are used to attract mosquitoes to the traps where they get sucked into collection bags by motor fans (Hoel *et al.*, 2009; Maciel-de-Freitas *et al.*, 2006; Njiru *et al.*, 2006; Farajollahi *et al.*, 2009). The most commonly used light traps are CDC and New Jersey light traps (Cheong 1985). Light traps are most effective if they are placed close to their preferred blood hosts (Odetoyinbo 1969). An advantage of this tool over human landing catches are that humans are not exposed to mosquito bites and many traps can be operated by one person (Nelson 1994). Drawbacks might be that traps are noisy to the house owners and expensive. Furthermore, they are unreliable to determine human biting rates (van den Bijllaardta *et al.*, 2009; Overgaard *et al.*, 2012) and the placement of the traps indoors interfere with peoples' privacy.

1.2.5.5.1 Gravid mosquito traps

Gravid traps that collect female mosquitoes ready to lay eggs were first developed by Reiter in 1983 for sampling culicine mosquitoes (Reiter 1983). This technique is unique in that it specifically targets egg-laying female mosquitoes though few mosquitoes in other physiological states can be collected (Allan and Kline 2004; Braks and Carde 2007; Harris *et al.*, 2011). The culicine gravid traps are usually baited with oviposition media containing various types of infusions (Scott *et al.*, 2001; Jackson *et al.,* 2005; Muturi *et al.,* 2007; Burkett-Cadena and Mullen 2008, 2007; Obenauer *et al.,* 2009; McPhatter and Debboun 2009). Gravid traps have been widely used for monitoring and studying *Culex* and *Aedes* mosquitoes and for isolation of West Nile virus from those mosquitoes (Scott *et al.,* 2001; Allan and Kline 2004; Barbosa *et al.,* 2007; Braks and Carde 2007). However, neither any of those traps were tested nor any standard gravid trap has been developed for *Anopheles* mosquitoes. The only trap developed so far is that of Harris *et al.,* (Harris *et al.,* 2011) where a sticky trap was prepared by applying glue on an acetate sheet which were placed at natural aquatic habitats. It caught culicines, anophlines and a large number of non-target insets. Although various types of gravid traps such as counter flow geometry gravid trap, sticky gravid trap, CDC gravid trap, Frommer updraft gravid trap and Box gravid trap have been developed for collection of oviposition site-seeking culicines, few are commercially available (Reiter *et al.,* 1986; Braks and Carde 2007; Allan and Kline 2004; Irish *et al.,* 2012). These include CDC gravid trap Model 1712 or more commonly referred to as CDC gravid trap, the CDC gravid trap Model 1719 or more commonly referred to as Frommer updraft gravid trap and the Box gravid trap (Irish *et al.,* 2012; Allan and Kline 2004). Some of the limitations of gravid traps include collection of less number of mosquitoes than other standard sampling tools such as CDC light traps and human landing collections, interference by rain, animals and potential damage to the collected mosquitoes by natural predators and\or extreme weather conditions (Russell and Hunter 2010; Meece 2002; Dennett *et al.,* 2007).

1.2.5.5.2 Ovitraps

The terms ovitrap (oviposition trap) and gravid trap are sometimes used interchangeably (Zeichner and Perich 1999). However, ovitrap in strict sense is a trap that targets mosquito eggs while a gravid trap is a trap that catches gravid female mosquitoes looking for an aquatic habitat (Barbosa and Regis 2011; Williams and Gingrich 2007; White *et al.,* 2009; Scott *et al.,* 2001; Jackson *et al.,* 2005). The ovitrap was first developed for monitoring *Ae. aegypti* in the United States (Fay and Eliason 1966; Perich *et al.,* 2003). It has been used for detection and monitoring dengue vector population (Perich *et al.,* 2003). Ovitraps are used to monitor container breeding mosquitoes and to control the vectors by applying chemicals that can affect both the egg-laying adults and their offspring (Zeichner and Perich 1999; Perich *et al.,* 2003). Some of the limitations of ovitraps include the ovitraps cannot be used to detect pathogens and they do not represent population density of an area as they trap egg-laying mosquitoes selectively.

1.2.5.6 Electric nets, sticky materials and detergents

Electric nets (E-nets) are devices made up of wires stretched in parallel, across an aluminium frame with aluminium rods fixed to two opposite sides of the frame (Vale 1974b; Torr *et al.,* 2008). They are prepared in various dimensions (0.5 m × 0.17 m to 1 m × 2 m) based on the purpose they are intended to serve (Brady and Griffiths 1993; Vale 1974b; Knols *et al.,* 1998; Torr 1988; Vale 1982b). The electricity that flows between the adjacent wires kills insects that touch the wires (Knols *et al.,* 1998; Vale 1974b). E-nets were developed to study the behaviour of tsetse flies. Recently, some researches were conducted using e-nets on host-seeking mosquitoes and demonstrated the potential use of e-nets for sampling and studying behaviour of mosquitoes (Torr *et al.,* 2008; Knols *et al.,* 1998).

Various sticky materials were used in oviposition behaviour studies and for trapping mosquitoes that look for egg laying sites (Harris *et al.,* 2011; Isoe *et al.,* 1995). Isoe *et al.* applied insect glue on screens of different mesh sizes (sticky screen) to study responses of gravid *Culex* mosquitoes to various semiochemicals (Isoe *et al.,* 1995). Insect glues were also applied in the internal walls of Ovitraps to catch adult mosquitoes in addition to the eggs laid (Chadee and Ritchie 2010; Lourenco-de-Oliveira *et al.,* 2008; Ritchie *et al.,* 2003; Zhang and Lei 2008). Detergents reduce surface tension of water and mosquitoes drown when they land due to lack of support. There are attempts to use surfactants for oviposition bioassays (Isoe *et al.,* 1995). However, electric nets, insect glues and detergents are not used for trapping or sampling wild mosquitoes.

1.2.6 Semi-field systems for studying insect behaviour

Studies in medical entomology are conducted in two different settings, laboratory-based studies of vectors and large scale-epidemiological surveys of their abundance and distribution in the natural field system. The laboratory setting is important for identification of biological mechanisms while the natural field setting is used for generating hypotheses from correlations. However, making conclusions from laboratory outcomes is hindered due to the gap with the realistic natural field condition (Ferguson *et al.,* 2008). A semi-field system, defined as an enclosed environment, ideally situated within the natural ecosystem of the target disease vector and exposed to ambient environmental conditions, provides a realistic transitional platform between the laboratory and the field (Ferguson *et al.,* 2008; Ritchie *et al.,* 2011). A semi-field system provides an environmentally secure experimental condition for assessing the effects of genetic modifications on vector biology, to test novel control agents such as Wolbachia and fungi to avoid accidental release and environmental risk assessment of chemicals (Schäffer *et al.,* 2008; Facchinelli *et al.,* 2011). A semi-field condition facilitates short-term behavioural or ecological studies based on a single cohort using a known number of vectors (Ferguson *et al.,* 2008). Moreover, the environmental parameters like temperature and relative humidity that affect adult mosquito biology and wind are partially controlled and regulated in semi-field systems (Ritchie *et al.,* 2011; Facchinelli *et al.,* 2011). There is least probability that the experiment is interfered by non-target organisms.

Surveillance of vector population is an important component of an integrated vector management that enables evidence-based decision making for malaria vector control. A trap that collects gravid females in search of an egg-laying site outdoor would complement the existing effective indoor sampling tools. Development of a gravid trap requires an understanding of the oviposition behaviour of the malaria vectors in order to properly target them. Semi-field systems provide useful settings to study behaviour of mosquitoes and to develop a trapping device.

1.2.7 Problem statement and justification

The currently prioritized intervention and the widely used vector sampling tools were developed based on early characterization of indoor biting and resting behaviour of the major malaria vectors, *An. gambiae s.l.* and *An. funestus* (Smith 1955; Gillies and DeMeillon 1968; Faye *et al.,* 1997; Harbison *et al.*, 2006; Ferguson *et al.,* 2010). Although ITNs/LLINs and IRS provided promising results in reduction of malaria cases and deaths, the long-term sustainability of these indoor intervention measures is threatened by the development of physiological insecticide resistance, as well as behavioural avoidance of adult mosquitoes of contaminated surfaces (Takken 2002; WHO 2006b; Mahande *et al.,* 2007; Malima *et al.,* 2009; Bayoh *et al.*, 2010; Okumu and Moore 2011; Sindato *et al.*, 2011). Moreover, in several places in Africa, a change in *An. gambiae s.l.* species composition from the more anthropophilic and endophagic *An. gambiae s.s.* to the more flexible and exophagic *An. arbiensis* (Tirados *et al.,* 2006; Russell *et al.,* 2011; Bayoh *et al.,* 2010; Derua *et al.*, 2012). A new malaria-transmitting mosquito species that bites humans outside houses in the early evening when people are not protected by the current control measures was recently discovered (Stevenson *et al.,* 2012). Taken together, sampling adult mosquitoes inside houses will be more and more difficult and unreliable (Okumu and Moore 2011; Bayoh *et al.,* 2010). Furthermore, the conventional indoor sampling and intervention methods require entry to peoples' houses which may create inconvenience to house owners (Reddy *et al.,* 2011; Takken 2002).

Outdoor sampling tools such as gravid traps can be an important complement to the existing monitoring tools. A gravid trap would add to the available monitoring and evaluation tools for estimating vector densities and provide a method for estimating mosquito numbers. Furthermore, in combination with other vector control strategies, such traps could potentially contribute to the control of malaria vectors in an 'attract and kill' strategy (Michaelakis *et al.,* 2007). An effective gravid trap could be used with an attractant to attract, trap and kill egg-laying mosquitoes. Trapping gravid mosquitoes has numerous advantages; reduction of the potential population build up by trapping the gravid vectors before they lay eggs, decrease of the survival of vector population and targeting both indoor biting and resting and outdoor biting and resting mosquito species. The traps could also be used for identification of the most productive larval habitats and selectively treat preferred sites with long-lasting and environmentally friendly larvicides to interrupt transmission cycle of the diseases and to assess the potential capacity of a vector population to increase stay the same or decrease. Moreover, acquisition and transmission of a parasite needs a minimum of two blood meals and completion of one oviposition cycle. Thus, the best estimate of infective vector population can be obtained through screening gravid mosquitoes for parasite infection (Williams and Gingrich 2007).

1.2.8 Hypotheses

2 Commercially available gravid traps deter gravid *An. gambiae s.l.* from approaching a pond with the traps.
3 Artificial ponds filled with water attract gravid *An. gambiae s.l.* females.
4 Gravid *An. gambiae s.l.* land on the water surface of an aquatic habitat to lay eggs.
5 Physical parts of a gravid mosquito trap (size, structure and design) are important factors that determine the collection efficacy of a trap.

1.2.9 Objectives of the study

General Objective

❖ To develop a trap for effective surveillance of gravid females of the malaria vectors, *Anopheles gambiae s.l.*

Specific Objectives

1. To develop and evaluate methods for using electrocuting nets (e-nets) and sticky materials for studying the behaviour of *An. gambiae* around oviposition sites and traps.

- To investigate if and where mosquitoes land to lay their eggs

2. To evaluate the influence of electric nets on the behaviour of oviposition site seeking *An. gambiae s.l.*
3. To develop an effective prototype gravid trap (OviART gravid trap) for the collection of gravid *An. gambiae s.l.*
    - To evaluate the trapping efficiency of 3 commercially available gravid traps for collecting gravid *An. gambiae* in a semi-field setting.
    - To investigate the factors impeding the trapping efficacy of commercially available traps.
4. To evaluate the performance of the OviART gravid trap in the field.
    - To study the effect of substrate volume (size of tray containing water) and fan speed on trapping efficacy of the new prototype OviART gravid trap.
    - To compare the efficacy of six collection tools targeting gravid *An. gambiae s.l.* (square of e-nets, detergent treated pond, spray glue applied on water, transparency with glue, sticky board and Box gravid trap) under field conditions.

CHAPTER 2: GENERAL METHODS

2.1 Study area

This study was conducted under semi-field, open field and natural field systems. These settings were introduced in this chapter and their detailed descriptions were given under the corresponding chapters.

2.1.1 Experiments under semi-field conditions

The majority of experiments in this study were conducted in semi-field systems (Chapters 3-5) located at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (ICIPE, TOC), Mbita, at the shores of Lake Victoria, Kenya (00^{o} 25’ 949’’ S, 34^{o} 12’ 412’’ E). Mbita area experiences a consistent tropical climate, a temperature of average minimum 18.3°C and average maximum 28.3°C (based on data from *icipe*-TOC meteorological station for 2010 – 2013). A long rainy season between March and June and a short rainy season between October and December are the two major rainy periods. Malaria is endemic in this area and transmitted in the order of abundance by *Anopheles arabiensis*, *Anopheles funestus* and *Anopheles gambiae s.s.*

The semi-field systems are screened building 7.1m wide, 11.4m long and 2.8m high at the wall and 4.0m high at the highest point of the roof (Figure 1). The two opposite shorter walls and roof were made of glass and the two longer walls were screened with black fibreglass netting gauze (1.7 × 1.5 mm) (Figure 1). The floor was covered with sand to a depth of 30 cm to ease digging in artificial ponds and retain water. Temperature and relative humidity are essential factors for mosquito biology (Facchinelli *et al.*, 2011). In order to regulate these two important parameters for the mosquito survival and activity, the semi-field systems were watered prior to the experiments. Care was taken to ensure that no pooling of water occurred on the floor and that the upper layer of sand was dry by the time mosquitoes were released into the system. The semi-field systems had been cleaned on daily basis to keep them free of natural mosquito predators like spiders and ants. Growth of grasses and weeds was also controlled to reduce any potential bias among the treatments due to plant type differences within the system.

Figure 1: The semi-field system at *icipe* Thomas Odhiambo Campus, Mbita, Kenya (Courtesy: Management, *icipe* TOC)

When treatments were positioned in the corners of the semi-field system (Site 1-4), the mosquitoes released in the centre and when the treatment was positioned in the centre (Site 5) mosquitoes were released 1.5 m from the wall at Site 6 (Figure 2). Treatments in the corners were always placed 1.5 m from the two adjacent walls).

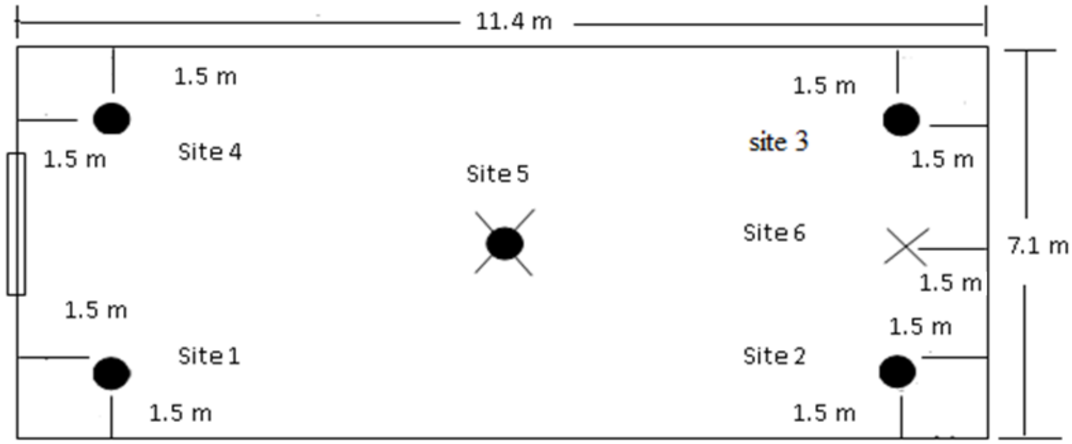


Figure 2: A schematic drawing of the dimension of the semi-field system, the treatment and release point sites. (.) Treatment sites, (×) Mosquito release point and (▯) Door.

2.1.2 Experiments under natural conditions

Two sets of experiments were conducted under natural conditions targeting natural mosquito populations. One set was implemented within the compound of ICIPE, TOC (00^o 25’ 949’’ S, 34^o 12’ 412’’ E) (Chapter 6) where movements of animals and people were limited resulting in low interference with the set up. The new OviART gravid trap was evaluated in the field. The study was carried out in Kombe village located between Mbita and Homa Bay town in the Homa Bay county of western Kenya (00^o 26’ 379’’ S, 34^o 13’ 295’’E). Treatment sites were located 300 m – 500 m from the shore of Lake Victoria which contributes to the formation of various aquatic habitats for mosquito larvae in the area. This site was selected due to availability of natural aquatic larval habitats, gravid mosquitoes and accessibility for field trial. The village is close to the main road to Homa Bay and ICIPE campus (150 m and 1.8 km respectively).

2.2 Design of experiments

When experiments are conducted in partially controlled or uncontrolled systems, the locations or sites of treatments, daily weather variation (wind, precipitation, humidity, temperature) and differences among batches of mosquitoes over time may have effect on the outputs. In order to adjust for the effects of position or site and other the other possible confounding factors, all experiments with more than one treatment followed a randomized complete block design (Appendix 3). The blocks had similar experimental units, the treatments, sites and days. In the randomized complete block designs the various experimental units were grouped into blocks according to expected variation which was isolated by the blocks. The treatments, the sites and the different experiment nights or days were the experimental units in each block. A lottery method was used to randomize the location of each treatment in the semi-field, open field and field systems on every experiment night. The experiments were sufficiently replicated.

2.3 Mosquitoes

2.3.1 Mosquito rearing

Mosquitoes from a laboratory colony of the Mbita strain of *An. gambiae s.l.* were used in all the semi-field studies. The mosquitoes were supplied for experiments by icipe’s mosquito insectary and reared following institutional standard operating procedures. The aquatic stages of the mosquitoes were reared under ambient conditions in screen houses. The eggs were placed in Lake Victoria water contained in plastic trays whose inside wall was lined with white filter paper. The larval instars were fed on Tetramin® baby fish food supplied three times a day. When the larvae pupated they were transferred to adult rearing rooms and kept in 30 × 30 × 30 cm cages covered by mosquito netting prior to adult emergence (Figure 3). 6% glucose solution was prepared in vials. Tissue paper was cut, rolled and soaked in the solution. The adults were provided with 6% glucose solution using a vial where the adsorbent tissue paper was soaked.

Gravid mosquitoes were prepared as follows. Two to three days old adult mosquitoes were selected. Both male and female mosquitoes were kept in 30 × 30 × 30 cm cages to ensure mating had occurred before or after they fed on blood meal. The mosquitoes were deprived of sugar starting from noon till evening 7.00 p.m. the time they were provided blood meal from human arm. The blood meal was provided for two consecutive nights. Moistened cotton wool was placed on top of the cages to keep them humid and 6% glucose was provided after each blood meal. They were kept in 30×30×30 cm netting cages *ad libitum* at 25-28°C and a relative humidity from 68-75%. Saturated cotton towels (50 × 25 cm) were folded and placed over the cages to avoid mosquito desiccation. 200 gravid females were selected and used for experiments two days after the second blood meal. In the experiment with host-seeking mosquitoes, two to three days old adult mosquitoes were selected from the insectary colony and kept in 30 × 30 × 30 cm cages. They were maintained in the insectary in similar condition as the blood-fed females. 200 host-seeking mosquitoes were selected and used for the experiments.

Figure 3: Adult mosquito rearing room located at icipe-Thomas Odhiambo Campus, Mbita, Kenya (Courtesy: Insectary personnel, *icipe* TOC).

2.3.2 Mosquito Identification

2.3.2.1 Identifying mosquitoes by morphology

The field collected mosquitoes were first separated from non-target insects and sorted to culicine and anopheline groups based on their morphological features. The anopheline mosquitoes were further identified into species level using keys developed by Gillies and Coetzee (Gillies and Coetzee 1987). The anophelines were identified into *An. gambiae s.l.*, *An. funestus*, *An. pharoensis* and *An. coustani.* Those specimens that were damaged during the collection process and could not be identified were recorded as non-identified anopheline mosquitoes. Members of the *An. gambiae s.l.* were distinguished by the following features: the legs specially the hind legs have speckles, the third pre-apical dark area on vein 1 has a pale interruption, and tarsi 1 – 4 have conspicuous pale bands. *An. funestus* are characterised by absence of speckles on the legs (entirely dark), absence of pale interruption on the third pre-apical dark area of vein, a pale spot on the second dark area of vein 1, a light spot between the two dark spots on vein 6 and two dark spots on vein 6 and absence of fringes on vein 6. *An. pharoensis* are characterised by abdominal segments with laterally projecting tufts of scales on segments II –VII, hind tarsus 5 and about apical half of the 4 pale. The three hind tarsus of *An. coustani* are entirely pale. Some mosquitoes lost their morphological features such as legs, wings and proboscis that are important for identification during the field collection. Those mosquitoes were recorded as non-identified anophelines. The culicine mosquitoes were not identified further into genus or species levels.

2.3.2.2 Identifying mosquitoes using PCR method

The members of the *An. gambiae s.l.*, *An. gambiae s.s.* and *An. arabiensis,* were further identified using a ribosomal DNA Polymerase Chain Reaction (PCR) method (Scott *et al.,* 1993). PCR was run by using the mosquitoes' legs as template. Positive controls for *An. gambiae s.s.* and *An. arabiensis* and a negative control were run together with the unknown samples. A master –mix was prepared in a 1.5 ml eppendorf tube from nuclease-free water, universal forward primer (10 pmol/µl), *An.*

gambiae reverse primer (10 pmol/µl) and *An. arabiensis* reverse primer (10 pmol/µl). The proportions of these substances in the mix were multiplied by the number of samples intended to be identified (Table 1).

Table 1: The substances used for preparation of a master mix and their proportions for identification of *An. gambiae s.l.* using PCR, where 'n' stands for the number of samples to be identified.

Substance	**n × 1 (µl)**
Nuclease-free water	23.2
Forward primer UN (10 pmol / µl)	0.6
Reverse primer GA (10 pmol / µl)	0.6
Reverse primer AR (10 pmol / µl)	0.6

The legs of the mosquitoes were put in PCR tubes with beads. 25 µl of the master – mix was pipetted into each of PCR tubes with beads and legs of the mosquitoes. No specimen was added into the control tube. The reaction that duplicates the DNA material was undergone in a thermo cycler with the program presented in Table 2.

Table 2: The program adjustment on thermo cycler for PCR

Program	**Adjusted value**
Heated lid	105 ºC
Pre-heat lid	Off
Initial denaturation	94 ºC for 5 minutes
No. of cycles	30
Denaturation	94 ºC for 30 seconds
Annealing	50 ºC for 30 seconds
Extension	72 ºC for 30 seconds
Final extension	72 ºC for 5 minutes
Hold	10 ºC

PCR products were separated by gel electrophoresis. The DNA molecule has a negative charge. Therefore, by applying a current to samples loaded in an agarose gel the molecules moved through the gel towards the positive terminal in a gel tank. The gel separated the DNA molecules according to their size. To visualise the DNA, a chemical called ethidium bromide (EtBr) was added to the gel. EtBr binds to DNA fluoresces under UV light. After electrophoresis the gel was observed and photographed under UV-light. The different *An. gambiae* species were identified from the size difference in the PCR products: *An. gambiae* gives a fragment that is 390 bp and *An. arabiensis* one that is 315bp.

2.3.2.3 Dissection of mosquitoes

The female mosquitoes collected in the field sites were dissected and classified as gravid and non-gravid based on the presence or absence of eggs in the ovaries. All the mosquitoes without eggs in their ovary were recorded as non-gravid.
The mosquitoes were dissected on a glass slide under a dissecting microscope using a set of dissection needles. A saline solution was dropped onto the specimen. The thorax was gently pressed by a needle with a left hand; a small tipped forceps was placed between the second last and the third last abdominal segments. The abdomen was nicked lightly and the lower (apical) part of the abdomen was gently pulled away with the forceps to expose the ovary. The ovaries were carefully separated from other material and the availability of eggs was examined as described elsewhere (Goodman *et al.*, 2003).

2.4 Gravid mosquito traps

Three commercially available gravid culicine traps were chosen for this study, CDC gravid trap, Frommer updraft gravid trap and Box gravid trap (Chapter 5, Figure 11). The way those traps were set up was modified to suit the breeding habitat of *Anopheles* mosquitoes. The basins were dug in to the sand to simulate natural aquatic habitats of the mosquitoes and no infusion was used with the traps. All the experiments with traps and electric nets were conducted using fully charged batteries.

2.5 Electric nets

1 m × 0.5 m electric nets were prepared for this study. Four-sided aluminium frames (1.0×0.5m), aluminium rods, screw bolts, small nylon loops (Damyl® fishing lines), , Fabory® zinc-plated draw springs (0.5×3.5×20 mm) and copper wires (diameter of 0.2mm) were used to construct the e-nets. The detail of the e-nets preparation was described under chapter 3. 12 V batteries were used to power the nets via inverter transformer (spark box) which amplifies the voltage supply. The e-nets were held upright by using clamps on metal stands with base in all the set ups (the detail is presented under Chapter 3, Figure 4). Opposite charges flow in opposite directions between the upper and lower sides of the e-nets through the copper wires.

No study has been conducted on the behaviour of gravid malaria vectors using e-nets to our knowledge. Electrocuted insects are conventionally collected in water filled trays under the e-nets to prevent them from attack by natural enemies and loss. However, this collection device could not be used in the studies on gravid mosquitoes as the water might attract gravid mosquitoes in search of an oviposition site and divert them from the presented target. Following optimization of the spark box settings using host-seeking mosquitoes, appropriate collection devices were devised for gravid females. Power settings on the spark boxes (spark energy and spark voltage) can be adjusted with the use of two separate dials. Proper arrangement of e-nets was devised to maximise chance of collecting gravid mosquitoes that approach a target.

2.6 Sticky materials and detergent

In this current study, sticky materials, insect glues and detergent (Chapter 3, Table 3) were used for two main purposes, for preparation of collection devices under e-nets and for making sticky ponds to study egg-laying behaviour of *Anopheles* mosquitoes. The sticky materials used include clear rollertrap (Oecos, UK), yellow rollertrap (Oecos, UK) and transparent sticky film (Barrettine, UK). The insect glues include spray glue (Oecos, UK) and a non-drip insect adhesive which does not set or dry (Oecos, UK). The sticky materials and insect glues were applied in different techniques based on the purpose intended for. The methods were discussed under the experiments. A detergent (Chemical Industries LTD, Nairobi) was used to drown landing mosquitoes by reducing surface tension.

2.7 Data analysis

The data were count data and as such not normally distributed (Seavy *et al.*, 2005). Therefore, all data were analysed untransformed using generalized linear models (O'Hara and Kotze 2010). Data analyses were done with R statistical software version 2.14.1 including the contributing packages MASS, effects, epicalc, multcomp, lme4, gee, geepack, aod (Team 2011). Experiments with one treatment tested per day were analysed using the glm.nb function (generalized linear model with negative binomial data distribution) and a log link function. Data collected for two or more treatments at the same day in the same semi-field system were not independent and were therefore analysed with gee or geepack function (generalized estimating equations). In the analyses that involved a generalised linear mixed effects model (GLMER), the effects of random variables on the result were accounted for by the model. In both GEE and GLMER, Poisson distribution with *log* link function was used in the models wherever counts were analysed. In the analysis involving proportions, GLMER model was used with binomial data distribution family and *logit* link function. The parameter estimates of the models were used to calculate the (predicted) mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.,* 2005). Similarly, multiple comparisons of treatments were calculated for more than two treatments based on the model parameter estimates.

CHAPTER 3: ELECTRIC NETS AND STICKY MATERIALS FOR ANALYSING OVIPOSITION BEHAVIOUR OF GRAVID MALARIA VECTORS

3.1. Background

Indoor resting populations of malaria vectors declined in many African countries with the massive scale-up of long-lasting insecticidal nets and indoor residual spraying (Bayoh *et al.,* 2010; Reddy *et al.,* 2011). This is due not only to the mortality of mosquitoes that contact the insecticides but also due to their behavioural avoidance of contaminated surfaces (Reddy *et al.,* 2011; Takken 2002; Malima *et al.,* 2009; WHO 2006b, 2007; Okumu and Moore 2011; Oyewole *et al.,* 2007). In areas where malaria transmission occurs outdoors at low densities (Fillinger *et al.,* 2008; Mbogo *et al.,* 1993), light traps and other indoor surveillance tools, may underestimate transmission. Consequently, there is need to develop novel surveillance and control tools targeting vector populations outdoors (Oyewole *et al.,* 2007; Mahande *et al.,* 2007; Yohannes and Boelee 2011; Sindato *et al.,* 2011; Tirados *et al.,* 2006). Sampling of gravid females may provide better opportunities to quantify the size of the vector population, and may be an approach that is more acceptable to local communities since monitoring does not require entering a house

The rational development of such tools is dependent on an understanding of the behaviour and ecology of vectors (Ferguson *et al.,* 2010). For instance, extensive studies of the processes involved in host seeking in *Anopheles gambiae s.l.* led to the development of a set of highly effective intervention strategies targeting indoor resting and feeding populations. Similarly therefore, an improved knowledge of how mosquitoes select an aquatic habitat in which to lay their eggs might provide the basis for new control strategies that exploit oviposition behaviour of *Anopheles*. For several culicine and aedine disease vectors, an understanding of oviposition behaviour has led to effective monitoring techniques and intervention strategies

(Reiter 1983, 1987; Russell and Hunter 2010; Barbosa and Regis 2011; Fay and Eliason 1966). By contrast, surprisingly little is known about the oviposition behaviour in *An. gambiae s.l.*, the major malaria vector in Sub-Saharan Africa. As a consequence, methods to monitor and control methods exploiting this behaviour are poorly developed.

To analyse oviposition behaviour, we need methods to quantify the flight, landing and egg-laying behaviour of gravid mosquitoes in the wild. Two approaches offer the prospect of being suitable. First, electric nets (e-nets) have been used to study the orientation and landing responses of insects towards visual and chemical cues (Vale 1974b, 1982a, b; Vale and Hargrove 1979; Knols *et al.*, 1998; Torr *et al.*, 2008). They were originally developed by Vale (Vale 1974b) to study the behaviour of tsetse flies and have been widely used to study odour- and trap-oriented behaviours of these flies (Vale 1993, 1974a, 1982b, a; Vale and Hargrove 1979). Whilst e-nets have been used to study the behaviour of host-seeking Mosquitoes (Knols *et al.*, 1998; Torr *et al.*, 2008), we are not aware of them being used for studying the behaviour of gravid malaria vectors.

Second, surfaces coated with a sticky substance have also been widely used to sample insects as they land on a surface (Facchinelli *et al.*, 2007; Facchinelli *et al.*, 2008; Maciel-de-Freitas *et al.*, 2006; Wallis and Shaw 2008; Brady and Griffiths 1993; Lourenco-de-Oliveira *et al.*, 2008; Zhang and Lei 2008; Ritchie *et al.*, 2003; Chadee and Ritchie 2010) and this same approach might be used to sample mosquitoes as they land. These traps are cheap, work without a battery and, providing the adhesive is sufficiently strong, will prevent trapped insects from being eaten by most common predators. Adding surfactants (e.g., detergents) to the water to reduce surface tension, insects can be sampled as they land on water (Isoe *et al.*, 1995).

The present study was carried out to explore the use of electric nets and sticky materials for analysing oviposition behaviour of gravid *An. gambiae s.l.* This study set out to develop a set of tools that can be used to study the attraction of gravid *An.*

gambiae s.l. towards visual or olfactory cues associated with aquatic habitats. Specifically, the aim was to dissect the behaviour into two components: (1) approaching an aquatic habitat and (2) the actual process of egg-laying.

3.2. Methods

3.2.1. Study site

The study was carried out in semi-field systems (Ferguson *et al.*, 2008) located at the International Centre of Insect Physiology and Ecology (description under section 2.1.1; Figures 1 and 2).

3.2.2. Artificial aquatic habitats

Two types of artificial habitats were used for experiments. For most experiments round ponds were constructed by positioning a black plastic bowl of 15 L capacity (36 cm diameter and 18 cm depth) into the ground so that the upper lip was at the same level as the sand floor. The pond was then filled with 9 L of water originating from Lake Victoria and filtered through a charcoal-sand filter (Palmateer *et al.*, 1999) henceforth called filtered lake water. Rectangular ponds were prepared by adding Lake Water to black rectangular plastic containers.

3.2.3. Electric nets (e-nets)

E-nets consist of high tension wires stretched in parallel, across an aluminium frame (1.0 m high×0.5 m wide) with aluminium rods fixed to the two shorter opposite sides of the frame (Figure 4a). Electricity flows between the two ends of each wire generating differentials of >2.5 kV between adjacent wires (Knols *et al.*, 1998; Vale 1974b), which kills insects that touch the wires. The wires are invisible to flying insects and they do not have significant impact on air movement (Vale 1993).

The rods had holes at a distance of 8 mm for fixing the wires into the rods, to enable the electric wires to be arranged in a vertical position. Small nylon loops (Damyl®

fishing lines) were tied of the same size as Fabory® zinc-plated draw springs (0.5×3.5×20mm). Copper wires (diameter of 0.2mm) 1m in length and tied to the fish line loops (insulator) from one end and to the springs (conductors) on the other end (Figure 4b). The ends of the wires with the springs and with the fish lines were alternately fitted to the holes 8-mm apart on rods to enable the flow of opposite charges in opposite directions (Figure 4a). Torr *et al.,* (Torr *et al.*, 2008) assessed different spacing of wires in the electric nets and observed no difference in mosquito catch size between 4 , 6 and 8 mm spacing. The e-nets were held upright by using clamps on metal stands with base (Figure 4a). Alternate wires in each row were charged by a 12V car battery via a transformer (spark box). In the spark box, the 12V direct current (DC) is first converted to an alternating current (AC) that is stepped up to 400 volts peak AC. It is then rectified and converted back to 400V DC. The 400 volt DC voltage is used to charge a bank of capacitors that are then discharged into the primary of the ignition coil. The voltage output to the e-nets can be reduced by an energy dial that is lowering the 12 DC input to the spark box, that in turn lowers the 400 volt output that charges the capacitors. The switch position roughly equates to the energy reduction not to a direct conversion of the voltage outputs to the nets. The output is 400V at 100% spark energy setting and approximately 300V at the 50% spark energy setting of the switch.

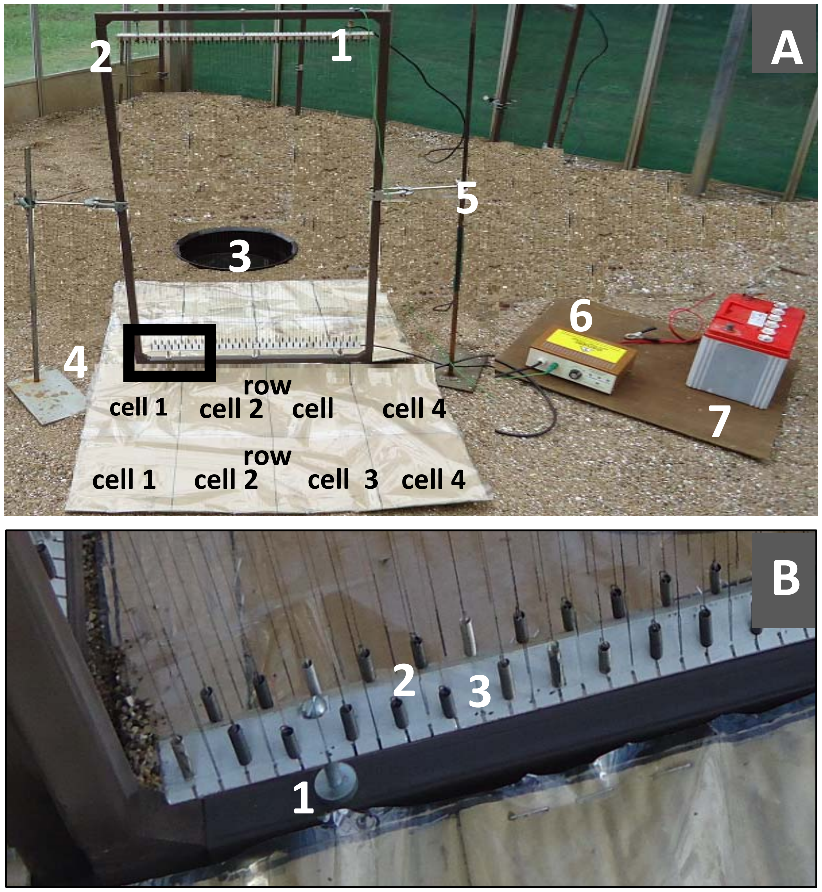


Figure 4: Electrocuting net with two mosquito collection boards made of transparent sticky film. (A) Overview of the set up: (1) aluminium rod, (2) aluminium frame, (3) artificial pond, (4) sticky boards on both sides of e-net, (5) stand and clamp to hold e-net, (6) spark box, (7) 12V battery. (B) Detail of wire connections: (1) bolt, (2) spring, (3) loop of fish line.

3.2.4. Sticky materials and detergent

A range of different materials were used as trapping devices for mosquitoes in the experiments. Throughout the manuscript reference is made to the materials listed in Table 3.

Table 3: Reference list of materials used in the experiments

Common name used	**Product name**	**Manufacturer**
Transparent double-sided sticky film	Clear rollertrap	Oecos, UK
Yellow sticky film	Yellow rollertrap	Oecos, UK
Transparent sticky film	FICSFIL(replacement glue boards)	Barrettine, UK
Insect glue	OecoTak A5	Oecos, UK
Spray glue	Oecos spray	Oecos, UK
Detergent	Teepol	Chemical Industries LTD, Nairobi
Black fly-screen	Polyester coated fibreglass mosquito netting (15×17 holes/ 2.54 cm^2)	Polytrex, China
Wire screen	Dark-green wire screen (9×11 holes/ 2.54 m^2)	Hebei Jimano, China
Transparency	A4 overhead projector transparency film (0.1 mm)	Ryman, UK

3.2.5. Mosquito preparation

For all the experiments under this chapter, insectary-reared *An. gambiae s.l.* mosquitoes were used. Host-seeking mosquitoes were prepared by selecting 300 two to three days old females on the day of experiment. Mosquitoes were starved for 6 h before the experiment commenced at 18.00 h (full description provided under section 2.3.1.).

3.2.6. Study design and experiments

Two hundred gravid female mosquitoes were selected from the holding cages based on their abdominal stages (whitish in colour and oval in shape) and were released into the semi-field system between 17.30 h and 18.00 h. Experiments were terminated at 08.00 h the following morning. Experiments with more than one treatment followed a randomized complete block design. Treatments were assigned randomly (using a random number generator) to the corners of the semi-field system and rotated randomly across corners until all treatments were run once in each of the corners included in the respective experiment. This block of experiments was then repeated. Experiments were carried out for 8 or 12 nights (3-4 blocks).

3.2.6.1 Evaluation of two spark box settings to optimize mosquito collections with e-nets

While e-nets hold promise for studying mosquito behaviour there are a few potential problems that needed to be investigated. High spark energy is used for collecting large insects like tsetse flies (Vale 1993; Torr *et al.,* 2008; Knols *et al.,* 1998), however, such high energy makes the net spark, creating a crackling sound, a burst of light and a burning smell, that may affect mosquito movement or it may destroy them by burning. Therefore, a modified transformer was used which allowed the moderation of the voltage to eliminate the sparking. Nevertheless, reduced sparking might also allow mosquitoes to escape. Accordingly, an experiment was designed to compare the catches of e-nets powered by a low-power or standard transformer.

This experiment was done using unfed *An. gambiae s.l.* females since previous research using e-nets used mosquitoes of this physiological stage and therefore a reliable response towards the target was expected (Knols *et al.*, 1998; Torr *et al.*, 2008). All consequent experiments were done with gravid females. Two e-nets were positioned in opposite corners of a semi-field system. E-nets were mounted over water-filled trays (45 × 85 × 6cm) that served to collect stunned mosquitoes that fell to the ground s (Knols *et al.*, 1998; Torr *et al.*, 2008). An odour source of carbon dioxide and a cotton sock worn for 8 hours was used as an attractant (Spitzen *et al.*, 2008; Schmied *et al.*, 2008; Jawara *et al.*, 2009; Smallegange *et al.*, 2010) and was positioned on the opposite side of the e-net, 70 cm from the e-nets and corner walls of the semi-field system. Power settings on the spark boxes can be adjusted. Two spark box settings were compared: 100% spark energy which produced sparks and 50% spark energy which was the highest energy setting that did not produce sparks. The experiment was carried out for 8 nights.

3.2.6.2 Assessment of sticky boards as collection device under e-nets

A second problem associated with e-nets is how to collect the stunned mosquitoes. Insects killed or stunned after colliding with the e-net fall to the ground. For ease of collection and to prevent them from being eaten by ants and other predators a catching device on the ground was needed. Water-filled trays under the e-nets worked well in experiments with host-seeking mosquitoes (Torr *et al.*, 2008; Knols *et al.*, 1998), however studying gravid mosquitoes, water-filled trays cannot be used since they might attract gravid mosquitoes in search of an oviposition site and divert them from the presented target. A series of experiments with e-nets positioned over sticky boards were carried out to find the most suitable material for collecting mosquitoes when stunned by the electric net.

3.2.6.3 Evaluation of cardboard mounted with transparent sticky films

One e-net was set up in a corner of a semi-field system (Sites 1-4) and a round pond was placed 70 cm from the e-net, between the net and the corner to attract gravid females. Transparent sticky film was mounted on two 50 x 80 cm cardboard rectangles. These were divided into two rows with 4 sections (20 x 25 cm) each and placed on each side of the e-net (Figure 2a). The e-net was charged using 50% spark energy and the experiment carried out for 8 nights. The number of mosquitoes that fell and got stuck on the film was counted separately for each section and direction towards the e-net.

3.2.6.4 Evaluation of potential attraction of gravid *An. gambiae s.l.* towards transparent sticky films

A collection device under an e-net should not attract gravid mosquitoes otherwise the number of mosquitoes approaching a target will be overestimated. Shiny sticky surfaces may itself look like a water body. Accordingly, studies were undertaken to assess whether gravid mosquitoes landed the shiny surfaces of the transparent sticky films. Four boards (50×80 cm) were prepared with transparent sticky film. Two of the boards were placed on the ground in one corner and the other pair of boards in the opposite corner. In order to test if landing on the sticky boards is associated with a resting behaviour close to a water source just prior or after egg-laying or if it is an actual attraction towards the surface we added an artificial pond to one of the two treatments. A round artificial pond was dug into the sand at a distance of 20 cm behind one of the pairs of the sticky boards. The experiment was carried out for 8 nights. The number of mosquitoes that landed on the boards was recorded.

3.2.6.5 Comparison of yellow, black and transparent film sticky boards for the collection of gravid An. gambiae s.l.

To find a non-attractive device for the collection of electrocuted mosquitoes, three sticky surfaces different in texture and colour were compared. Three cardboard squares of 50 x 50 cm were covered with one of the following treatments (Figure 5): (1) transparent sticky film; (2) black netting painted with 100 g insect glue dissolved in 25 ml hexane; and (3) yellow sticky film. Boards were positioned in three different corners of the semi-field system. One corner remained empty but was included in the random allocation of treatment location. Round artificial ponds were dug into the sand at a distance of 20cm behind each of the boards (Figure 5). The experiment was carried out for 12 nights. The number of mosquitoes that landed on the boards and the number of eggs laid in their respective ponds were recorded.

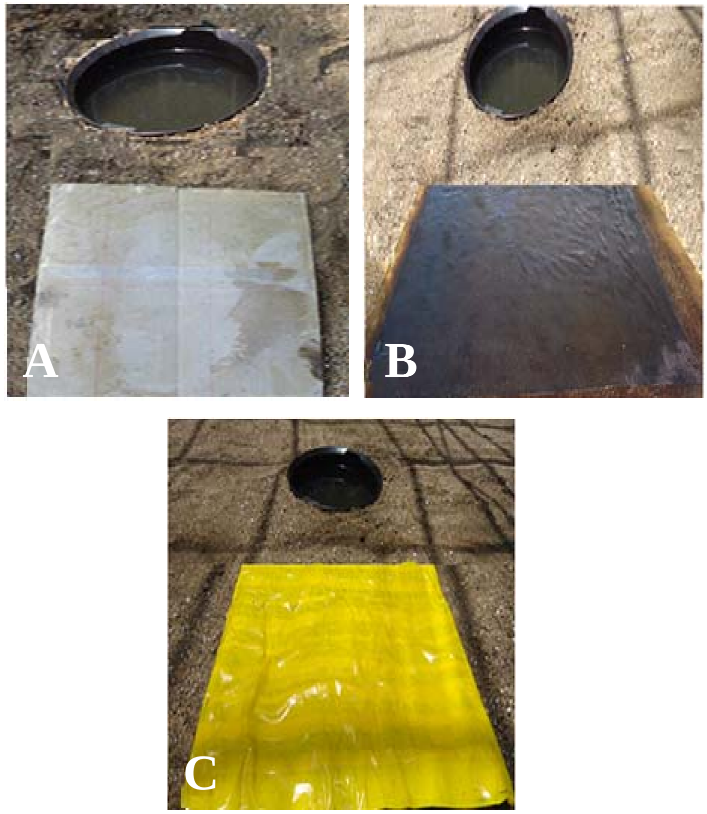


Figure 5: Three sticky boards evaluated in comparison to assess the attraction of gravid mosquitoes towards their surfaces: (A) transparent sticky film, (B) sticky black fly-screen, (C) yellow sticky film.

3.2.6.6 Collection efficacy of a square of e-nets surrounding an artificial oviposition site

A complete square of four e-nets was mounted around a rectangular pond set up in the centre of the semi-field system in order to estimate the number of gravid females approaching water. Adjacent e-nets were held together by clamps on stands and two of them shared one battery and a spark box (Figure 6). E-nets were charged with 50% spark energy. Four yellow sticky boards of 50×50cm were placed in front of each of the e-nets. Any open space inside the square of e-nets was also covered with yellow sticky board (Figure 6). The boards were divided into two horizontal rows (25×50cm) for further evaluation of the efficacy of the net and of the board as a collection device. The number of mosquitoes collected in the two rows of the board and inside the square of e-nets was recorded separately. Any eggs in the ponds were counted.

Figure 6: A complete square of four electrocuting nets surrounding an artificial aquatic habitat. Yellow sticky boards serve as collection device for stunned mosquitoes.

3.2.6.7 The use of sticky materials and detergents to assess if and where *An. gambiae s.l.* land on aquatic habitats when laying eggs

A prerequisite for the development of new monitoring control tools targeting oviposition site seeking mosquitoes e.g. with gravid traps (Reiter *et al.,* 1986; Reiter 1986b, 1983, 1987; Braks and Carde 2007; Allan and Kline 2004; Russell and Hunter 2010; Muturi *et al.,* 2007; Ritchie *et al.,* 2003; Ritchie *et al.,* 2009; Williams *et al.,* 2006) is to know if and where gravid females land during oviposition. Very few studies have assessed this particular behaviour and a variety of different modes of oviposition have been described. Here the use of different sticky materials and a detergent are evaluated to analyse potential landing of gravid females on the water surface or habitat edge for laying eggs.

3.2.6.8 Assessment of landing on the habitat edge

The edges of three round artificial ponds were made sticky to trap any landing mosquito by applying one of the three treatments: (A) yellow sticky film, (B) spray glue or (C) transparent double-sided sticky film to their inner walls. The sticky edge was 7cm wide and bordered the water surface. The ponds were set up in three corners of a semi-field system. The empty corner was included in the randomization of the treatments. The experiment was run for 12 nights. The number of mosquitoes stuck to the sticky edges and the number of eggs laid in the ponds were recorded.

3.2.6.9 Assessment of landing on the water surface

Four round artificial ponds were prepared. One of the following four treatments were applied on the water surfaces: (1) two A4 overhead projection transparencies were overlain on each other with colourless adhesive tape to form a cross-shaped surface area; the transparencies were coated on one side with 100 g insect glue dissolved in 30 ml hexane and placed on the water surface leaving 8 areas of approximately 105 cm^2 free water access at the edges (Figure 7a); (2) a circle of dark-green fly-screen of the same area as the pond was prepared and coated with 100 g insect glue dissolved in 30 ml hexane; the screen was mounted on a square of wire and placed horizontally inside the pond 5 cm below the edge of the pond and 2 cm above the water surface (Figure 7b); (3) 225 ml detergent was added to the water (2.5% solution; Figure 7c); (4) insect spray glue was uniformly sprayed on the water surface (Figure 7d). The ponds were set up in the corners of the semi-field system and the experiment carried out on 12 nights. The number of mosquitoes caught and the number of eggs laid in each pond were recorded.

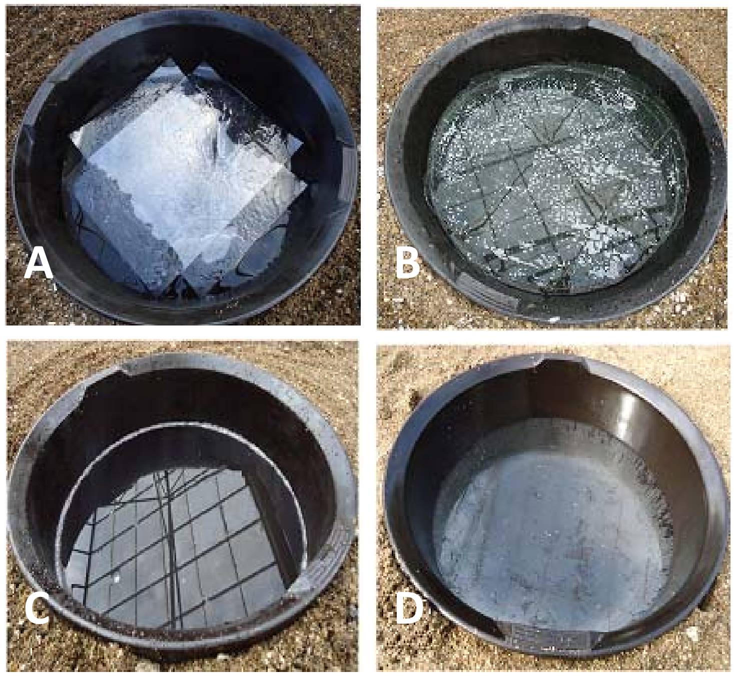


Figure 7: Surface treated artificial ponds with (A) sticky transparency, (B) sticky wire screen (C) detergent, (D) spray glue

3.2.6.10 Evaluation of the landing behaviour using a combination of detergent in the water and spray glue on the edge of the pond

Finally the best catching tools from the previous two experiments were combined, to assess whether there is a sequence in the landing behaviour (e.g. landing on surface for egg-laying and then resting on the edge of the pond). One round artificial pond was prepared. On the edge spay glue was applied and 225 ml detergent added to the water. The artificial pond was set up in the centre of a semi-field system. The experiment was carried out for 8 nights. The number of mosquitoes caught and the number of eggs in the pond were recorded.

3.2.7. Data analysis

The data generated in this study were count data, i.e. either the number of gravid mosquitoes recollected or the number of eggs laid in artificial ponds, and were not normally distributed. Data were analysed untransformed using generalized linear models (Seavy *et al.*, 2005; O'Hara and Kotze 2010). Data analyses were done with R statistical software version 2.13.1 including the contributing packages MASS, effects, epicalc, multcomp, lme4, gee, geepack, aod (Team 2011). Experiments with one treatment tested per day were analysed using the glm.nb function (generalized linear model with negative binomial data distribution) and a log link function. Data collected for two or more treatments at the same day in the same semi-field system were not independent and were therefore analysed with gee or geepack function (generalized estimating equations). The repeated measure was the day of experiment, a Poisson distribution of the data and an exchangeable working correlation matrix were assumed. The factor variables included in the model were the treatments of interest and the corner of the semi-field system in which a treatment was placed which was considered a potential confounding factor. If the effect of the corner was insignificant this variable was removed from the final model.

The parameter estimates of the models were used to predicted the mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.,* 2005). Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

3.3. Results

3.3.1. Evaluation of two spark box settings to optimize mosquito collections with e-nets

With the low energy setting, twice as many *An. gambiae s.l.* mosquitoes were collected than with the high energy setting (Table 4). Thus, the low energy setting was chosen for all subsequent experiments with gravid females.

3.3.2. Evaluation of cardboards mounted with transparent sticky films

In the first e-net experiment with gravid females, an average of 104.1 females (95% CI 78.0-138.9) were collected per night on the transparent film of the collection boards, representing around 50% of all the females released. Similar numbers were caught on both sides of the e-net, with greatest numbers close to the net in the centre (Figure 8, Table 4). This distribution indicated that most mosquitoes were electrocuted by the net but many females on the row furthest from the e-net appeared to 'sit' on the board rather than lay on the side as was the case when stunned, some were even still alive in the morning. This suggested that some females were not stunned by the net but had been attracted by the shiny film and landed on it. If this was true the number of mosquitoes on the collection board overestimated the number attracted by the water and stunned by the e-net. It was, therefore, necessary to evaluate the potential attractiveness of the collection device in the next experiments.

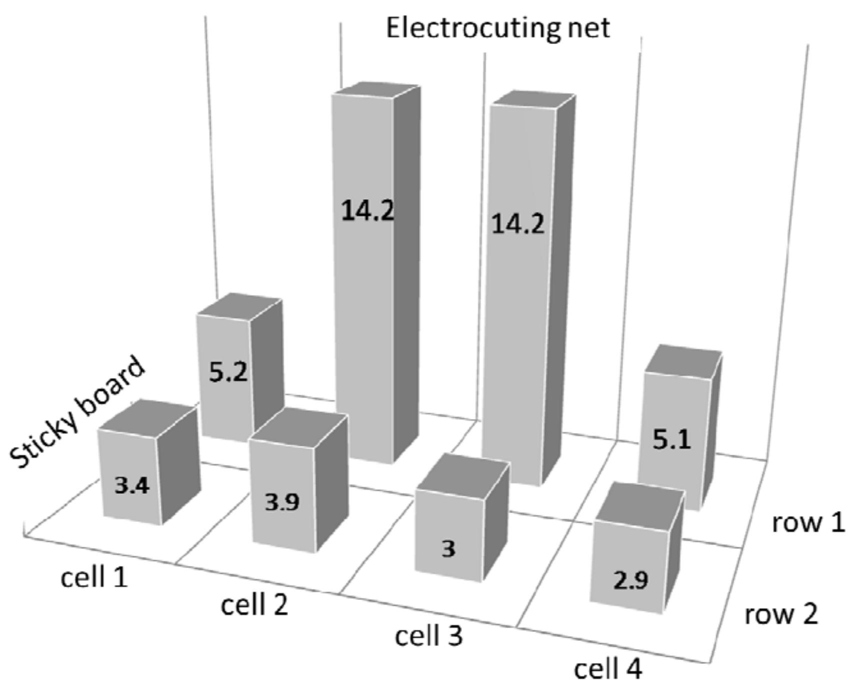


Figure 8: Distribution of electrocuted mosquitoes on the transparent sticky film collection board. The height of the columns show the average number of mosquitoes collected per cell of the grid drawn on the board.

3.3.3. Evaluation of attraction of gravid *An. gambiae s.l.* to transparent sticky films

In this experiment mosquito collections were significantly confounded by the corner in which the treatments were presented in the semi-field system, with one corner being 2.5 times more attractive than the other (Table 4). After adjusting for corner, the analyses showed that the sticky board alone caught approximately 15% of the released mosquitoes, while 24% were collected when the sticky board was placed next to water. These results show that the sticky board alone was attractive to gravid females and their landing on it was not associated with resting around a potential habitat otherwise females should not have been trapped by the boards without pond. This experiment also confirms that water vapour is a strong attractant for oviposition site seeking mosquitoes.

Table 4: The results of experiments on evaluation of spark energy settings and transparent sticky films as collection devices under e-nets

Treatment	Mean no. of mosquitoes (95% CI)	OR (95% CI)	p-value
Experiment: Evaluation of high and low energy settings for electrocuting nets			
100% spark energy	9.0 (5.9 - 13.7)	1	-
50% spark energy	19.9 (13.2 - 30.0)	2.2 (1.8 – 2.8)	<0.001
Experiment: Comparison of average mosquito collections on transparent sticky film boards close and away from one e-net			
row 2 (>25 cm)	26.5 (16.15 – 43.49)	1	-
row 1 (<25 cm)	77.63 (62.36 – 96.63)	2.93 (2.08 – 4.13)	< 0.001
Experiment: Evaluation of attraction of gravid *An. gambiae s.l.* to transparent sticky films			
without pond	29.4 (21.5- 40.4)	1	-
with pond	47.1 (37.4- 59.3)	1.6 (1.1- 2.3)	0.012
site 4	15.3 (11.2- 20.7)	1	
site 2	38.4 (30.0- 49.1)	2.5 (1.6- 4.1)	<0.001

CI=confidence interval, OR=odds ratio, *Multiple comparisons of treatments: treatments denoted with same letter are not significantly different

3.3.4. Comparison of yellow, black and transparent sticky boards for the collection of gravid *An. gambiae s.l.*

Gravid females were equally attracted by the transparent sticky film and the sticky black fly-screen, yet few were collected on the yellow sticky film. Furthermore, a significantly higher number of eggs were laid in the pond behind the yellow boards than in the ponds behind the other sticky materials (Figure 9, Table 5). Yellow sticky boards did not interfere with the approach of the gravid female towards a pond and consequent egg-laying and were therefore chosen as routine collection device under e-nets.

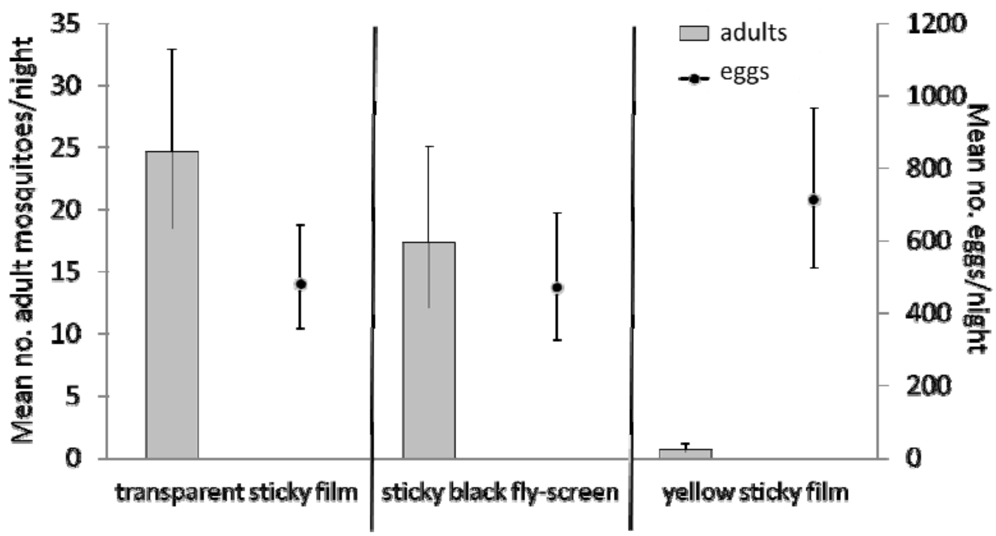


Figure 9: Mean number (error bars: 95% confidence intervals) of gravid females collected on three types of sticky boards and the mean number of eggs laid in the ponds associated with the boards.

3.3.5. Collection efficacy of a square of e-nets around an artificial pond

In order to estimate the number of gravid females approaching an aquatic habitat a complete square of e-nets was used that surrounded an artificial pond. Yellow sticky boards were used as collection devices for the stunned mosquitoes (Figure 6). On average 65.3 (95% CI 55.9 – 76.10) of the 200 released mosquitoes were collected. Over 81% of these were found on the outside of the ring indicating that few gravid females might have approached the oviposition site from a height of above 1m from the ground or passed through the 8 mm gaps between the vertical aluminium frames and the wires. Eggs were found on 6 out of 12 days. The average number of eggs was 80.3 (95 % CI 43.6 – 147.8). On average over nine times as many mosquitoes were found stuck in the first 25 cm of the sticky board close to the e-nets than further away (the second 25 cm) showing that they were stunned by the electric nets and hence fell close to the base of the net (Table 5). Here, the square of e-nets was adapted and optimised as a tool to study oviposition behaviour of malaria vectors for the first time.

Table 5: Results obtained from evaluation of three sticky boards and a square of e-nets with yellow collection boards

Treatment	**Mean no. (95% CI)**	**OR (95% CI)**	**p-value**
Experiment: Comparison of yellow, black and transparent sticky boards for the collection of gravid *An. gambiae s.l.*			
Mosquitoes*			
Transparent sticky film	24.6 (18.4-32.9)	1[a]	-
Sticky black fly-screen	17.3 (12.0-25.1)	0.71 (0.39-1.26) [a]	0.240
Yellow sticky film	0.58 (0.32-1.09)	0.02 (0.01-0.05) [b]	<0.001
Eggs*			
Transparent sticky film	478 (356-643)	1[a]	-
Sticky black fly-screen	469 (326-469)	0.98 (0.80-1.20) [a]	0.841
Yellow sticky film	712 (525-712)	1.50 (1.18-1.92) [b]	0.001
Experiment: Comparison of average mosquito collections on yellow sticky film boards mounted under a square of e-nets			
row 2 (>25 cm)	5.1 (3.9-6.8)	1[a]	-
row 1 (<25 cm)	48.1 (40.7-56.9)	9.4 (7.7-11.4)[b]	<0.001
inside the square	12 (9.6-15.0)	2.3 (1.8-3.0)[c]	<0.001

CI=confidence interval, OR=odds ratio, *Multiple comparisons of treatments: treatments denoted with same letter are not significantly different.

3.3.6. The use of sticky materials and detergents to assess if and where *An. gambiae s.l.* land on aquatic habitats when laying eggs

On average the number of females trapped on the water surfaces was over four times higher than on the edges (103.3, 95% CI 93.0-115 and 23.7, 95% CI 20-28.2, respectively) irrespective of collection device differences (Table 6 and Figure 7). The detergent and the spray glue caught about three times more mosquitoes than the sticky screen or transparencies (Table 3). The detergent lowered the water tension to such an extent that mosquitoes that landed on the water surface sunk, presenting little opportunity to lay eggs. On the other hand, a large proportion of the mosquitoes stuck on the surface with spray glue laid eggs, leading to more than 11 times higher mean egg numbers than other treatments (Table 6).

From those treatments applied to the edge of the pond, the yellow and transparent films trapped similar numbers of mosquitoes but less than half of the spray glue (Table 6). Similar egg numbers in all the treatments indicate that a similar number of gravid females approached these ponds and laid eggs. It is unlikely that all these eggs were laid by the few mosquitoes trapped on the edge. The mean number of eggs in these ponds is comparable with the mean number laid by mosquitoes stuck on the spray glue on the water surface (Table 6).

Finally, when detergent in the water was combined with spray glue on the edge of a pond, most mosquitoes were drowned in the water with only 15% stuck on the pond edge (Table 6). Notably, approximately a quarter of released mosquitoes were collected with this method. This corresponds well with the figures obtained from the square of e-nets. Eggs were not found in the pond throughout the test nights showing that oviposition did not take place in flight.

Table 6: The use of sticky materials and detergents to assess if and where *An. gambiae s.l.* land on aquatic habitats when laying eggs. Results were obtained from generalized linear models for individual experiments.

Treatment	**Mean no. of (95% CI)**	**OR (95% CI)**	**p-value**
Experiment: Assessment of landing and egg-laying on the habitat surface			
Mosquitoes			
wire screen	11.9 (8.1 -17.6)	1 [a]	-
spray glue	35.2 (27.4 – 45.1)	3.0 (2.1 - 4.1) [b]	<0.001
detergent	41.7 (32.6 – 53.2)	3.5 (2.0 – 6.1) [b]	<0.001
transparency	14.6 (10.8 – 19.7)	1.2 (0.7 – 2.0) [a]	0.460
site 1	20.4 (14.3 – 29.2)	1 [a]	-
site 2	16.4 (12.5 – 21.6)	0.8 (0.6 - 1.0) [a]	0.070
site 3	37.5 (26.1 – 54.0)	1.8 (1.3 – 2.5) [b]	<0.001
site 4	29.0 (21.0 – 40.0)	1.4 (1.2 – 1.7) [b]	<0.001
Eggs			
wire screen	39 (23 - 65)	1 [a]	-
spray glue	464 (344 – 628)	11.9 (6.8 - 20.9) [b]	<0.001
detergent	12 (4 – 34)	0.3 (0.1 - 0.8) [c]	0.018
transparency	23 (10 – 52)	0.6 (0.3 - 1.1) [ac]	0.109
site 1	105 (42 - 259)	1 [a]	-
site 2	79 (29 - 219)	0.8 (0.4 – 1.6) [a]	0.546
site 3	173 (68 - 439)	1.8 (1.1 – 3.1) [b]	0.026
site 4	181 (79 - 419)	1.8 (1.3 – 2.5) [b]	0.001
Experiment: Assessment of landing on the habitat edge for egg-laying			
Mosquitoes			
spray glue	13 (9.4 – 18.0)	1 [a]	-
yellow sticky film	5.4 (3.0 – 10.0)	0.4 (0.2 - 0.8) [b]	0.012
transparent double-sided sticky film	5.3 (4.0 – 7.3)	0.4 (0.2 - 0.6) [b]	<0.001
site 1	4.9 (3.1 – 7.7)	1 [a]	-

Treatment	Mean no. of mosquitoes/eggs (95% CI)	OR (95% CI)	p-value
site 2	10.8 (6.7 – 17.5)	2.5 (1.5 – 4.1) [b]	<0.001
site 3	9.0 (6.0 – 13.6)	1.9 (1.0 – 3.5)[b]	0.036
site 4	7.1 (4.1 – 12.2)	1.7 (0.8 – 2.9) [a]	0.062
Eggs			
spray glue	358 (232 - 552)	1[a]	-
yellow sticky film	363 (240 - 549)	1.0 (0.6 - 1.7) [a]	0.930
transparent double-sided sticky film	297 (178 - 497)	0.8 (0.5 – 1.4) [a]	0.420
Experiment: Evaluation of sequence of landing on habitat during oviposition			
surface catch (detergent)	42.5 (37.7 – 47.9)	1	-
edge catch (spray glue)	7.4 (5.1 – 10.8)	0.17 (0.12 – 0.25)	<0.001

CI=confidence interval, OR=odds ratio, Multiple comparisons of treatments: treatments denoted with same letter are not significantly different.

3.4. Discussion

Electric nets have been successfully used for the development of control tools for tsetse flies for nearly 40 years (Omolo *et al.,* 2009; Vale 1993, 1974b), yet have been used little for mosquito research (Knols *et al.,* 1998; Torr *et al.,* 2008). Our results show that e-nets can be used to study the oviposition behaviour of malaria vectors. Importantly, we found that reducing the voltage to prevent sparking doubled the catch, which confirms earlier observation by Torr and colleagues (Torr *et al.,* 2008). We are uncertain whether it is the visual, acoustic or chemical cues associated with the sparking that reduces the catch. When a single e-net was used next to an artificial pond, similar numbers of mosquitoes were collected on both sides of the net indicating that the mosquitoes approached the target from both directions. In order to quantify the total number approaching an attractive source, such as a water body, a complete square of e-nets surrounding the water was found useful.

Sticky boards proved to be a simple method for collecting mosquitoes that were stunned after colliding with the net and fell to the ground since they effectively retained specimens and protected them from predation by ants. However, we found that a transparent film was also attractive to gravid mosquitoes, even when used as sole collection device without any e-nets and without any water source associated. Adding an artificial pond behind the transparent film sticky board increased the number of females trapped on the board confirming that water vapour is a strong attractant for oviposition site seeking mosquitoes (Isoe *et al.,* 1995; Huang *et al.,* 2005; Clements 1999).

In search of an alternative collection material under e-nets, the black fibreglass gauze coated with insect glue proved as attractive to gravid mosquitoes as transparent film. Both surfaces were conspicuously shiny for the human eye compared to the yellow film that appeared matt. Experiments in cages with individual mosquitoes implemented at night under ambient moon light conditions and in complete darkness as well as experiments in an airflow olfactometer with 100 females in complete darkness confirmed that the attraction was a visual response and not a response to chemicals in the insect glue or water content (data not published). Many insects, including mosquitoes respond to reflectance of water surfaces (McCrae 1984; Bentley and Day 1989; Bernáth *et al.*, 2004; Bernáth *et al.*, 2008; Horvath 1995). Attraction of insects to shiny surfaces has been shown before, for example black flies of all physiological stages have been successfully trapped with glue coated aluminium plates (Bellec 1976; Mutero *et al.*, 1991; Orndorff *et al.*, 2002) . Recently, Harris and colleagues (Harris *et al.*, 2011) utilized this principle to collect gravid mosquitoes from water surfaces using glue-coated transparencies.

The low number of mosquitoes on the yellow sticky film and the high number of eggs laid in the adjacent pond suggest that this material does not have the same visual properties for a mosquito as the transparent film and black glue boards and does not attract mosquitoes for landing on it. Oviposition site seeking females fly straight to the pond to lay their eggs, and then fly off again, without landing close to the aquatic habitat before or after egg-laying. This might be due to the light colour (Huang *et al.*, 2007) and the lack of reflectance. It is unlikely that it has to do with the actual colour of the board since mosquitoes have dichromatic vision, which results in good contrast sensitivity but poor colour resolution (Collins and Blackwell 2000). It is known that mosquitoes respond to contrasts (McCrae 1984; Huang *et al.*, 2007) and gravid females are attracted by dark surfaces rather than light coloured ones (McCrae 1984).

The number of mosquitoes collected with transparent sticky boards was approximately twice the number collected with yellow sticky boards. It is likely that transparent films overestimated the number of mosquitoes that approached the pond when they were used in combination with e-nets but sticky boards made of the yellow film can serve as effective collection device. On the other hand, the attractiveness of the boards mounted with transparent sticky film might be exploited further in future for the development of new trapping devices for gravid malaria vectors.

For the development of new interventions (e.g. auto-dissemination of larvicides (Gaugler *et al.,* 2012; Chism and Apperson 2003; Itoh *et al.,* 1994)) and monitoring tools (e.g. ovi-traps and gravid traps (Reiter *et al.,* 1986; Reiter 1983, 1987; Braks and Carde 2007; Russell and Hunter 2010; Muturi *et al.,* 2007; Ritchie *et al.,* 2003; Ritchie *et al.,* 2009; Williams *et al.,* 2006; Allan and Kline 2004) targeting gravid malaria vectors it is important to know if and where gravid females land during oviposition. Notably, very few studies have been implemented to date and all these studies used relatively small cages (less than $1m^3$) except one which was implemented under field conditions (Harris *et al.,* 2011). Gravid females were most commonly observed laying their eggs directly seated on the water surface and on the lip of the oviposition cup irrespective of the floor colour of the cup or size of cage (McCrae 1984; Miller *et al.,* 2007; Clements 1999). Occasionally, oviposition from flight has been described when the oviposition cup was placed over a black surface (McCrae 1984). Here, for the first time, experiments in large semi-field systems are described that investigate if and where *An. gambiae s.l.* lands to lay her eggs. The results indicate that gravid females primarily land directly on the water surface to lay eggs. Since no eggs were found in ponds with both detergent and sticky sides, which prevents directly egg-laying on the water surface, there is no evidence for eggs being dropped in flight onto the water from these experiments. The relatively large number of eggs found associated with females caught on the spray glue applied on the water surface was probably due to stress induced oviposition on the surface (Harris *et al.,* 2011).

Similar numbers of eggs were laid in the ponds treated with different sticky materials at their edges. Although the number of adults caught on the edges differed, the number caught there was small. This suggests that even mosquitoes caught at the edge might have landed there to rest before or after laying eggs, rather than to lay whilst seated on the edge of the pond. In the case of the pond with spay glue at the edge attraction of female mosquitoes cannot be excluded since the numbers were significantly higher than for the other two treatments and the glue made the pond edge appear very shiny.

Some caution must be exercised when interpreting the data since the artificial ponds used in this study did have a vertical edge which was not utilized by gravid females to sit on and lay eggs. This might have been different if ponds with a slope would have been used. Previous cage experiments have shown that *An. gambiae s.s.* and *Anopheles arabiensis* laid a large proportion of eggs on water saturated slopes rather than the free-standing water when given a choice (Miller *et al.,* 2007; Huang *et al.,* 2005; Balestrino *et al.,* 2010). Nevertheless, even then it was observed that these eggs were laid whilst seated rather than during flight (Miller *et al.,* 2007).

The finding of this study that *An. gambiae s.l.* lays its eggs directly on the water surface supports the observations made on *An. arabiensis* by Harris and colleagues (Harris *et al.,* 2011) in the field using transparencies floating on the water on the edge of natural habitats. The finding that gravid *An. gambiae s.l.* lay eggs directly on the water surface is encouraging for two reasons. Firstly, it lends support to the principle that gravid females could be used to transfer larvicides from a resting site to a breeding site (Chism and Apperson 2003; Gaugler *et al.,* 2012; Itoh *et al.,* 1994; Devine *et al.,* 2009). Secondly, it may lead to the development of a gravid trap where mosquitoes are attracted to a water source and trapped there (Russell and Hunter 2010).

Sticky materials and the detergent used in this study were shown to be useful methods for collecting mosquitoes when landing to lay eggs. Of all the tools tested the detergent and the spray glue directly applied to the water surface was most effective at collecting gravid females under semi-field conditions. Transparencies and sticky screens did not work as well which might be due to obstruction of water vapour coming off the pond by the transparency or due to visual obstruction of the water surface area. The latter two might have been useful tools for testing the attraction of female vectors towards a water source that was treated with putative oviposition semiochemicals or natural infusions (Isoe *et al.,* 1995) but due to their reduced trapping efficiency e-nets might be the best alternative for analysing such odour-oriented behaviour. Detergents and spray glue, though powerful in arresting approaching females, might interfere with the presented chemical or infusion.

Therefore, further research would be required to present these in combination for attracting and trapping gravid female mosquitoes. The use of these tools under natural conditions also needs to be further evaluated.

3.5. Conclusion

This study demonstrated that electric nets are suitable devices for studying the egg-laying behaviour of *An. gambiae s.l.* when used in combination with yellow sticky boards for collecting stunned mosquitoes. Shiny sticky surfaces attract gravid females possibly because they are visually mistaken as breeding sites. These materials might be developed further as gravid traps. *Anopheles gambiae s.l.* primarily land on the water surface for oviposition. This behaviour can be exploited for the development of new trapping and control strategies.

CHAPTER 4: SYSTEMATIC EVALUATION OF THE INFLUENCE OF ELECTRIC NETS ON THE BEHAVIOUR OF OVIPOSITION SITE SEEKING *Anopheles gambiae s.l.*

4.1 Background

A square of electric nets (e-nets) was one of a range of the tools developed for analysing the behaviour of gravid malaria vectors in a recent study (Dugassa *et al.*, 2012). Detergent and a square of e-nets were the most effective tools to catch gravid mosquitoes responding to an artificial aquatic habitat prepared in semi-field systems. In the study, 2.5% detergent (Teepol, Chemical Industries LTD, Nairobi) was used to drown mosquitoes landing on the water by lowering the surface tension. This helps quantify the number of mosquitoes that actually visit a given pond. It was also found that a square of e-nets is an effective tool to estimate the number of mosquitoes responding to and approaching a potential oviposition site. The squares of e-nets can particularly be used for evaluation of chemical and physical cues of an oviposition site without interfering with the water. Furthermore, they could help figure out what happens to mosquitoes that do not reach the end chain of behavioural responses (SACEMA ; Irish *et al.*, 2012). A study of oviposition behaviours of gravid malaria vectors would be useful for development of novel sampling methods and possibly alternative and complementary intervention tools to control malaria. This necessitates tools such as a square of e-nets that could be used to study various chemical and physical cues directing mosquitoes to aquatic habitats. For several culicine and aedine disease vectors, an understanding of oviposition behaviour has led to effective monitoring techniques and intervention strategies (Reiter 1983, 1987; Russell and Hunter 2010; Barbosa and Regis 2011; Fay and Eliason 1966).

Every tool used to collect mosquitoes has its own limitations and trap design affects the catch size (Kweka and Mahande 2009; Nelson 1994; Southwood 1966). Choice and efficacy of trapping tools depends on the target mosquito species, sex, physiological state, biting and resting behaviours and developmental stages (Nelson 1994). Since each method has built-in bias, none can 100% represent nature (Nelson 1994). Nelson (Nelson 1994) recommends a combined use of more than one method for estimation of population parameter. A detailed understanding of a tool intended to be used is important for correct interpretation of results. A suction gravid trap might not provide as accurate measure of attraction to or repulsion away from aquatic habitats (Irish *et al.*, 2012) as the square of e-nets. This is because, 1. It would be difficult to quantify the number of mosquitoes that approach or visit the trap and escape after or without laying eggs, 2. The number of mosquitoes visiting the trap depends on the relative abundance of competing natural breeding ponds in the field. Therefore, trap catches may lead to either over estimation or under estimation of the strength of an attractant (chemical or physical cue). The square of e-nets provides an opportunity to fill this gap in the behaviour studies.

However, there should be an understanding of how the mosquitoes behave in the presence of this tool around a presented aquatic habitat. Knowledge of if and how the responses of mosquitoes are affected are important to reduce potential extra noise that might affect the result of a study. For instance efficacy of a trap can be assessed in terms of estimated number of mosquitoes approaching it. To do this two choice assays can be implemented where the actual catch in a trap is compared to the total number of mosquitoes ready to lay eggs or visit the second trap within a square of e-nets. This is applicable if the presence of the square of e-nets at the alternative side does not modify the mosquitoes' response towards an aquatic habitat (pond) surrounded by the square of e-nets in any way. The square of e-nets in combination with yellow sticky boards presents a highly visible structure to a human eye even if the actual electric wires might not be seen by the mosquito. In previous studies it was noted that electric nets are not visible to the flying insects as they accidentally collide with it and the nets do not affect air flow or odour plumes (Vale 1993; Torr *et al.*, 2008; Rayaisse *et al.*, 2011; Laveisère *et al.*, 1987). Consequently, the flight of the flies around the source is less likely to be affected by the electric nets. Nevertheless, previous studies have shown that gravid *An. gambiae s.l.* are highly sensitive to visual cues when searching for an oviposition site and landscape structures and contrasts might guide their orientation flight towards or away from a site (Bentley and Day 1989; McCrae 1984; Huang *et al.*, 2007).

This study was intended to analyse if the presence of a functional square of e-net or any of its components has any impact on the behavioural responses of gravid *An. gambiae s.l.* to a potential oviposition site within the square of e-nets. It also served to investigate if the catching efficacy of a functional square of e-nets around a pond and a detergent pond can directly be compared.

4.2 Materials and Methods

4.2.1 Study area

The study was carried out in 80 m^2 semi-field system located at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (see section 2.1.1.; Figures 1 and 2). The weather condition is favourable for mosquito survival and reproduction. This area is one of the malarious regions of western Kenya.

4.2.2 Mosquito preparation

Insectary-reared *An. gambiae s.l.* mosquitoes were used throughout this study (description provided under section 2.3.1.).

4.2.3 Study design and experiments

Binary choice assays were conducted in semi-field systems in all the experiments under this chapter. The treatments were positioned in the corners of the semi-field system at a distance of 1.5m from the two adjacent walls (Site 1-4) and the mosquitoes were released from the centre or site 5 (Figure 2). Artificial ponds were prepared by digging black plastic bowls of 15 L capacity (36 cm diameter and 18 cm depth) into the ground so that the upper lip was at the same level as the sand floor. 9L Lake Water was added to the bowls. Two treatments were set up in opposite corners to reduce a chance of interaction between the ponds. 2.5% (225ml) detergent was added in all the ponds to quantify mosquitoes visiting the ponds. The comparisons were done one at a time. The number of mosquitoes drowned was recorded and availability of eggs in the ponds was checked. The experiments were conducted using randomized complete block designs (RCBD) in 12 nights' replicates. Each treatment was assigned to each corner three times.

The new terms used in this chapter are briefly described in Table 7 in order to simplify following up the content.

Table 7: Description of terms used in the chapter

	Terms	**Description**
1	Detergent pond	An artificial pond in which a detergent (Teepol, Chemical Industries LTD, Nairobi) was added
2	Functional square of e-nets & yellow collection boards	Four e-nets joined to form a complete square around a pond where the yellow collection boards were placed under the nets. The nets were connected to a 12 V battery (power source) to electrify them.
3	Non-powered square of e-nets	Square of e-nets without the yellow collection boards and not connected to a battery (power source).
4	Non-powered square of e-nets with large gaps without yellow boards	Square of e-nets where the gaps between the wires were increased to 24 mm by removing every two consecutive wires leaving one wire. Yellow collection boards were not included and no power was supplied.
5	Non-powered square of e-nets with larger gaps with yellow boards	Square of four e-nets with larger gaps (the wires 24 mm apart) like number 4 but the yellow collection boards were included. The power was not supplied.
6	Non-sticky yellow boards	A square of four non-sticky yellow boards surrounding an artificial pond.

4.2.3.1 Evaluation of the oviposition response of gravid *An. gambiae s.l.* when presented with two equal habitat choices in a semi-field system

This first experiment was to serve as a proof of principle that when presented with two equal habitats, equal proportions of gravid females approach these sites. In this experiment, two similar ponds with detergents were prepared using black round basins of the same size. This control-control test was conducted to examine if similar distribution of the mosquitoes can be attained in the semi-field system.

4.2.3.2 The behavioural responses of the egg-laying females towards functional square of e-nets and its parts

Here the aim was to evaluate if the response of gravid females towards an aquatic habitat is affected by the square of e-nets or by any of its parts. Firstly, a response towards two similar ponds was assessed as a control-control experiment to test if the mosquitoes show similar distribution in two similar set ups. Following this, series of experiments were carried out to study how mosquitoes behave when a square of e-nets or its components are set up around a pond compared to the control (open pond). Accordingly, a pond surrounded by: 1. a functional square of e-nets with yellow boards collection devices, 2. a non-powered square of e-nets without collection boards, 3. a non-powered square of e-nets with wider gaps among the wires (boards not included), 4. a non-powered square of e-nets with wider gaps among the wires (boards included) and 5. four non-sticky yellow boards were compared with a pond without any of those structures.

4.2.3.3 Functional square of e-nets & yellow collection boards

A square of e-nets and detergent were found to be effective tools for catching gravid females in a semi-field system. This experiment was intended to test if a functional square of e-nets surrounding a pond and a detergent treated pond catch similar number of gravid mosquitoes. A powered square of e-nets with yellow collection boards was set up around a detergent pond (Figure 10). The e-nets were powered by connecting them to the batteries via spark boxes. Another detergent pond was prepared as alternative treatment (control).

Figure 10: A complete set up of a square of e-nets

4.2.3.4 Non-powered square of e-nets

The previous experiment indicated that significantly more gravid *An. gambiae* females approached the functional e-net than the detergent pond. To assess whether this has anything to do with the structural component of the functional square of e-nets a square of e-nets without collection boards was set up around a detergent pond (Figure 11). The e-nets were not supplied with power with the aim to allow mosquitoes access the water through the nets. Again this treatment was compared to the catching efficacy of a detergent pond alone.

Figure 11: A square of e-nets set up alone around a pond

4.2.3.5 Non-powered square of e-nets with larger gaps without yellow boards

The distance between adjacent wires of the e-nets is 8 mm. If the tip of a wire is tied to a spring, the next or the adjacent wire is tied to a knot of fishing line (Dugassa *et al.,* 2012). This alternation allows flow of electricity between the two sides of the nets. The preceding experiment showed that the non-powered e-net reduced the number of mosquitoes that would visit a pond surrounded by the nets. Here it was intended to test if the wires create a barrier to mosquito's entry. Two third of the 8 mm spaced wires of the 1 × 0.5 m e-nets were removed leaving a distance of 24 mm between the adjacent wires. Yellow collection boards were not included in this test and the e-nets were not powered. The intention of opening the gaps was to allow entry of the mosquitoes to the aquatic habitat. A pond surrounded by e-net towers and a pond alone were compared.

4.2.3.6 Non-powered square of e-nets with larger gaps with yellow boards

Although there was slight improvement on the number of mosquitoes entering to the pond when the gaps were wider, it was still less than the pond alone. This experiment was intended to assess if inclusion of yellow collection boards in the e-nets with the wider gaps makes any difference on the number of mosquitoes approaching the site. The e-nets were set up along with the yellow boards but they were not connected to a power. The alternative treatment (control) was a detergent pond alone (without e-nets).

4.2.3.7 Non-sticky yellow cardboards

It was evident from the above experiments that the presence of the yellow boards with the e-net setups increased the catch of the detergent pond surrounded by the tool. This experiment was intended to test the effect of the yellow boards (excluding e-nets) arranged around a pond on the mosquitoes' response. In the first treatment, four non-sticky yellow cardboards were placed around a detergent pond (Figure 12). A second treatment was a detergent pond alone. The pond with yellow collection boards and the pond alone were set up in the opposite corners of a semi-field system randomly. The catch sizes of the ponds were compared.

Figure 12: Non-sticky yellow boards placed around a detergent pond

4.2.4 Data analysis

The data were analysed using generalized linear mixed effects model. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht, multcomp, (Team 2011). The night of experiment (same batch of mosquitoes) and location (site) where the traps were placed in the semi-field system were included in the models as random effects. The control (the detergent pond alone) and the treatments (the detergent pond surrounded by a square of e-nets or its components) were entered as fixed effects. Here, the proportions of mosquitoes collected in the control and the treatments were modelled and the odds ratios of finding a mosquito in the treatment were compared with the odds ratio of the two similar ponds. A binomial distribution of the data and a *logit* link function were used in the model. The excess variation between data points (overdispersion) that remained after adjustment for all other factors was adjusted by creating a random factor with a different level for each row of the data set (rowid<-factor(1:nrow(rdataset))). The parameter estimates of the models were used to predict the mean percentages per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.,* 2005). Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

4.3 Results

4.3.1 Response of the gravid mosquitoes towards two similar detergent ponds

This study validates the implementation of two choice experiments in the semi-field system by confirming that when presented with two equal choices randomly labelled as treatment and control gravid *Anopheles* females select both ponds in an equal proportion (Figure 5). It also confirms previous observations (Dugassa *et al.,* 2012) that a detergent pond is an effective tool to study the oviposition behaviour of *An. gambiae s.l.* Approximately one quarter of the released mosquitoes drowned per pond when settling on the water surface for oviposition.

4.3.2 Functional square of e-nets

Comparing the proportion of gravid females approaching a pond using a square of e-nets with the proportion of females caught in a detergent pond significantly differed from the fifty-fifty distribution obtained in the two similar ponds (Figure 10). A gravid female was 1.7 times more likely to be collected in the treatment in this comparison than it was when a choice of two detergent ponds was given. Two reasons could have been responsible for that. Either gravid females explore aquatic habitats first before they make a decision to land, therefore we would measure a larger number approaching with the e-nets than landing with the detergent pond, or the square of e-nets includes structural components that make the potential oviposition site more visible or attractive for gravid females. To explore this, the following tests were implemented.

4.3.3 Non-powered square of e-nets

The proportional distribution of mosquitoes collected in the comparison of non-powered square of e-nets surrounding a detergent pond and a detergent pond alone was significantly different from the distribution in the two similar ponds. A significantly lower proportion of gravid females were collected in the treatment suggesting that the square of e-net surrounding the detergent pond either deterred the gravid females or prevented them from entering through the wires to the water (20.6% 95%CI 14.6% – 28.2%) (Figure 10).

4.3.4 Non-powered square of e-nets with larger gaps without yellow boards

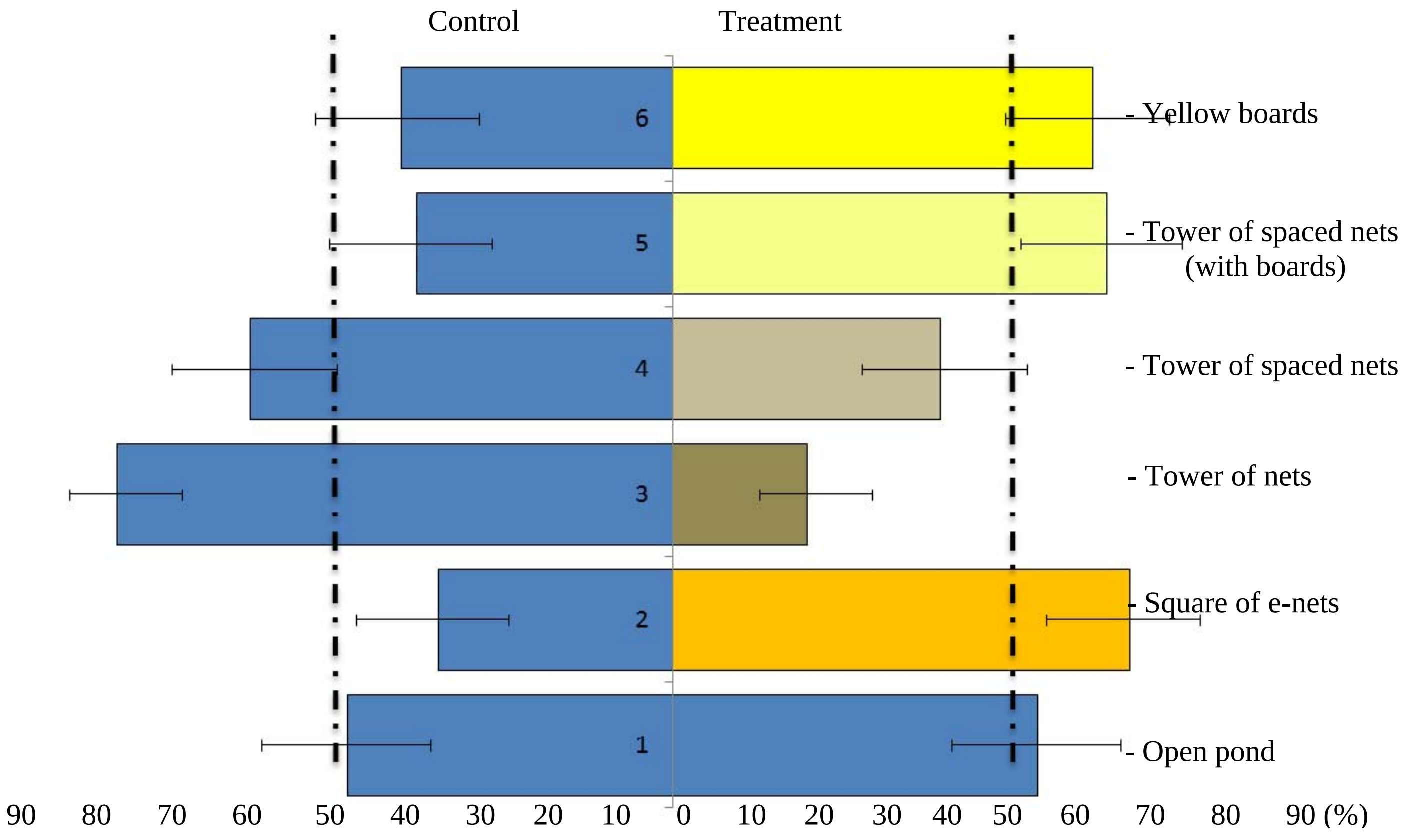
Increasing the gaps among the wires of the e-nets increased the probability of a female being collected in the detergent pond within the square of e-nets 2.5 fold (OR 2.6 95%CI 1.6 – 4.2; $p<0.001$) as compared to the previous setting. However, the proportional distribution between treatment and control in this experiment was still significantly different from the control-control experiment suggesting that the presence of the wires still lead to a certain amount of avoidance by gravid females (Figure 10).

4.3.5 Non-powered square of e-nets with larger gaps with yellow boards

The inclusion of the yellow boards to the previous setup increased the proportion recollected in the treatment by three fold (OR 2.7 95%CI 1.7 – 4.3; $p < 0.001$) as compared to when the boards were not included. This suggests that more mosquitoes were attracted towards the pond that included the square of e-nets and the yellow boards and consequently entered through the wires to reach the pond for oviposition. In this experiment the yellow boards were sticky and when including the females that were collected sitting on the yellow boards outside the square of e-nets the proportion of females collected in the treatment sites increased even more compared to the control (OR 1.9 95%CI 1.2 – 3.0).

4.3.6 Non-sticky yellow boards

The previous experiments indicated the yellow boards associated with the e-net setup to be responsible for the increased approach towards a pond. Therefore, in a final step only yellow boards (non-sticky) were tested. Even though the distribution of collected females did not significantly differ from the control-control experiment due to a high variability in catch rates between sites (variance of random factor = 0.2) it is important to note that with yellow boards alone the same response was recorded towards the treatment as in the previous experiment with the square of e-nets present and in the experiment with a functional e-net. A higher proportion of females (60.6% 95%CI 48.1 – 71.8) were collected in the pond surrounded by non-sticky yellow boards compared with the detergent pond alone (Figure 13).



Mean (95%CI)	**OR(95% CI)**	**p-value**
60.6 (48.1 – 71.8)	1.4 (0.9 – 2.2)[ab]	0.173
62.8 (50.3 – 73.7)	1.5 (1.0 – 2.4)[ab]	0.079
38.7 (27.4 – 51.2)	0.6 (0.4 – 0.9)[d]	0.015
19.4 (12.5 – 28.9)	0.2 (0.1 – 0.3)[c]	<0.001
66.0 (54.0 – 76.3)	1.7 (1.1 – 2.7)[b]	0.017
52.8 (40.3 – 64.9)	1[a]	–

Figure 13: The percentage of mosquitoes collected in the detergent ponds and the various treatments

4.4 Discussion

The proportion of gravid females collected in randomly allocated control and treatment sites over a 12 night experimental period was similar when two similar choices were presented to the released females. The functional square of e-nets collected about twice the proportion of mosquitoes collected in the detergent pond alone. A non-powered square of e-nets without collection devices set up around one of the ponds reduced the number of mosquitoes visiting the artificial habitat by over three quarters. Although opening the gap of the wires improved entry of the mosquitoes, it still reduced the proportion of mosquitoes that would visit the pond. However, inclusion of the yellow collection boards in the setups increased the catch size of the squares of e-nets. Here, it was investigated that a combination of yellow cardboards and a black pond in the centre of these boards attracted more mosquitoes to the site.

Based on the results it is most likely that the wires of non-powered e-nets surrounding a pond prevented the mosquitoes from entering through the wires. When there is no power on the e-nets, the mosquitoes hit the wires while flying towards the pond and then fly away between the wires in a zigzag fashion (personal observation). Torr *et al.,* (Torr *et al.,* 2008) also suggested that the fine nets (0.2 mm in diameter and 8 mm apart) prevent insects from flying straight through the e-nets. The wires more likely obstruct the mosquitoes from accessing the artificial pond. Even increasing the gaps among the wires did not result in similar distribution in the pond surrounded by the e-nets and the detergent pond alone. The sparse wires might still create barrier to the mosquitoes. However, inclusion of the yellow collection boards with the setup and surrounding a pond with non-sticky yellow boards apparently increased the proportion of mosquitoes responding to the artificial habitats. It is more likely that the combination of the yellow boards and the central black pond formed a contrast that was preferred by the egg laying females. The surrounding grey sandy floor of the semi-field system might have also contributed to the contrast formation. Previous studies showed that these mosquitoes are attracted to contrasts of colours (Bentley and Day 1989; Huang *et al.,* 2005; Huang *et al.,* 2007). They might be visually directed towards the combination of the yellow boards and the pond from distance but went for the pond when they reached the aquatic habitat. Reports also show that visual cues are among the essential cues for many mosquitoes especially for the day active species to choose a habitat for egg-laying or to locate hosts (Collins and Blackwell 2000; Bentley and Day 1989; Huang *et al.,* 2006b; Bernáth *et al.,* 2012; Huang *et al.,* 2007). Those mosquitoes might be attracted to the contrast from distance and then landed on the pond not on the yellow boards up on arrival at the site. It could be questioned here if mosquitoes show similar behaviour to tsetse flies that are attracted to a blue colour of a trap from distance and then to the black colour for landing when they arrive at the trap. The current study is consistent with previous work (Dugassa *et al.,* 2012) which showed that these mosquitoes laid greatest number of eggs in the pond next to the yellow board as compared to the shiny sticky board and the board with netting and insect glue. However, they hardly landed on the yellow boards unlike the latter two which seem to mimic a water body due to their reflective surface. Although the number of eggs laid in the ponds next to the boards mounted with transparent sticky film and black netting gauze with glue could be reduced due to landing and sticking of the mosquitoes on the surfaces, the mosquitoes seemingly approached all the three ponds similarly. The yellow boards might have formed a contrast that attracts the mosquitoes. The importance of reflectance of a surface in attracting gravid mosquitoes was reported in various studies (Kennedy 1942; McCrae 1984; Bentley and Day 1989; Bernáth *et al.,* 2004; Bernáth *et al.,* 2008; Bernáth *et al.,* 2012). There is also possibility that these boards serve as 'oviposition markers' (i.e. informative of the presence of preferred egg-laying site) (Bentley and Day 1989; Huang *et al.,* 2007; McCrae 1984) when they are placed next to a pond. Studies showed that mosquitoes in the mating physiological state use markers for swarming (Charlwood *et al.,* 2002). Mosquitoes in host-seeking physiological states also to some extent rely on visual cues to locate hosts (Bentley and Day 1989; Allan *et al.,* 1987; Bowen 1991).

With the use of a functional square of e-nets the mosquitoes may not get a chance of approaching the site and later explore other alternative sites because they get electrocuted once they touch the wires. In addition to this the setup attracts more mosquitoes. These might be the factors that led to the high catch size with the functional square of e-nets. The non-powered e-nets with wider gaps (no boards included) recollected less proportion of mosquitoes than the detergent pond alone in contrast to the non-powered square of e-nets with wider gaps with boards which caught twice that of the pond alone. The mosquitoes might frequently visit the site because of its attractiveness of the contrast.

It should be noted that a bias could be introduced when a square of e-nets is used to study behavioural responses of mosquitoes. If efficacy of a trap is intended to be compared with a square of e-nets catch, it will not be a fair comparison as the square of e-nets setup attracts more mosquitoes. Thus, if a given trap's efficacy is required to be evaluated in terms of estimated number mosquitoes approached the trap; a square of e-nets may lead to over estimation of the mosquitoes approaching the trap. However, like in the study by Dugassa *et al.* (Dugassa *et al.*, 2013) the way mosquitoes behave around a trap could be studied using similar setups of e–nets. A catch size of a treatment surrounded by the square of e-nets without power would be different from a treatment with the square of e-nets with power. Therefore, choice experiments could result in bias if a non-powered square of e-nets is used with one treatment and a powered square of e-nets is used with the other treatment. A choice experiment with square of e-nets with one treatment only would have similar limitation. However, the attractiveness or repellence of two different treatments could be assessed by using a functional setup of square of e-nets with power in all involving treatments.

4.5 Conclusion

The setup of a square of e-nets enhances attractiveness of a pond for gravid *An. gambiae s.l.* due to the contrast introduced by surrounding the dark habitat with light-coloured boards. This effect of the trapping tool must be taken into consideration when designing experiments to avoid introducing collection bias. In choice experiments, both habitats must be surrounded by the same setup for mosquito collections to be comparable. When the aim is to compare the approach versus landing and the e-nets on one side would not be electrified it is important to reduce the gaps between the wires on those e-net to reduce the physical barrier created by those wires. Both habitat choices need to be surrounded by light-coloured boards to make both habitats equally visual. Whilst the attractiveness of the boards might present an obstacle is experimental studies, it could be highly beneficial for mosquito collections in the field. Conspicuous functional squares of e-nets might be an effective tool in the field trying to intercept the gravid females on their way from their resting site close to the host to the aquatic habitat. Furthermore, contrasting elements might be included in the previously developed OviART gravid trap to increase their visual attraction.

CHAPTER 5: DEVELOPMENT OF A GRAVID TRAP FOR COLLECTING LIVE MALARIA VECTORS *Anopheles gambiae s.l.*

5.1. Background

Vector control plays a central role in the prevention of malaria (Greenwood 2008; Enayati *et al.,* 2009; Mendis *et al.,* 2009; Mutero *et al.,* 2012; WHO 2006b). Monitoring vector populations and assessment of disease risk are among the key elements of vector management strategies (WHO 2009a, 2004, 2012c; Beier *et al.,* 2008). So far various tools have been developed and utilized for sampling mosquito vectors and the pathogens they transmit (Service 1977; Oyewole *et al.,* 2007; Mahande *et al.,* 2007; Odiere 2007). In sub-Saharan Africa (SSA) the most commonly used sampling methods for mosquitoes are human landing catches, CDC light traps, and pyrethrum spray collections which are excellent for sampling mosquitoes indoors (Dia *et al.,* 2005; Ndiath *et al.,* 2011; Duo-quan *et al.,* 2012). Effective vector control targeting indoor host-seeking mosquitoes has resulted in a reduction in the number of mosquitoes entering and resting in houses (Bayoh *et al.,* 2010; Derua *et al.,* 2012; Tirados *et al.,* 2006; Russell *et al.,* 2011; Takken 2002; Oyewole *et al.,* 2007; Reddy *et al.,* 2011; Malima *et al.,* 2009; Stevenson *et al.,* 2012) rendering these tools less effective for monitoring potential vector populations (Fillinger *et al.,* 2008).

Outdoor vector collections become increasingly important as surveillance tools; and effective traps might even be used for control purposes (Mukabana *et al.,* 2012a). To date outdoor vector collections target either resting populations with pit traps (Muirhead-Thomson 1958; Andrianaivolambo *et al.,* 2010), pot traps (Odiere 2007), resting boxes (Kweka *et al.,* 2009) and aspirator collections or host-seeking mosquitoes with animal-baited traps (Odiere 2007; Knols and Farenhorst 2009; van den Bijllaardta *et al.,* 2009), human baited tent traps (Dia *et al.,* 2005) and, more recently, odour-baited MM-X traps (Qiu *et al.,* 2007). In general, outdoor sampling is far more challenging than indoor sampling since the outdoor vector population is more dispersed over the landscape. Resting catches often underestimate actual vector densities (Tirados *et al.,* 2006) and odour-baited traps only target a proportion of the host-seeking population. Furthermore, animal or human-baited traps are complicated to organise and are inappropriate for using on a large scale.

Gravid traps are used routinely for the surveillance of *Culex* and *Aedes* vectors (Scott *et al.,* 2001; Williams and Gingrich 2007) but few have attempted to develop a gravid trap for *Anopheles gambiae s.l.* (Harris *et al.,* 2011) the major malaria vector in SSA. Gravid mosquito traps are designed to catch gravid females in search of an aquatic habitat. The first was developed by Reiter in 1983 (Reiter 1983) to collect gravid *Culex* and *Aedes* mosquitoes for West Nile virus isolation (Allan and Kline 2004; Scott *et al.,* 2001; Mboera *et al.,* 2000) and many gravid mosquito traps have been developed and modified since then (Braks and Carde 2007; Allan and Kline 2004; Dennett *et al.,* 2007). Among these, the CDC gravid trap Model 1712, commonly referred to as CDC gravid trap, the CDC gravid trap Model 1719 commonly referred to as Frommer updraft gravid trap and the Box gravid trap (also referred to as Reiter-Cummings gravid trap) (Allan and Kline 2004) are commercially available and widely used for the collection of gravid culicines (Allan and Kline 2004; Braks and Carde 2007). However, to my knowledge, none of these have been purposely evaluated for collecting neither *An. gambiae s.l. nor* anophelines in general. Trapping gravid malaria vectors may be an alternative method for sampling both endophilic and exophilic vector populations in search of an oviposition site and here we aimed to investigate the factors that impact on the catching efficiency of these commercially available traps. Based on the results a new prototype gravid trap for the collection of malaria vectors was developed.

5.2. Materials and Methods

5.2.1 Study area

The study was carried out in a semi-field system (described under section 2.1.1.; Figures 1and 2).

5.2.2 The study design

Experiments were conducted using randomized complete block designs (RCBD) and replicated for 12 nights. The number of replications was based on sample size considerations for comparing proportions of clustered data (Hayes and Bennett 1999). The preliminary data for this were generated by setting two identical artificial ponds in opposite corners of the semi-field system. The water in the ponds was treated with detergent as previously described in detail (Dugassa *et al.,* 2012) . Two hundred gravid females were released in the evening and the number of mosquitoes drowned in each pond was counted the next morning. This was done for 12 nights. The results showed that when presented with an identical treatment the gravid females approached both ponds in an equal proportion (p1=0.5). The variability of the nightly catches was used to calculate the coefficient of variation (ratio of standard deviation/mean) which was 0.26. At this variation replication of the experiment over 12 nights assuming 100 responders out of 200 released mosquitoes per night had 80% power to detect an increase or decrease in the catch rate of 20% (p2=0.7) at the 5% level of significance. This level of accuracy was deemed appropriate for developing new traps for gravid *An. gambiae s.l.*

5.2.3 Mosquitoes

Gravid mosquitoes were prepared from insectary-reared *An. gambiae s.l.* (description provided under section 2.3.1.).

5.2.4 Gravid Traps

The Box gravid trap (BioQuip, Rancho Dominguez, CA), CDC gravid trap (John W. Hock Company, Gainesville, FL)) and Frommer updraft gravid trap (John W. Hock Company, Gainesville, FL) were used in this study. These traps attract egg-laying females to a water-based oviposition medium added to bowls below the trapping device. The bowls size varied slightly between different trap models. According to the manufacturers’ recommendations oviposition medium was filled in the bowls to a level of about 3 cm below the opening of the intake ducts. This is equivalent to 8 L of oviposition medium in the Box gravid trap, 6 L in the CDC and 5 L in the Frommer trap. All traps operate by drawing air from the surface of the bowls and distributing any volatile chemicals associated with the oviposition medium, including water vapour, to the surrounding of the traps. Depending on the design of the trap and the location of the air intake duct, the air plume varies amongst the traps (Braks and Carde 2007; Irish *et al.,* 2012). Mosquitoes are sucked into a collection chamber while they evaluate the potential oviposition site and prepare to lay eggs. The collection chambers are found on top of a suction tube in the Box and Frommer updraft gravid traps to avoid exposure of mosquitoes to the aspiration fan. However, the collection chamber of the CDC gravid trap is placed above the aspiration fan (Allan and Kline 2004; Braks and Carde 2007) so that some mosquitoes are damaged by the rotating fan (Russell and Hunter 2010; Reiter 1987). The water basins of the traps are usually placed on the ground for collection of *Aedes* and *Culex* mosquitoes (Figure 14A, B, C) which will readily lay eggs in containers. However, *An. gambiae s.l.* usually prefers natural habitats so here we aimed to reduce the container impression by sinking the bowls in to the ground for all experiments, where we refer to the water-filled bowls as ponds (Figure 14D). The Box gravid trap was set up by fitting the anti-spread bars that are found under the horizontal exhaust tube to the black bowl (16.5 L volume, 44 cm long, 34 cm wide and 12 cm deep). The black conducting duct with the large ‘O’ ring around it was placed into the hole at the bottom of the case. The stockinet of the collecting chamber was placed on the outside of the collecting duct with the intake screen facing the exhaust tube. The CDC gravid trap was set up by placing the aluminium supports of the trap on the rim of the pan (24 L volume, 44 cm long, 34 cm wide and 17 cm deep) and slipping the collection bag over the upright tube. The sleeve was slipped downwards towards the aluminium supports until the bottom of the bag rested on the top end of the trap. Setting up the Frommer updraft gravid trap involved fitting the rain shield with aspiration fan to the trap and setting it into the base stand. The parts were attached tightly. The base stand was placed in the black pan (24 L volume, 44 m long, 34 cm wide and 17 cm deep) so that the feet rested inside the tray.

For all experiments piped non-chlorinated water from Lake Victoria was filtered through a sand-charcoal filter and used in the containers of the traps as oviposition medium. All the gravid traps were operated by a fully charged 6 volt 12 Ah battery (Universal battery UB6120). Experiments were started at 17.30 h by releasing 200 gravid mosquitoes at the centre (site 5) of the semi-field system and stopped at 8.00 h the following day. The collection chambers from the traps were collected in the morning and kept in a freezer for 30 minutes to kill the mosquitoes.

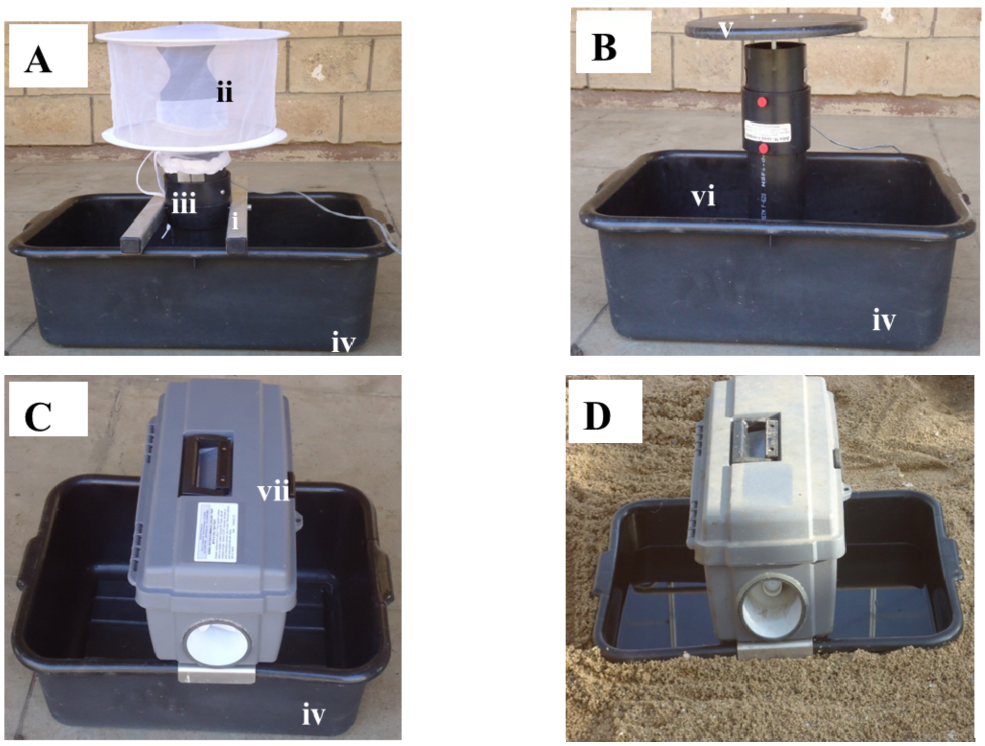


Figure 14: Set up of three gravid traps: A) CDC gravid trap; i. aluminium supports, ii. collection bag, iii. upright tube, iv. pan B) Frommer updraft gravid trap; v. rain shield, vi. base stand, iv. pan and C) Box gravid trap; vii. horizontal exhaust tube, viii. anti-spread bar, iv. Pan D) Box gravid trap setup

5.2.5 Experiments

5.2.5.1 Trap comparison

In the first experiment the trapping efficacies of the Box, CDC and Frommer updraft gravid traps were compared. In addition to the three traps an open pond made of a similar bowl but without a trapping device was set up in the semi-field system. The open pond was positioned in the same site each night (site 4). Traps were rotated over the 12 nights between sites 1, 2 and 3. The purpose of the open pond was to serve as a reference to compare mosquito responses from night to night and to compare the relative attractiveness of a ‘natural’ water body with ones that had gravid traps. The number of mosquitoes trapped in the collection chambers of the traps and the number of eggs laid in each pond was recorded nightly.

5.2.5.2 Trapping efficacy of the Box gravid trap

After the first experiment the Box gravid trap was chosen for further evaluation to investigate factors that might affect catch size. Experiments were designed to assess if the position of the Box trap on top of the pond or the sound of the fan affected the number of gravid females that approach the trap. In the following experiments, 8L filtered-lake water was used to prepare the ponds.

The first experiment had a fully-functional Box gravid trap in one corner of the semi-field system (Figure 15A) and an open pond (pond alone without trapping device) in the opposite corner (Figure 15B). In the second experiment the fan of the Box gravid trap was switched off to assess if the sound of the trap affected the number of gravid *Anopheles* mosquitoes approaching the pond. Here we compared a non-functional Box trap with an open pond in the opposite corner of the semi-field system. In both experiments treatments were rotated between all sites (site 1-4). To analyse the orientation of gravid females towards either of the ponds they were surrounded by a complete square of electrocuting nets (Figure 15). The adjacent wires of the nets were powered by a 12 V 50 Ah lead acid battery (Chloride Exide Ltd, Kenya) via a spark box (Alan Cullis, South Africa) adjusted to a low spark energy setting that did not produce any sound or spark but killed the mosquitoes that touched the net while approaching the pond (Dugassa *et al.,* 2012). Yellow sticky films mounted on strips of cardboard served as collection boards (Figure 15). These were placed under each net outside the closed square (50 x 60 cm) and in the gaps between the two longer sides of the bowls and the net (53 x 7 cm). The number of mosquitoes approaching either pond was estimated by counting the number on the net and on the collection boards.

Figure 15: Set up of the fully operating Box gravid trap (A) and the open pond (B) surrounded by a square of electrocuting nets

5.2.5.3 Development of a new gravid trap (OviART gravid trap)

Results from the previous experiments indicated that the presence of a Box on top of a pond affected the number of gravid females approaching this pond. Therefore, we moved the Box trap from directly above the pond to one side of the pond. The trap was positioned 50 cm from the edge of a pond and compared with an open artificial pond. The trap was not switched on as the aim was to test if the presence of a box close to the pond would also deter mosquitoes (Dugassa *et al.,* 2012). To quantify the number of mosquitoes visiting the ponds 200 ml detergent (Teepol Industries LTD, Nairobi) was added to the water.

Based on our findings we constructed a new prototype gravid trap (named 'OviART gravid trap) where the collection chamber and fan were positioned on one side of the pond (Figure 16). A black round bucket (20 cm high and 30 cm in diameter) filled with 8 L of filtered-lake water served as oviposition site. An oval slit (13 cm wide and 5 cm high) was cut 5 cm below the lip of the bucket into which a collapsible pipe (30 cm long and 10.2 cm in diameter) was inserted. This pipe was connected to a collection chamber made out of a water plastic bottle as described below. At the end of the collection chamber another 30 cm collapsible pipe and a fan of 12 V and 0.38 Ah current output (as opposed to 6 V and 0.1 Ah of the Box gravid trap) was fixed to create strong air suction. A strong suction of air from the entire water surface was needed to compensate for the reduction in air flow as a result of moving the suction point from above the pond to the side. The fan sucked air into a collection chamber (20 cm long and 10 cm in diameter) which was prepared from a plastic water bottle of 1 L volume and black fiberglass netting gauze (1.7 mm × 1.5 mm mesh size). A piece of netting gauze (15 cmx15 cm) was cut and prepared into a conical funnel with a 2.5 cm wide hole at the narrower end and 10 cm at the wider end. It was then fixed at the inlet side of the water bottle with the narrower opening of the funnel positioned inside the bottle. This narrow inlet minimized risk of escape of mosquitoes even when the power stopped due to battery failure. Another piece of the gauze (18 cm × 18 cm) was cut and tied to the opposite side of the bottle towards the fan (Figure 16).

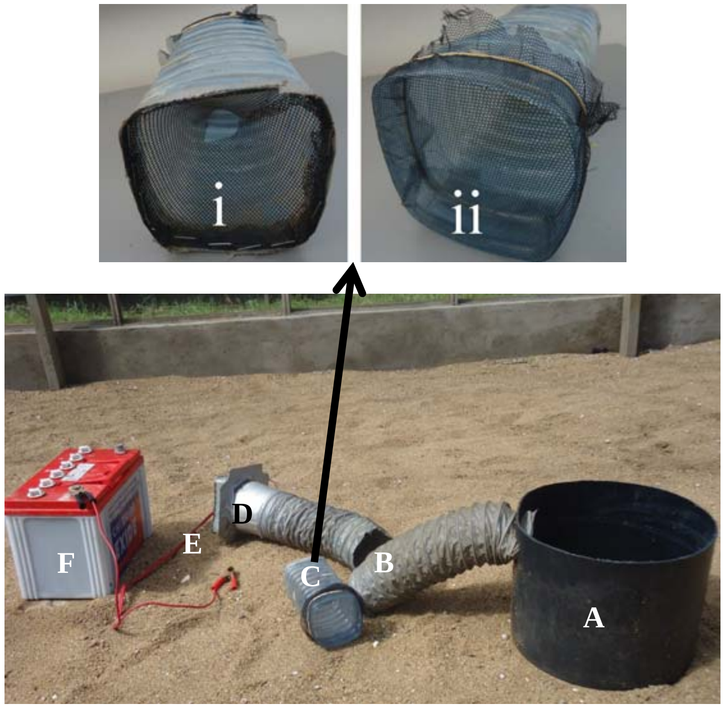


Figure 16: Ovi-ART gravid trap prototype: A) Round black bucket B) Suction tube prepared from collapsible plastic pipe C) Collection chamber (i. conical net at inlet side ii. removable net at outlet side) D) Fan (12 V, 0.38 A) E) Electric cable F) 12 V battery

The OviART trap was set by sinking the water-filled bucket into the ground so that the lip of the bucket was flush with the sand surface. The suction tube was buried in the sand leaving only the end with the fan exposed above the soil in order to let air flow freely (Figure 17). Gravid mosquitoes passed through this tube into the collection chamber. The fan was powered by a 12 V 50 Ah lead acid battery (Chloride Exide Ltd, Kenya). The trapping efficacy of the OviART gravid trap was compared with the Box gravid trap in two choice bioassays. The number of mosquitoes caught in the traps and the number of eggs in the ponds was recorded. In a final step a single OviART prototype trap was tested nightly for 12 nights in a semi-field system. The intention was to determine the proportion of released *An. gambiae s.l.* that could be collected by the trap. The trap was rotated randomly between all four sites in the semi-field system. The number of mosquitoes trapped was recorded.

Figure 17: The OviART gravid trap set up, A) the sedge of the bucket at the level of sand B) Water level and inlet of the suction duct

5.2.6 Data analysis

The data were analysed using generalized linear mixed effects models. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht and multcomp (Team 2011). Previous experiments (Dugassa *et al.,* 2012) have shown that mosquito responses are highly variable between different batches of mosquitoes and between different sites in the greenhouse irrespective of the test treatments. Therefore, the night of experiment (same batch of mosquitoes) and location (site) where the traps were placed in the semi-field system were included in the models as random factors. To adjust for excess variation between rows (data points) recording the number of trapped mosquitoes (over dispersion) a factor was created with a different level for each row of the data set and also included as a random factor in the model. The experimental treatments were entered as fixed effects. A Poisson distribution of the data and an exchangeable working correlation matrix were used. All mean counts per treatment and their 95% confidence intervals (CIs) were calculated using the parameter estimates of the models by removing the intercept from the models (Seavy *et al.*, 2005). Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

5.3. Results

5.3.1 Trap comparison

An. gambiae s.l. females were caught in all three traps in the semi-field system (Figure 18), but the total mean number trapped per night was low (59.3, 95% CI 50.3 – 70.0) i.e. <30% of released mosquitoes were recovered by the three traps. The Box gravid trap and the CDC gravid trap collected similar numbers of mosquitoes (OR 0.8, 95% CI 0.6 – 1.2; p= 0.284). In contrast, it was 70% less likely to collect a mosquito with the Frommer updraft gravid trap (OR 0.3, 95 % CI 0.2 – 0.5; p < 0.001) compared with the other two traps (Figure 6). On average, 858 (95% CI 570 - 1291) eggs were collected from the open pond per day, indicating that the low catch numbers in the traps were not because released mosquitoes did not search for oviposition sites. From this result it appears that the females preferred to approach the open pond rather than a pond with a trap on top. Only few and similar numbers of eggs were found in ponds with traps (average for all three traps combined 125.1 (95% CI 82.8 – 189.0)). This implies that most females that approached the pond were sucked into the trap before getting an opportunity to lay eggs, so low catch sizes are probably not due to weak suction of the fans.

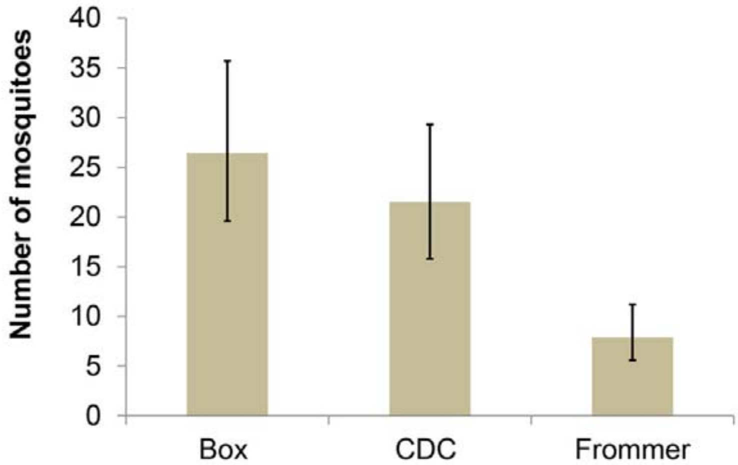


Figure 18: Mean *Anopheles gambiae s.l.* catch sizes of CDC, Frommer updraft and Box gravid traps.

5.3.2 Trapping efficacy of the Box gravid trap

Based on the trap comparisons the Box gravid trap was selected for further evaluation in the following series of experiments since it caught the greatest number of mosquitoes and provided protection for battery, cables and mosquitoes which would be an added advantage when used in the field during wet weather (Allan and Kline 2004; Braks and Carde 2007).

The number of mosquitoes approaching the Box gravid trap was reduced by 30% compared to the number that approached the open pond, irrespective of whether the trap was switched on, creating a distinct sound, or switched off and silent (Table 8). This suggested that the presence of the Box on top of the pond deterred mosquitoes from approaching the site and led to the next experiment.

5.3.3 Development of a new gravid trap

A test was designed where the number of mosquitoes that visited a pond with a Box gravid trap set next to it was compared with a pond without a trap. Similar numbers of females visited the two ponds (Table 8).

Based on the analyses of factors that affected the approach of gravid *An. gambiae s.l.* to a Box trap a prototype of a new trap (OviART gravid trap) was developed. Here the catching device was moved to the side of the pond; 60% more *An. gambiae s.l.* were collected by the OviART gravid trap prototype than the Box gravid trap (Table 8). A large difference was found in the egg numbers recovered from the ponds of the two traps. Eggs were only found on three out of the 12 collection nights in the pond of the new OviART trap (in total 87 eggs). In contrast, eggs were found nightly and nearly 19 times more eggs (total 1652) were laid in the pond of the Box gravid trap over the 12 night's period. When evaluated alone, the OviART prototype recollected approximately one third of the released of mosquitoes in the one choice bioassay (31.9%, 95% CI 20.4- 46.4%).

Table 8: Results of the statistical analyses of the individual experiments implemented to develop a new gravid trap for *Anopheles gambiae s.l.*

Treatment	**Mean (95% CI)***	**OR (95% CI)**	**p-value**
Response of gravid *An. gambiae s.l.* to a pond with functional Box gravid trap			
Pond only	62.1 (40.2 – 95.8)	1	-
Trap over pond	40.1 (25.9 – 62.1)	0.7 (0.6 – 0.7)	< 0.001
Response of gravid *An. gambiae s.l.* to a pond with a soundless trap			
Pond only	51.2 (39.6 – 66.1)	1	-
Soundless trap over pond	36.9 (28.4 – 47.9)	0.7 (0.6 – 0.8)	<0.001
Response of gravid *An. gambiae s.l.* to a pond with a Box gravid trap next to it			
Pond only	32.5 (22.8 – 46.5)	1	-
Trap next to pond	33.4 (23.4 – 47.7)	1.0 (0.9 – 1.2)	0.693
Comparison of the prototype OviART gravid trap and the Box gravid trap			
Box gravid trap	25.2 (19.1 – 33.3)	1	-
New gravid trap	41.3 (31.6 – 53.9)	1.6 (1.2 – 2.2)	0.001

CI=confidence interval, OR=odds ratio

*predicted by using the parameter estimates of the mixed effects model

5.4. Discussion

In the situation where gravid *An. gambiae s.l.* females had a choice to oviposit in an open pond or in ponds with a trap on top <30% of the released mosquitoes were collected by the three commercially available gravid traps in the semi-field system. The Box and CDC gravid traps showed similar efficacy whilst the Frommer updraft gravid trap trapped relatively few mosquitoes. The extremely low efficacy of the Frommer updraft gravid trap may be due to its physical features since the base of this trap stands inside the water-filled basin. To be trapped, mosquitoes have to fly under the base of the trap that is only about 3 cm above the water surface. The lower volume of water as compared to the other traps might have also contributed to the lower catch size. The low efficacy of this trap is consistent with recent observations by Irish *et al.* for *Culex quinquefasciatus* (Irish *et al.*, 2012).

It is interesting to note that the overall catching efficacy of the CDC and Box gravid trap under semi-field conditions falls into the same range as reported for gravid culicine mosquitoes where recollections were highly variable and between 22% and 63% of the released mosquitoes (Allan and Kline 2004; Ritchie 1984; Reiter *et al.*, 1986). However, it is difficult to make direct comparisons between the efficacy of these traps for culicine and anopheline mosquitoes since (1) our semi-field system had a much greater volume than those used for culicines (Braks and Carde 2007; Allan and Kline 2004), (2) unlike the experiments for culicines which use attractive infusions there are no known attractants for *An. gambiae s.l.*, and (3) in our trap comparison the open pond competed with the traps and therefore might have diverted a proportion of the mosquitoes and reduced the number of mosquitoes approaching each trap.

There are very few reports of the trapping efficacy of gravid traps for collection of *An. gambiae s.l.* in the field and surprisingly none of these were set intending to collect malaria vectors. Nevertheless, there is a notion that commercially available gravid traps might be less suitable for anophelines than for culicines based on the actual trapping results (Irish *et al.*, 2012; Muturi *et al.*, 2007). This has to be cautiously interpreted because most gravid traps are used to collect culicine females and were baited with a range of fermented plant infusions which might repel malaria vectors which are generally associated with less strongly polluted water (Minakawa *et al.,* 1999; Mutuku *et al.,* 2006; Sumba *et al.,* 2008). The only indication that it might be possible to sample malaria vectors with commercially available gravid traps comes from the work of Muturi and colleagues (Muturi *et al.,* 2007). In outdoor collections in a rice agro-ecosystem they collected approximately 5-6 gravid anophelines each night with a grass-infusion baited CDC gravid trap compared with 18-20 host-seeking anophelines in a CO_2-baited CDC light trap (Muturi *et al.,* 2007). Since host-seeking collections are usually higher than others [19], this ratio is encouraging for the development of a gravid trap for malaria vectors. Fresh water instead of grass-infusion might have increased the trapping result for anophelines in this study.

In our study the Box gravid trap was selected for further evaluation since its compact design meant that the internal parts were well protected from the elements, which would be an advantage during the rainy seasons, but it was found that the approach of gravid females was significantly reduced when the Box trap was positioned directly over a pond, compared to a pond alone. This effect was not due to the sound of the trap and the removal of the trapping device off the pond confirmed that the females were visually deterred by the presence of the trapping device directly on the oviposition medium.

Previous work has shown that *An. gambiae* mosquitoes first evaluate a potential larval habitat before making a decision to lay eggs (Sumba *et al.,* 2008; Huang *et al.,* 2005; Munga *et al.,* 2005; Warburg *et al.,* 2011; Wachira *et al.,* 2010; Muirhead Thomson 1946). The decision to lay eggs might be based on visual or chemical cues or a combination of both (Bentley and Day 1989). Mosquitoes in flight depend on optical inputs to orient themselves, identify and access a target (Allan *et al.,* 1987; Bidlingmayer 1975; Browne 1981; Gillies and Wilkes 1982). Visual cues are believed to be long range cues important for gravid mosquitoes to identify different habitats and specific oviposition site characteristics before they evaluate the habitat using chemical signals received by olfactory receptors, hygroreceptors and contact chemoreceptors (Bentley and Day 1989; Hoel *et al.,* 2011; McCrae 1984; Kennedy 1942; Eguchi and Tominaga 1999). The visual parameters include shape, size, contrast, light quality and intensity, texture and colour of a pond (Muirhead Thomson 1946; Bentley and Day 1989; Collins and Blackwell 2000; Huang *et al.,* 2005; Huang *et al.,* 2006b; Bernáth *et al.,* 2004; Bernáth *et al.,* 2008; Bernáth *et al.,* 2012). *An. gambiae s.l.* prefers open sunlit habitats (Gillies and DeMeillon 1968; Gimnig *et al.,* 2001; Minakawa *et al.,* 2002; Munga *et al.,* 2005) and avoids habitats densely covered by vegetation that create obstacles to oviposition (Fillinger *et al.,* 2009; Minakawa *et al.,* 1999; Muirhead Thomson 1946). A recent study suggested that shiny sticky film attracted *An. gambiae s.l.* due to its close resemblance to water (Dugassa *et al.,* 2012).

The most likely reasons that the Box trap does not attract many gravid females are because the pond is too shady and the large trap over the water surface impedes their pre-oviposition flight. The new OviART gravid trap provided female *An. gambiae s.l.* with an open oviposition site which improved the catch size by 60% compared to the Box gravid trap. When the OviART trap was evaluated alone, approximately one third of all released mosquitoes were trapped. This corresponds well with observations we made when studying gravid females' approach towards an artificial pond with a complete square of electrocuting nets [42]. The absence of eggs in the pond of the new trap during most of the nights also indicates that the great majority of the females that approach the ponds with the intention to lay eggs got sucked into the collection chamber.

The disadvantage of the OviART prototype trap is that the collection device and the battery are less protected from the elements compared to the Box trap. Nevertheless, modifications might be possible to improve the design by providing a casing for both the collection device and battery. Furthermore, to power the stronger fan needed to suck mosquitoes from the entire water surface a larger battery was required that makes the trap more difficult to transport and increases the risk of theft when used outdoors. The strong suction in the trap, forcing mosquitoes into the netting at speed, probably contributed to the large number of dead mosquitoes found in the trap. Since collection of undamaged gravid females is advantageous for the isolation of a number pathogens (other than malaria parasites that can be identified from dry specimen) (Russell and Hunter 2010; Reiter 1987; Braks and Carde 2007; Allan and Kline 2004) future modifications should aim to improve the survival of mosquitoes in the trap. Future work should also evaluate the airflow of the trap and its impact on attracting mosquitoes. Sucking the air from above the water surface through the collapsible pipe channels potential volatile chemicals from the water surface to the side of the pond, which might affect the response towards the trap especially if attractant semiochemicals were used (Irish *et al.*, 2012; Michaelakis *et al.*, 2007). To increase user safety and longevity a gel battery should be considered in future instead of a lead acid battery. Whilst the required battery is expensive (approximately $ 100-120) all the other parts can be made from locally available plastic ware and electronic supplies. Costs for the entire trap are estimated to be less than $150, which is still cheaper than the commercially available Box gravid trap ($192) (BioQuipProducts.).

5.5. Conclusion

The three commercially available gravid traps tested in this study were specifically developed for collecting culicine mosquitoes that differ greatly in their oviposition behaviour from the malaria vector *An. gambiae s.l.* (Munga *et al.,* 2005; Sumba 2004). Nevertheless, the Box and CDC gravid trap caught consistent numbers of this species under semi-field conditions but their performance was not considered satisfactory enough to evaluate them under field conditions. The present work revealed that gravid *An. gambiae* females were visually deterred by the presence of the trapping device directly on the oviposition medium. Based on these investigations, a gravid trap was developed that provides open landing space for egg-laying female mosquitoes which improved the catch size by 60% compared to the Box gravid trap. The efficacy of this prototype trap under semi-field conditions is promising and warrants further investigations to: (1) further improve the catch size by modifying the fan suction, the size of the oviposition bowl, and physical characteristics of the trap (e.g. include visual contrast), (2) improve the physical structure of the trap and its components to reduce costs and increase durability and (3) evaluate the trap under field conditions.

CHAPTER 6: FIELD EVALUATION OF OviART GRAVID TRAP AND OTHER DEVICES FOR COLLECTION OF GRAVID MOSQUITOES

6.1 Optimising the OviART gravid trap

6.1.1 Background

An effective suction gravid trap, the OviART gravid trap, was developed in a semi-field system for collection of live malaria vectors (Dugassa *et al.*, 2013). The OviART gravid trap was designed considering the importance of colour of a pond and water body to direct gravid mosquitoes to a habitat. Visual cues such as colour of a pond, reflectance of the water, shape, size, contrast, light quality and intensity, and texture (Kennedy 1942; Bentley and Day 1989; Horvath 1995; Muirhead Thomson 1946; Collins and Blackwell 2000; Huang *et al.*, 2005; Huang *et al.*, 2006b; Bernáth *et al.*, 2004; Bernáth *et al.*, 2008; Bernáth *et al.*, 2012) are among important cues that ovipositing female mosquitoes use to locate a breeding habitat. Humidity coming off from a habitat is another important factor that attracts gravid mosquitoes (Isoe *et al.*, 1995; Huang *et al.*, 2005; Clements 1999). The trap might face strong competition in the natural field system. Here, it was hypothesized that the competitiveness of the OviART gravid trap could further be improved by increasing the surface area of the basin to enhance visualization of the water surface, the amount of humidity coming off the pond and the strength of the fan. This study was intended to improve the collection efficacy of the OviART gravid trap prior to field testing.

6.1.2 Materials and Methods

6.1.2.1 Study site

The study was carried out in one of the semi-field systems located at the International Centre of Insect Physiology and Ecology (description under section 2.1.1; Figures 1 and 2).

6.1.2.2 Experimental design and procedure

Two choice assays were conducted in the semi-field system. The treatments were positioned in the corners of the semi-field system at a distance of 1.5 m from the two adjacent walls (section 2.1.1.; Figures 2). The prototype and the modified OviART gravid traps were set up in opposite corners to reduce a potential interaction. Charcoal filtered Lake Water was used as oviposition medium.

The prototype OviART gravid trap was developed from a black bucket (height = 20 cm, diameter = 30 cm, Volume = 8L), collapsible pipes, 12V battery and 12 V, 0.38A fan. Two modifications were made on the prototype OviART gravid trap (Figure 19A): 1. A basin of bigger surface area (height = 20 cm, diameter = 50 cm, volume = 16 L) was prepared 2. A stronger fan (12 V, 0.75A) was fitted on the bigger basin (Figure 19B). These two modified traps were compared with the prototype OviART gravid trap one at a time in a semi-field system. The two treatments were set up in opposite corners randomly each experimental night. The traps were set up based on the protocol developed in the preceding study (Dugassa *et al.,* 2013). 16L water was used in the modified trap and 8L was used in the prototype design till the water reaches the mouth of the intake duct.

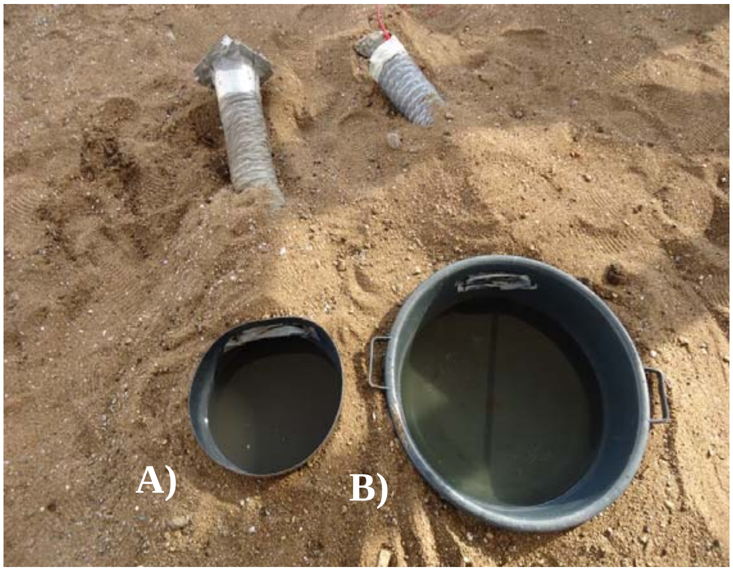


Figure 19: A) The prototype OviART gravid trap, B) The OviART gravid trap with bigger basin

6.1.2.3 Data analysis

The number of mosquitoes recollected and the eggs laid were analysed. The data were analysed using generalized linear mixed effects model. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht, (Team 2011). The blocks of experiments and the trap sites were included in the models as random effects and the treatments were modelled as fixed effects. A Poisson distribution of the data and an exchangeable working correlation matrix were used. The excess variation between data points (over dispersion) that remained after adjustment for all other factors was adjusted by creating a random factor with a different level for each row of the data set (rowid<-factor(1:nrow(rdataset))). The parameter estimates of the models were used to predict the mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.,* 2005).

6.1.3 Results

The OviART gravid trap with bigger basin and the original fan recollected twice the number of mosquitoes as the prototype trap (Table 9). Increment of the surface area and water volume increased the catch size of the trap. Whilst hardly any egg was found in the prototype trap, eggs were regularly found in larger trap indicating either that the fan was not strong enough to collect all approaching mosquitoes letting some of them lay and fly off, or some of the trapped mosquitoes got a chance to lay before they eventually got close enough to the suction to be collected. Consequently, a stronger fan was tested in the second experiment to see if more mosquitoes could be collected.

Table 9: Comparison of the OviART gravid trap with bigger basin and the prototype OviART gravid trap

Treatment	Mean (95% CI)	Odds ratios (95% CI)	p-value
Adults recollected by the traps			
Prototype trap	20.4 (13.5 – 30.9)	1	-
Trap with bigger basin and original fan	38.8 (26.0 – 57.9)	1.9 (1.1 – 3.4)	0.029
Eggs laid in each ponds of the traps			
Prototype trap	0.7 (0.1 – 3.2)	1[a]	-
trap with bigger basin and original fan	71.4 (19.4 – 262.7)	109.8 (25.3 – 476.3)[b]	<0.001

Test of the stronger fan fitted on the bigger basin

The OviART gravid trap with bigger basin and stronger fan recollected 40% more than the prototype design (Table 10). Using stronger fan did not further change the catching efficacy. The mean numbers of mosquitoes collected by the OviART gravid trap with bigger basin and original fan and the OviART gravid trap with bigger basin and stronger fan were similar (Table 9 and 10). Even though, the less powerful fan was sufficient in combination with the larger basin to achieve similar efficacy, the stronger fan was selected for the final trap evaluation under natural field conditions since stronger air movements in the field might interfere with the weaker suction.

Table 10: Collection efficacies of the OviART gravid trap with bigger basin and stronger fan and the prototype OviART gravid trap

Treatment	Mean (95% CI)	Odds ratios (95% CI)	p-value
Adults recollected by the traps			
Prototype trap	26.3 (21.1– 32.7)	1	-
Trap with bigger basin and stronger fan	36.2 (29.4 – 44.6)	1.4 (1.0 – 1.9)	0.037
Eggs laid in each ponds of the traps			
Prototype trap	6.5 (2.5 – 17.3)	1	-
Trap with bigger basin and stronger fan	124.3 (50.5 – 306.4)	19.0 (5.0 – 71.8)	<0.001

6.1.4 Discussion

Increase in the surface area and hence water volume of the OviART gravid trap improved the trap's catch size. However, similar mean numbers of mosquitoes were recollected with the stronger fan as the original fan. In both cases, higher numbers of eggs were recorded in the bigger traps than the prototype trap.

Visual cue is one part of a complex set of signals and cues that anopheline mosquitoes use to choose an oviposition site (Huang *et al.,* 2007; McCrae 1984; Bentley and Day 1989; Huang *et al.,* 2005). Some scholars believe that visual cue is long range cue that mosquitoes use to locate breeding sites from distance (Bidlingmayer 1975; Bentley and Day 1989). As the egg laying mosquitoes come closer to the habitat, other cues such as volatile and non-volatile chemicals (from dissolved organic matter, microorganisms, conspecific larvae, predators etc.), PH, temperature become very important to make the last decision to lay or not to lay eggs in the habitat approached (Bentley and Day 1989). The increase in catch size of the modified trap with the bigger basin suggests that the increase in volume of water more likely resulted in the increase in the amount of water vapour that comes off the habitat which makes the trap more attractive (Isoe *et al.,* 1995; Huang *et al.,* 2005;

Clements 1999). In addition, the bigger surface area of the water might have been easily located by the mosquitoes. The stronger fan did not perform better than the original fan in the semi-field system and the original fan might be sufficient to suck in approaching females of the larger trap. Nevertheless, since under natural conditions stronger movement of the air is expected a stronger fan was incorporated in the final trap design to be tested under natural conditions.

6.2 Open field evaluation of six gravid mosquito catching devices

6.2.1 Background

Five tools that catch gravid females at an aquatic habitat were recently developed to study oviposition behaviour of malaria vectors (Harris *et al.,* 2011; Dugassa *et al.,* 2012). The tools were developed using e-nets, detergent, spray glue, insect glue applied on transparency and transparent sticky film. A square of e-nets, detergent treated pond and spray glue applied on water surface were effective methods to collect gravid *An. gambiae s.l.* in a semi-field system (Dugassa *et al.,* 2012). However, there is usually a gap between results obtained in the laboratory, semi-field and natural field systems (Ferguson *et al.,* 2008; Hewitt *et al.,* 2007). Moreover, sampling mosquitoes outdoors is very challenging as complex variable environmental factors may influence the process (Bhatt *et al.,* 1989; Hewitt *et al.,* 2007). Some of these factors include wide range and scattered target sites, wind direction and strength, rain condition that can affect activities of mosquitoes. Therefore, it was important to test and compare the efficacy of these tools in the open field before embarking on the natural field study. The tools were compared with the Box gravid trap. This study was intended to identify the best catching device to which the OviART gravid trap can be compared in the field as there was no standard tool for collecting *An. gambiae*.

6.2.2 Materials and Methods

6.2.2.1 Study area

This study was conducted in the agricultural fields located at ICIPE, TOC (0^{o} 26' 06.19'' S, 34 ^{o} 12' 53.13''E). There are limited movement of humans, organisms and the formations of breeding sites in this compound. This makes it different from natural field system although it is more natural than a semi-field system. They were referred to as 'open field' in this document to differentiate them from the actual natural field system.

6.2.2.2 Study design

Three field sites were prepared (50×35m each) by clearing tall vegetation. Six sites were marked 15 m away from each other in the three fields to set up six treatments; spray glue, transparency with glue, detergent, square of e-nets, sticky board and Box gravid trap (Figure 20). Within each trapping location in each time period there were replications. The time periods were weeks and response data were recorded for six nights per week. Therefore, there were six data points per site per week. Three blocks of this experiment were implemented in parallel in three different fields in the campus following similar procedures. Random assignment of the treatments into locations and weeks was done separately for each field.

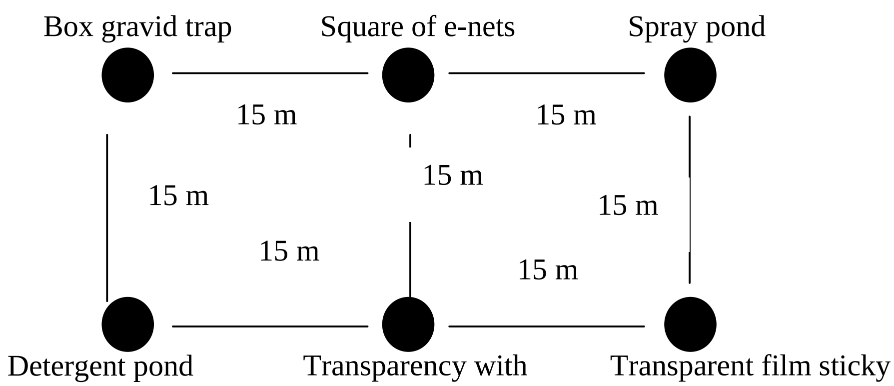


Figure 20: A graphical representation of the placement of the six catching devices in a field

6.2.2.3 Procedure of setting up the treatments

In order to attract gravid females to the tools, artificial aquatic habitats were prepared for all the treatments except for the sticky board. The ponds were prepared by adding 9L Lake Water to the basins. After the experiments, the water in the ponds was discarded and the basins were cleaned daily and the treatments were assigned to new sites after six days. All these procedures were followed in all the fields. The treatments were prepared and set up between 15:00-17:00h and the electric nets and the trap were switched on daily at 18:00h.

Square of e-nets

A square of e-nets surrounding a pond electrocute mosquitoes flying to the pond. Yellow sticky sheets were prepared to collect mosquitoes falling under the e-nets after electrocuted. For this the strips of the yellow rollertrap (Figure 21) were mounted on 12 iron sheets (50×60cm) till they entirely covered the sheets. Three other pieces of yellow sticky sheets were prepared by cutting out a circle of 43cm diameter in a rectangular iron sheet (53×53cm) that can fit to the inside gaps between the e-nets and the round basin. Here, iron sheets were used instead of cardboards to avoid damage by rain. The square of e-nets was then set up around the pond and the yellow collection devices were placed under the nets. Two adjacent e-nets were powered by a 12V battery via a spark box adjusted at 50% spark energy setting.

Transparency with glue:

Transparency on which insect glue (Figure 21) is applied catches mosquitoes when they land on the surface. 100g non-dripping insect glue (Oecos, UK; Figure 21) was dissolved in 30ml hexane and applied uniformly on an A4 transparency (Ryman, UK) prior to the experiment. The transparency with the glue was placed on water surface of the pond. Care was taken not to tilt it to avoid sinking from one side.

Detergent treated pond:

A detergent reduces surface tension of water and mosquitoes get drowned when landed on the surface due to lack of support. 225ml (2.5%) detergent was added into one of the prepared ponds. Black fly screen was tied to a circular wire (36 cm in diameter) and placed on the bottom of the pond assigned for this treatment to fish out the drowned mosquitoes. Three small stones were placed on the screen (two on the edges and one at the centre) to avoid floating.

Spray glue applied to water surface:

When spray glue (Figure 21) is applied on water, it floats like oil. It sticks landing mosquitoes. The bottle of the spray glue was shaken well and the spray glue was evenly sprayed on the water of the pond assigned for the treatment. After counting and recording the mosquitoes caught, the water was discarded and the glue was washed to reuse the basins.

Sticky board

Transparent sticky film mounted on a board or iron sheet resembles water surface. This might be why it attracted mosquitoes in the semi-field system. A sticky iron sheet was prepared by mounting transparent sticky film (Figure 21) on the 50×80cm iron sheet. The sticky sheet was placed on the floor at the site assigned for this treatment. A pond was not prepared for this device.

Box gravid trap

A Box gravid trap was set up on the rectangular pan by fitting the anti-spread bars to the longer sides of the pan. The collecting chamber was placed on the top of the suction tube. The cables were connected to the 6V battery with correct polarity (negative or the black to the black terminal and positive or the red to the red terminal). The battery was switched on and the box was closed at the start of the experiment.

Figure 21: The various sticky materials and detergent used in the open field study

6.2.2.4 Data analysis

The number of mosquitoes collected per device per site was pooled for the six consecutive sampling nights presenting one data point (the mosquitoes collected for six days were summed up). Therefore, a total of 24 nights were constituted in four blocks and analysed. Pooling was done because there were several zero catch rates in the open field which would result in zero inflated errors. In addition, the positions of the treatments were changed after six nights' collection at a given site.

The data were analysed using generalized linear mixed effects model. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht, multcomp, (Team 2011). The blocks of experiments and location (field sites) were included in the models as random effects and the treatments were modelled as fixed effects. A Poisson distribution of the data and an exchangeable working correlation matrix were used. The excess variation between data points (over dispersion) that remained after adjustment for all other factors was adjusted by creating a random factor with a different level for each row of the data set (rowid<-factor(1:nrow(rdataset))). The parameter estimates of the models were used to predict the mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.*, 2005). Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

6.2.3 Results

6.2.3.1 Open field evaluation of six gravid mosquito catching devices

The overall collection efficacy of the six devices

A total of 1,582 mosquitoes were collected by the six tools over the 24 sampling nights. The work was implemented at the beginning of the dry season, the 2nd week of June and overall mosquito density was very low with an average 22 mosquitoes per trap night. 6.6% (105) of these mosquitoes were *An. gambiae s.l.* and 93.4% (1,477) were culicines. 96.6% (1,536) of the sampled mosquitoes were females and 3.0% (46) were males. The Box gravid trap and the pond treated with spray glue on the water surface caught similar numbers of *An. gambiae s.l.* whereas the e-nets collected over three times the number of mosquitoes trapped by each of these tools (Table 11). The sticky transparency caught 16 times less *Anopheles* mosquitoes than the e-net and five times less number of *Anopheles* mosquitoes than the Box gravid trap and spray pond. Despite their effectiveness in the semi-field system, the detergent pond and the transparent sticky board did not collect any *Anopheles* under field conditions. The e-nets and the sticky transparency floating on a pond collected nearly four times as many culicines as the Box trap, the detergent pond and the transparent sticky board (Table 11).

Table 11: The mean number of both gravid and non-gravid female *An. gambiae s.l.* collected by the devices

Treatment	Mean (95% CI)	*Odds ratios (95% CI)	p-value
The female *An. gambiae s.l.* collected			
Box gravid trap	1.0 (0.3 – 2.7)	1[a]	-
E-nets	3.1 (1.2 – 8.0)	3.3 (1.4 – 7.6)[b]	0.006
Spray	0.9 (0.3 – 2.5)	0.9 (0.4 – 2.4)[a]	0.864
Transparency with glue	0.2 (0.1 – 0.9)	0.2 (0.1 – 0.9)[c]	0.029
The female Culicines collected			
Box gravid trap	5.6 (2.3 – 13.9)	1[a]	-
Detergent	4.3 (1.8 – 10.6)	0.8 (0.4 – 1.3)[a]	0.348
E-nets	21.2 (8.9 – 50.6)	3.8 (2.3 – 6.2)[b]	<0.001
Spray	30.9 (13.0 – 73.5)	5.5 (3.4 – 9.0)[b]	<0.001
Sticky board	5.0 (2.0 – 12.4)	0.9 (0.5 – 1.5)[a]	0.693
Transparency with glue	20.8 (8.7 – 49.6)	3.7 (2.3 – 6.1)[b]	<0.001

***** Values sharing similar letter were not statistically different. **Note:** Detergent and sticky boards did not collect *An. gambiae s.l.* mosquitoes. Therefore, they were not included in the model of *An. gambiae s.l.* analysis.

6.2.3.2 The proportion of gravid females collected in the open field

Some of the collected mosquitoes were either damaged or highly dipped in the glues upon removal from the sticky surfaces. 27.8% (427) of the total collected 1,536 female mosquitoes were successfully removed and dissected to determine the proportion of gravid females collected in the open field. Accordingly, 87.9% (95% CI 82.1% - 93.6%) of the total female mosquitoes collected by the six tools were gravid. There was no significant difference in the proportion gravid between species and sampling tools.

6.2.3.3 Species composition of *An. gambiae s.l.* collected in the open field

Specimens of the *An. gambiae s.l.* were identified to species level by Polymerase Chain Reaction (PCR) method. Some mosquitoes could not be removed from the sticky surfaces and some of the successfully removed mosquitoes were highly contaminated by the sticky materials and dusts. Therefore, 64 *An. gambiae s.l.* mosquitoes were stored and identified. Out of the 64 only three (4.7%) were *An. gambiae s.s.* while the rest (95.3%) were *An. arabiensis* (Appendix 1).

6.2.4 Discussion

Low mosquito density and high variability were observed over the different sampling nights. The square of e-nets was the most effective device tested followed by the Box gravid trap and the spray glue applied on the water surface. On the other hand, the transparency with glue hardly caught *Anopheles* mosquitoes while no *Anopheles* mosquito was caught by detergent and the transparent sticky boards. The majority of the mosquitoes collected in the open field by all the tools were gravid (84.4%). The percentages of gravid females collected by all the tools were similar. The square of e-nets and the spray glue were equally effective for sampling culicines followed by the transparency with glue. *An. arabiensis* constitute 95.3% of the *An. gambiae s.l.*

The efficacies of the square of e-nets and the spray glue were consistent with the semi-field study (Dugassa *et al.*, 2012). The e-nets catch not only those mosquitoes that reach the pond it surrounds but also any mosquito that approaches it (Irish *et al.*, 2012; Dugassa *et al.*, 2012; SACEMA). This suggests that electrocution before mosquitoes explore and evaluate the water results in collection of high number of mosquitoes. In this case, mosquitoes do not get a second chance to evaluate and leave the pond if they do not prefer it. In addition, there is a possibility that the setup attracts mosquitoes more than the other treatments. Our recent study (Chapter 4) in a semi-field system indicated that *An. gambiae s.l.* were attracted to a combination a square of yellow boards, a central black pond and the surrounding grey sand which might have created a preferable contrast for the mosquitoes. However, it remains a question how this affects mosquitoes' responses in the field. Various reports showed that these mosquitoes are attracted to contrasting colours for oviposition (Bentley and Day 1989; Huang *et al.,* 2005; Huang *et al.,* 2007). The spray glue was found to be the best for collection of gravid culicine mosquitoes. The spray glue spreads and floats on water surface like oil. This might give the water an appearance of polluted water preferred by the culicines (Ishii and Sohn 1987; Lampman and Novak 1996; Laporta and Sallum 2008). Furthermore, the spray glue might have had attractive effect on gravid culicine mosquitoes. With this efficacy, it might have a potential to replace suction gravid culicine traps which were effective for collection of these mosquitoes (Braks and Carde 2007; Irish *et al.,* 2012).

Although the transparency with glue collected high number of culicines consistent with a previous study (Harris *et al.,* 2011), it relatively caught very low number of *An. gambiae s.l.* in this study. However, it should be noted that the design of this tool was different from the previous study which might have given it a different appearance. It was floated on the surface of natural habitats in the previous study (Harris *et al.,* 2011) whereas it was placed on artificial fresh ponds that have about 5 cm deep edges in the current study. The transparency with glue placed on water surface was visible as a foreign material to human eyes. It likely reduces the volume of water vapour coming out of the water by blocking the surface. Although its catch size seems relatively satisfactory, these factors might have resulted in the reduction of the number of mosquitoes collected as compared to the spray glue and e-nets. On the other hand, it seems that the Box gravid set up on a pond has an impact on both anopheline and culicine mosquitoes' choice of a pond (Dugassa *et al.,* 2013; Irish *et al.,* 2012). However, it should be noted that no infusion was used in this study unlike the previous studies targeting culicine mosquitoes (Irish *et al.,* 2012; Scott *et al.,* 2001; Burkett-Cadena and Mullen 2007, 2008; Kesavaraju *et al.,* 2011). This might be one reason for relatively low culicine catch but greater number of *Anopheles* than the other treatments except the e-nets.

The pond with detergent and the sticky board caught no *Anopheles* mosquitoes in contrast to the semi-field results. These devices also collected a relatively very low numbers of culicines. The sticky board sampled similar number of *Culex* mosquitoes to the Box gravid trap. This suggests that there is some sort of visual attraction created by this shiny surface in the absence of water with the board. The detergent treated pond did not collect any *Anopheles* mosquito and the number of culicines drowned was also very low relative to other devices for unknown reason. This result contrasts semi-field study where it was consistently found to catch relatively high number of *An. gambiae s.l.* (Dugassa *et al.*, 2012). The mixture of detergent and water might have produced a chemical that repel the wild mosquitoes. There might be a difference between the insectary-reared and the field mosquitoes in sensing chemicals. Detergents or soaps are used to wash rearing basins in the larval insectary and to wash cages, vials that are used to provide sugar solution and towels that are put on top of the cages to keep the cages humid in the adult insectary. Thus, the insectary-reared *An. gambiae s.l.* used in the semi-field study might have selected for the odours coming from the combination of water and the detergent. On the other hand, the wild mosquitoes have access to various odours emanating from various larval habitats, vegetation and flowers i.e. blend that are more natural than the artificial insectary condition. It should also be noted that there may be far more complex interacting factors that could influence the more realistic field studies. Here, the square of e-nets was selected to serve as a reference tool for field evaluation of the OviART gravid trap. Spray glue and transparency with glue were found to trap a relatively high number of mosquitoes following the square of e-nets in the open field system. There could be possibility of optimising them as alternative trapping tools.

6.3 Field evaluation of OviART gravid trap

6.3.1 Background

The indoor sampling and control measures have been effective against the mosquitoes biting and/or resting inside houses. Nevertheless, the mosquito species that bite and rest outdoors remain less affected by these tools (Mahande *et al.*, 2007;

Bayoh *et al.*, 2010). Furthermore, sampling host-seeking adult mosquitoes inside houses becomes increasingly difficult and unreliable as mosquitoes increasingly avoid contaminated surfaces indoors (WHO 2007; Bayoh *et al.*, 2010; Okumu and Moore 2011). Malaria vector species that traditionally bite and rest indoors are shifting their biting and resting behaviour to outdoors (Reddy *et al.*, 2011; Sindato *et al.*, 2011; Gatton *et al.*, 2013; Yohannes and Boelee 2011). The wide use of insecticides inside houses also resulted in *An. gambiae s.l.* species composition shift from the more anthropohilic and endophilic *An. gambiae s.s.* to the more flexible and exophagic *An. arabiensis* (Bayoh *et al.*, 2010; Derua *et al.*, 2012). Hence, the OviART gravid trap that collects gravid females looking for oviposition site has been developed (Dugassa *et al.*, 2013) and optimised in a semi-field system.

However, the efficacy of the trap needs to be evaluated under natural field condition because studies show that there is usually a gap between results obtained in the laboratory, semi-field and natural field systems (Ferguson *et al.,* 2008; Hewitt *et al.*, 2007; Ritchie *et al.,* 2011). Although a semi-field system provides a realistic transitional platform between laboratory and field results, there is still a huge difference to the actual field situation (Ferguson *et al.*, 2008; Ritchie *et al.*, 2011). This study was intended to evaluate efficacy of the OviART gravid trap for sampling wild gravid *An. gambiae* in the natural field system.

6.3.2 Materials and Methods

6.3.2.1 Study area

The field evaluation of the OviART gravid trap was conducted in Kombe, between Mbita and Homa Bay (00^0 26.379’ S 034^0 13.295’ E; elev 1,150 m), western Kenya (Figure 22). Animal hoof prints contribute to formation of breeding sites in this field area. When Lake Victoria increases in volume and retreats it gives rise to formation of numerous small breeding habitats along the edge. Numerous small aquatic habitats are found within the agricultural fields of the area (Figure 22). It is one of the malaria endemic areas of western Kenya. The main economic activities of the local inhabitants include fishing and livestock keeping. There is also small scale farming activities. Maize, millet and sorghum are the common cereal crops produced in the area. Many of the houses are mud-walled with open eaves, have corrugated iron-sheet roofs, and have no ceiling. The eaves are used to increase ventilation in the houses. However, they provide entrance sites for mosquitoes (Snow 1987; Lindsay and Snow 1988).

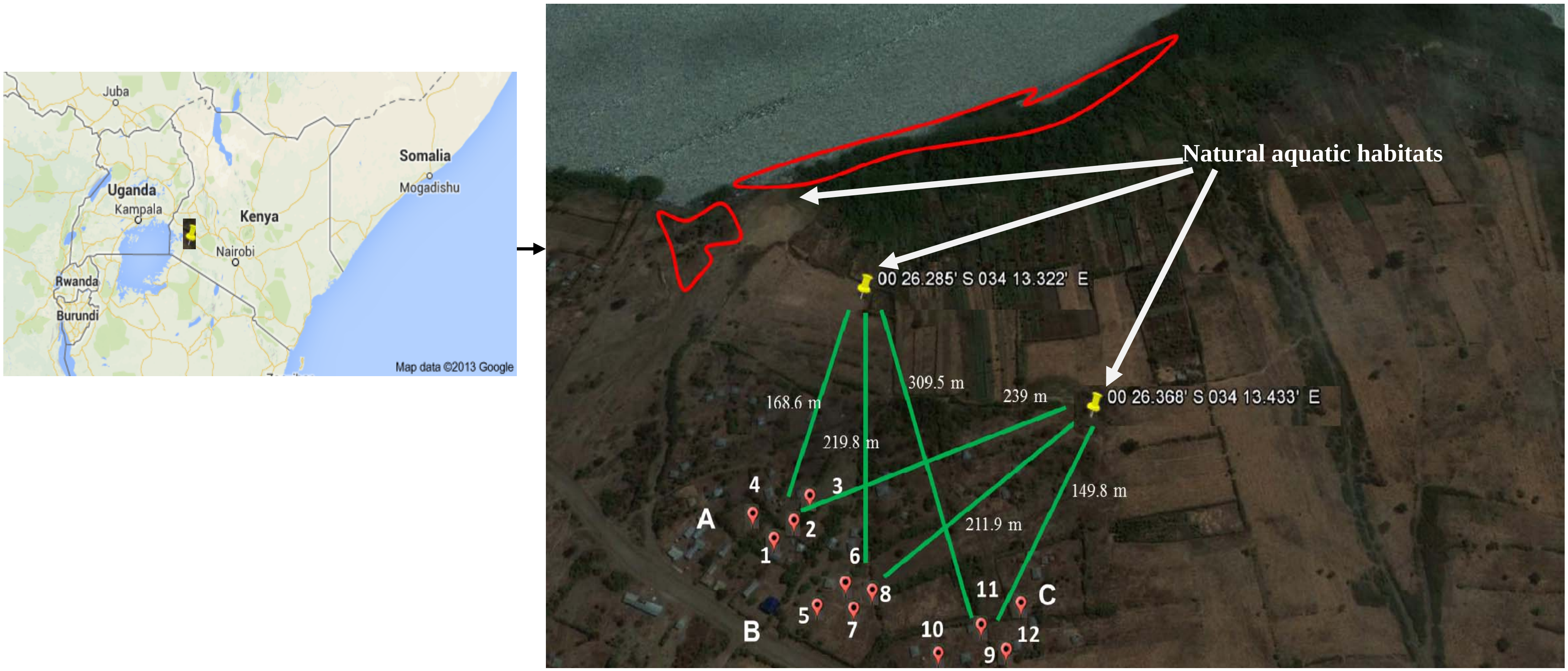


Figure 22: Location of treatements and natural aquatic habitats for field evaluation of the OviART gravid trap

6.3.2.2 Study design and procedure

12 houses which were 200 – 400 m from potential breeding sites were selected and the house owners were contacted for permission. The houses were grouped into three clusters and they were labelled as house 1, house 2, house 3 … etc. and house 12, i.e. houses 1 – 4 were in cluster 1, 5–8 in cluster 2 and 9-12 in cluster 3 . The adjacent houses selected within the same cluster were at least 50 m apart and the adjacent clusters were at least 100 m apart. The treatments were set up in private compounds 12 m away from a house to protect the materials from theft. A complete randomized block design was used in this study. The four treatments were assigned to each compound randomly within each cluster by a generator lottery method and rotated randomly across trapping locations after every 4 nights experiment.

A preliminary assessment was conducted on the presence of *An. gambiae* in the study area for four nights. This was done by sampling adult mosquitoes and monitoring appearance of larvae in artificial ponds provided in the area. The mosquitoes were sampled using two MM-X traps. The traps were suspended on the outside roofs of the residential houses so that their lowest parts were 15 cm above the ground (Okumu *et al.,* 2010). The availability of gravid mosquitoes was assessed by setting up two artificial ponds to monitor the appearance of eggs and early instar larvae in the ponds. The water was discarded and the larvae were killed after monitoring. On average 2.5 *Anopheles* mosquitoes were collected per trap night by the MM-X trap and an average of 7 *Anopheles* larvae which could be filtered from the water were recorded per artificial ponds.

Four trapping devices, the OviART gravid trap, Box gravid trap, square of e-nets and MM-X trap were set up in the field (Figure 23). Except the Box gravid trap which uses 6V battery, all the three tools were powered using 12 V batteries. All the 12 V batteries were placed inside houses and connected to the traps and the e-nets using electric cables (15 m long). The treatments were set up as previously described elsewhere (Dugassa *et al.*, 2012; Dugassa *et al.*, 2013) and the experiments were started at 18.00h. Lake water was used to prepare the ponds. The traps were stopped in the following morning at 8:00h and the collection chambers were put in a freezer for 30 minutes. The mosquitoes that fell on the yellow collection device under the square of e-nets were removed using forceps. The mosquitoes were morphologically identified into culicines and anophelines. The members *An. gambiae s.l.* were identified into species by using Polymerase Chain Reaction (PCR) method.

OviART gravid trap

Box gravid trap

Square of e-nets

MMX trap

Figure 23: Photos of the four trapping tools setups in the field

6.3.2.3 Data analysis

The number of mosquitoes collected per trapping tool per site (house) was pooled for the four consecutive sampling nights presenting one data point (mosquito numbers per four nights). Therefore, a total of 24 replicates were grouped into six blocks and analysed. Pooling was to avoid zero inflated errors in the analysis. In addition, the positions of the treatments were changed after four nights' collection at a given site.

The data were analysed using generalized linear mixed effects model. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht, multcomp, (Team 2011). The blocks of experiments and location (field sites) were included in the models as random effects and the treatments were modelled as fixed effects. A Poisson distribution of the data and an exchangeable working correlation matrix were used. The excess variation between data points (over dispersion) that remained after adjustment for all other factors was adjusted by creating a random factor with a different level for each row of the data set (rowid<-factor(1:nrow(rdataset))). The parameter estimates of the models were used to predict the mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models (Seavy *et al.*, 2005). Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates. The proportions of gravid females collected during the field evaluation of the OviART gravid trap were analysed using generalized linear mixed effects model. Here, binomial data distribution was used with logit link function.

6.3.3 Results

6.3.3.1 The female mosquitoes collected in the field in each trap

A total of 2,698 mosquitoes were collected in the field during the study period, 93.3% (2,518) were female and 6.7% (180) were male mosquitoes. The majority 84.6% (2,282) were culicine mosquitoes and 15.4% (416) were anophelines (35.8% (149) *An. gambiae s.l.*, 34.9% (145) *An*. *funestus*, 12.0% (50) *An*. *pharoensis*, 7.9% (33) *An*. *coustani* and 9.4% (39) non-identified anophelines). The OviART gravid trap collected a significantly greater number of female *An. gambiae s.l.* than the Box gravid trap (Table 12). The OviART gravid trap collected similar number of *An. gambiae s.l.* as the square of e-nets. However, the *An. funestus* and other anopheline catch sizes of the OviART and the Box gravid traps were similar and less than that of the square of e-nets. The MM-X trap collected similar number of female *An. gambiae s.l.* with the OviART gravid trap and the square of e-nets but less culicines than the two.

6.3.3.2 The proportion of gravid mosquitoes collected in the natural field system

Gravid mosquitoes collected during the study period constitute 64.1 % (1,613) of the total 2,518 female mosquitoes collected. The greater proportions of *An. gambiae s.l.* and culicine mosquitoes trapped by the OviART gravid trap, e-nets and the Box gravid trap were gravid (Figure 24). However, the percentage of gravid females was relatively less for *An. funestus* and other anophlines (Figure 24). The MM-X trap also collected few gravid females.

Table 12: The mean total female mosquitoes collected during the field study

Treatment	Mean (95% CI)*	OR (95% CI)	p-value
An. gambiae s.l.			
Box gravid trap	0.5 (0.2 – 1.2)	1^{a}	-
OviART gravid trap	1.7 (0.9 – 3.4)	3.3 (1.5, 7.1)^{b}	0.003
E-nets	1.2 (0.6 – 2.4)	2.3 (1.0, 5.1)^{b}	0.041
MM-X trap	2.2 (1.1 - 4.3)	4.2 (1.94, 8.9)^{b}	<0.001
***An. funestus* complex**			
Box gravid trap	0.3 (0.2 – 0.8)	1^{a}	-
OviART gravid trap	0.7 (0.4 – 1.3)	2.0 (0.8 – 5.4)^{ab}	0.156
E-nets	1.4 (0.9 – 2.3)	4.2 (1.7 – 10.3)^{b}	0.002
MM-X trap	4.2 (2.9 – 6.2)	12.6 (5.4 – 29.3)^{c}	<0.001
Other anophelines			
Box gravid trap	0.2 (0.1 – 0.6)	1^{a}	-
OviART gravid trap	0.2 (0.1 – 0.5)	0.8 (0.2 – 3.3)^{a}	0.722
E-nets	2.7 (1.5 – 4.9)	11.8 (3.9 – 35.4)^{b}	<0.001
MM-X trap	1.0 (0.5 – 1.9)	4.1 (1.3 – 13.0)^{b}	0.016

culicine mosquitoes			
Box gravid trap	16.0 (11.0 – 23.1)	1[a]	-
OviART gravid trap	29.2 (20.3 – 41.9)	1.8 (1.3 – 2.7)[b]	0.002
E-nets	31.8 (22.2 – 45.6)	2.0 (1.4 – 23.1)[b]	<0.001
MM-X trap	19.6 (13.6 – 28.2)	1.23 (0.8 – 1.8)[a]	0.284

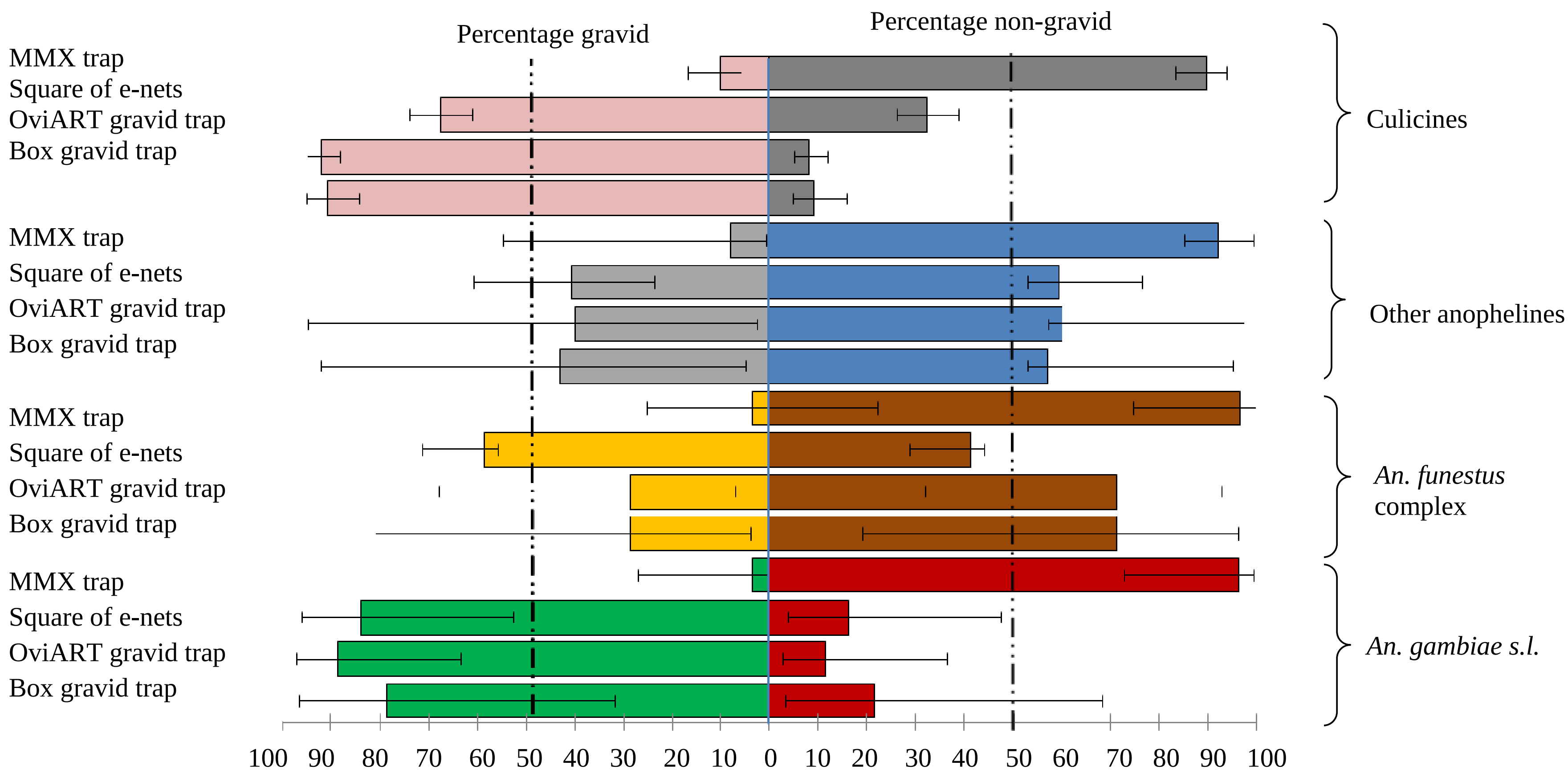


Figure 24: The percentage of gravid females collected in each treatment

6.3.3.3 Species composition of *An. gambiae s.l.* collected in the field

Majority of the *An. gambiae s.l.* were *An. arabiensis.* Of the 130 *An. gambiae s.l.* mosquitoes amplified in the gel electrophoresis 96.2% (125) mosquitoes were found to be *An. arabiensis* and 3.8% (5) were *An. gambiae s.s.* (Appendix 2).

6.3.4 Discussion

OviART gravid trap and e-nets collected similar number of *An. gambiae s.l.* and culicines. The catch sizes in both tools were greater than the Box gravid trap. The MM-X trap also collected gravid females in very low number. High number of other anopheline mosquitoes (other than *An. gambiae s.l.*) was collected by e-nets. However, the larger proportion of other anopheline species (*An. funestus*, *An. pharoensis*, *An. coustani* and unidentified anophelines) collected in the field was non-gravid unlike the *An. gambiae s.l.* and culicine mosquitoes. The majority of these mosquitoes were collected by the MM-X trap and e-nets.

The OviART gravid trap design provides greater opportunities for the gravid mosquitoes to explore the pond than a trap set up on top of a pond (Dugassa *et al.*, 2013; Irish *et al.*, 2012). This might have resulted in an increased chance of trapping the gravid mosquitoes as they hover over the pond during pre-oviposition habitat evaluation (Bentley and Day 1989). The catches with the square of e-nets could be considered as a reference to estimate the maximum trap catch per night in an area as it collects mosquitoes approaching its vicinity (SACEMA ; Dugassa *et al.*, 2012). The relatively high number of mosquitoes collected by e-nets might be an indication that the nets are not visible to mosquitoes like other flying insects (Vale 1993) creating no obstruction around the pond. Although our recent work suggested that the contrast created by a combination of the central black pond, the yellow collection device and the surrounding floor may somehow invite more mosquitoes, we are not sure to what extent this contributes to the catch in the natural field system. There are two important observations that might help as precaution in a future work, 1. Wing scales and broken appendages of insects probably mosquitoes but difficult to identify were observed on some heavy rainy days on the yellow boards and 2. There were some cases where birds picked the mosquitoes very early in the morning before collection. Culicine gravid traps are baited with various infusions (Scott *et al.,* 2001; Burkett-Cadena and Mullen 2008; Ponnusamy *et al.,* 2010b). However, no infusion was used in this study because *An. gambiae s.l.* which lay eggs in a relatively fresh water with less impurities (Munga *et al.,* 2005) were targeted. The odour source and CO_2 generated in the MM-X trap were cues for host seeking malaria vector mosquitoes. That might resulted in the least number of culicine females collection by the MM-X trap compared to the other three tools.

The proportion of other gravid *Anopheles* species such as *An. funestus, An. pharoensis* and *An. coustani* was low compared to the *An. gambiae s.l.* and culicines. The first reason could be the outdoor hot-seeking and animal biting behaviour of these mosquitoes. The community keep their cattle outside within their compound. It seems that the probability of catching the host seeking mosquitoes increased for most of these mosquitoes feed outdoors either on animals or on humans and predominantly rest outdoors (Muriu *et al.,* 2008; Mendis *et al.,* 2000). Although *An. funestus* is classified as more endophilic species (Aniedu 1993), exophily has also been reported (Fontenille *et al.,* 1990). The members of *An. funestus* complex predominantly feed on animals except *An. funestus s.l.* (Muturi *et al.,* 2009). Moreover, a report by Moiroux *et al.*, (Moiroux *et al.,* 2012) indicated that there was an increase of outdoor biting proportion of *An. funestus* from 45% to 68.1% one year after universal coverage by LLINs. Githeko *et al.,* (Githeko *et al.,* 1996) reported that *An. funestus* shifted to more feeding on cattle in response to permethrin use indoors. It might not be easy to generalise exophily/endophily and exophagy/endophagy of anopheline mosquitoes as both were observed in different places and are subject to change over time (Faye *et al.,* 1997; Mahande *et al.,* 2007; Ameneshewa and Service 1996; Paaijmans and Thomas 2011; Fontenille *et al.,* 1990; Githeko *et al.,* 1996). Not surprisingly, the majority of these mosquitoes were collected in the MM-X trap which attracts and traps host-seeking mosquitoes followed by the square of e-nets which catches any mosquito flying around. The host-seeking mosquitoes might have approached the e-nets more than the other treatments for resting as the roofing used to protect the wires from rain probably marked the structure. The second reason for the collection of less proportion of gravid females of the other anopheline species in the traps could be the different nature of their larval habitat from that *An. gambiae*. The aquatic habitats of *An. funestus, An. pharoensis* and *An. coustani* are swampy areas with dense and tall vegetation while the small sunlit fresh water habitats are preferred by *An. gambiae s.l.* (Gillies and DeMeillon 1968; Gimnig *et al.,* 2001; Minakawa *et al.,* 2002; Munga *et al.,* 2005; Muirhead Thomson 1951). On the other hand, the culicine mosquitoes are more flexible and have wide range of aquatic habitats (Bentley and Day 1989; Azari-Hamidian 2007).

An. funestus were collected in low number in the nearby study site (Lwanda village) in a previous study (Mukabana *et al.,* 2012b). Other *Anopheles* species such as *An. funestus, An. pharoensis, An. coustani* that were trapped in Kombe village during this field study were not trapped by the six tools within the *icipe* compound during the study period. Communities in the villages keep cattle inside or around their houses and spend relatively more time outdoors. However, only humans live within the *icipe* compound. The mosquitoes that prefer humans might have been relatively abundant while the others that predominantly feed on other animals outdoors (Muriu *et al.,* 2008) remain within the surrounding villages.

6.4 Conclusion

The bigger basin with larger water volume improved efficacy of the prototype trap by attracting more mosquitoes to the trap. This optimised OviART gravid trap was effective tool for trapping gravid mosquitoes in the natural field system. The catch size was similar with the square of e-nets that catches any approaching mosquito. This trap can be deployed in mosquito surveillance programs. Square of e-nets, spray glue and transparency with glue could be optimised as alternative tools for surveillance of malaria vectors.

CHAPTER 7: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 Introduction

Development of a gravid trap provides an opportunity of collecting both endophilic and exophilic malaria vectors at a common site, aquatic habitat. This tool has substantial advantage for the success of malaria vector surveillance programs because the proportion of outdoor biting and resting mosquitoes are increasing. The initial experiments focused on the development of tools for studying oviposition behaviour of malaria vectors in order to understand how the egg-laying mosquitoes could effectively be targeted for trapping at an aquatic habitat. Accordingly, behaviours of gravid *An. gambiae* were studied. Two of these tools which were the most effective, square of e-nets and detergent, were used for evaluating the catching efficiency of commercially available gravid culicine traps and for investigating the factors that are responsible for a reduced acceptability of gravid malaria vectors to approach these traps. Based on the results a new prototype trap, OviART gravid trap was designed for the collection of gravid malaria vectors. This is the first suction gravid trap for the collection of live *An. gambiae s.l.* It trapped about twice the number of gravid *An. gambiae* females collected by the Box gravid trap (the commercially available gravid culicine trap) under semi-field system.

Here, findings and key discussion points of each chapter will be provided. At the end the limitations and implications of the current study will be discussed, and the possible future work will be indicated.

7.2 Tools to study oviposition behaviour of gravid *An. gambiae*

Studying oviposition behaviour of gravid malaria vectors allows develop a mechanism for proper targeting of the mosquitoes by surveillance or control tools. This study has two major sections. The first section involved developing a method of using electric nets to analyse behaviour of gravid females around oviposition sites while the second section focused on the development of tools using sticky materials and detergent to study landing behaviour of the mosquitoes during oviposition.

The arrangement of electric nets in a complete square form was found to be an effective setup to study behavioural responses of gravid females to a certain aquatic habitat. The lower energy setting that does not create spark, sound and smell was more effective than the high energy setting. The square of e-nets effectively catches mosquitoes approaching a pond surrounded by the e-nets. Either a single e-net or incomplete rings of e-nets were used in previous studies on tsetse flies and host-seeking mosquitoes (Rayaisse *et al.,* 2011; Torr *et al.,* 2008; Vale 1982b; Vale and Hargrove 1979). It should be noted that different setups of e-nets could be used based on the objective of a given study. When insects touch electric nets they fall under the nets after electrocution. Water filled trays were used in the studies with tsetse flies and host-seeking mosquitoes (Rayaisse *et al.,* 2011; Torr *et al.,* 2008; Knols *et al.,* 1998). Since the water may divert the gravid females away from a target, water filled tray was replaced by a yellow sticky film collection device on which the mosquitoes hardly landed. During the experiments of optimising this alternative collection device, it was found that the transparent sticky film was attractive resulting in landing of high number of mosquitoes on its surface even when it was not placed next to an artificial pond. This is more likely due to reflective surface of the material which makes it appearance like water. Previous studies indicated that gravid mosquitoes were attracted to land on shiny surfaces resembling water bodies such as mirrors (McCrae 1984; Bentley and Day 1989; Bernáth *et al.,* 2004; Bernáth *et al.,* 2008; Horvath 1995; Kennedy 1942). However, the transparent film did not work well in the field unlike in the semi-field system. Its competitiveness might have decreased in the field. This study also suggested that the combination of the yellow boards, the black pond at the centre and the surrounding greyish sand floor attracted mosquitoes more than the comparison pond prepared alone. This contrast might be preferable by gravid *An. gambiae*. Many studies indicated that contrast is important for these mosquitoes to choose a habitat (McCrae 1984; Huang *et al.,* 2007; Bentley and Day 1989; Huang *et al.,* 2005).

Novel application methods of various sticky materials (spray glue, yellow sticky film, and clear roller trap and non-drip insect glue) and detergent were devised to study behaviour of gravid mosquitoes during oviposition. Particularly, they were devised to determine how gravid *An. gambiae s.l.* deposit their eggs into a habitat. It is important to know if and where gravid females land during oviposition in order to effectively target them at aquatic habitats. This allows development of effective monitoring tools (e.g. ovi-traps and gravid traps (Reiter *et al.,* 1986; Reiter 1983, 1987; Braks and Carde 2007; Russell and Hunter 2010; Muturi *et al.,* 2007; Ritchie *et al.,* 2003; Ritchie *et al.,* 2009; Williams *et al.,* 2006; Allan and Kline 2004) and intervention measures (e.g. auto-dissemination of larvicides (Gaugler *et al.,* 2012; Chism and Apperson 2003; Itoh *et al.,* 1994)). The current study is the first to be conducted in a bigger setting, the semi-field system. The few studies conducted on how the malaria vectors lay eggs used relatively small cages (less than $1m^3$) except one which was implemented under field conditions (Harris *et al.,* 2011). Some of these studies reported that gravid females were most commonly observed laying their eggs directly seated on the water surface and on the lip of the oviposition cup regardless of the cup's colour and cage size (McCrae 1984; Miller *et al.,* 2007; Clements 1999). Oviposition from flight has also been described when the oviposition cup was placed over a black surface (McCrae 1984). In this study, it was indicated that gravid females primarily land directly on the water surface to lay eggs. Since no eggs were found in ponds with detergent treated ponds, which prevents egg-laying while landed on the water surface, this particular study does not support eggs being dropped in flight onto the water. Even if there might existed female mosquitoes that would drop eggs in flight, it seems that they must touch the water surface before they lay eggs. The relatively large number of eggs found associated with females caught on the spray glue applied on the water surface was probably due to stress induced oviposition on the surface (Harris *et al.,* 2011). The detergent, spray glue and the transparency with glue were shown to be useful methods for collecting mosquitoes when landing to lay eggs.

7.3 Development of a prototype gravid trap

Development of a gravid trap provides an opportunity to sample mosquitoes with various biting and resting behaviours. First, the catching efficacies of commercially available gravid culicine traps were evaluated and the factors that are responsible for a reduced acceptability of gravid malaria vectors to approach these traps were investigated. It was investigated that the trap placed on top of oviposition medium reduces the number of mosquitoes by visually obstructing them from visiting the pond. Visual cue is important for mosquitoes to locate a breeding site or host (Bentley and Day 1989; Allan *et al.,* 1987; Bidlingmayer 1975; Browne 1981; Gillies and Wilkes 1982). These traps may cover the pond and gives it a shady appearance while *An. gambiae s.l.* prefers open sunlit habitats (Gillies and DeMeillon 1968; Gimnig *et al.,* 2001; Minakawa *et al.,* 2002; Munga *et al.,* 2005; Muirhead Thomson 1951). In addition, a trap set up on top of a pond might hinder pre-oviposition flight of the mosquitoes in the arena of the site. Although the gravid culicine traps have not been purposely evaluated for collecting *An. gambiae s.l.*, reports from field studies on culicines show that either very few or no *Anopheles* mosquitoes were trapped in the gravid culicine traps (Irish *et al.,* 2012; Muturi *et al.,* 2007). There is an idea that commercially available gravid traps might be less suitable for anophelines than for culicines based on those actual trapping results. This has to be cautiously interpreted because the gravid traps that are used to collect culicine females are baited with a range of fermented plant infusions which might repel malaria vectors which are generally associated with less strongly polluted water (Minakawa *et al.,* 1999; Mutuku *et al.,* 2006; Sumba *et al.,* 2008).

The OviART gravid trap was designed to draw mosquitoes from the side while providing open landing space for egg laying females. The suction duct fitted to the side draws air from wide area which reduces suction strength unlike the Box gravid trap where placement of the suction duct above the water surface more likely creates a better confined area of suction. This necessitated stronger fan (12V) and battery (12V) than the Box gravid trap. The OviART gravid trap recollected 60% more mosquitoes than the Box gravid trap in the semi-field system.

7.4 Field evaluation of the OviART gravid trap

Field evaluation is important to validate semi-field findings. Usually, a gap exists between semi-filed and field studies. Prior to field evaluation of the OviART gravid trap, two studies were conducted. Firstly, the prototype trap was optimised by increasing the pond size and the water volume. This was intended to increase the trap's competitiveness in the natural field system where there are strong competing natural habitats. Secondly, the tools used in semi-field systems to study oviposition behaviour of the mosquitoes were tested in an open field condition to screen a device that can serve as a reference. This was required because there was no standard gravid trap for anophlines to compare the OviART gravid trap with.

The open field system study suggested that square of e-nets and spray glue could be optimised for field sampling of malaria vectors. However, much more critical thinking and work remain to ensure their use in the natural field condition in monitoring programs. A square of e-nets was found to be the most effective catching devices tested in the open field. Therefore, it was used as a reference for field testing of the OviART gravid trap. The field result indicated that the OviART gravid trap is effective for trapping gravid *An. gambiae s.l.* and culicines. The proportion of gravid mosquitoes trapped was higher than the non-gravid females. However, relatively greater proportions of the other anophelines (*An. funestus, An. pharoensis,* and *An. coustani*) were non-gravid. This might be due to their biting and resting behaviours. The greater proportion of these mosquitoes predominantly feed on cattle outdoors and some may bite humans outside houses (Muriu *et al.,* 2008; Mendis *et al.,* 2000; Muturi *et al.*, 2009). The communities in the study area keep their cattle outside within their compound. It seems that the probability of catching the host seeking mosquitoes increased. Another possible reason is that these mosquitoes colonise aquatic habitats different from the ponds used in this experiment. *An. funestus, An. pharoensis* and *An. coustani* breed in swampy areas with dense and tall vegetation while *An. gambiae s.l.* breeds in small sunlit fresh water habitats of (Gillies and DeMeillon 1968; Gimnig *et al.,* 2001; Minakawa *et al.,* 2002; Munga *et al.,* 2005; Muirhead Thomson 1951). On the other hand, the culicine mosquitoes exhibit more flexible oviposition behaviour and colonise a wide range of aquatic habitats (Bentley and Day 1989; Azari-Hamidian 2007). This might have increased the chance of trapping culicines in the OviART gravid trap, the square of e-nets and the Box gravid trap.

7.5 The OviART gravid trap as surveillance tool

The main aim of this study was to develop a trap that collects female malaria vectors in search for an aquatic habitat outdoors. The OviART gravid trap could complement the existing indoor sampling tools. There are growing evidences that the population of mosquitoes biting and resting outdoors is increasing (Tirados *et al.,* 2006; Russell *et al.,* 2011; Bayoh *et al.,* 2010; Derua *et al.,* 2012; Okumu and Moore 2011). This challenges the effectiveness the indoor sampling tools. Endophilic and exophilic mosquitoes eventually fly to aquatic habitats for oviposition and can be targeted at the site common to both. Furthermore, trapping mosquitoes at this crucial stage of their lifecycle increases chances of parasite detection and breaks the transmission cycle, and reduce potential population growth by killing the mosquitoes before they lay hundreds of eggs.

The mosquitoes collected by the OviART gravid trap are protected from rain and sun and damage from natural enemies such as ants and spiders. This maximum protection was attained because the collection chamber was fully covered with the horizontal pipe. Removal, identification, storage and further analysis (physiological condition, parasite examination, blood meal analysis etc.) of the mosquitoes collected in such suction traps are more practical than any of the other catching materials used in this study; the square of e-nets, the sticky substances and the detergent. These devices were developed to study behaviour of egg laying females in semi-field system. Although there might be possibility of optimising some of these devices, the suction trap is far more practical and effective surveillance tool.

7.6 Limitations of the present study

There are some limitations in the current study. The first one relates to the mosquitoes used for the semi-field studies. *An. gambiae s.s.* was used for the semi-field experiments. Although *An. arabiensis* were also reared in the insectary, very low proportions inseminate and get gravid. This would have resulted in responses that do not represent actual behaviour in nature. However, it should be noted that the results could be interpreted or work for *An. arabiensis* as well since both *An. gambiae s.s.* and *An. arabiensis* exhibit similar oviposition behaviour and share the same habitat (Minakawa *et al.*, 2002; Minakawa *et al.*, 1999; Gimnig *et al.*, 2001).

The second limitation is lack of special attractant chemical to bait the OviART gravid trap. Gravid culicine traps are usually baited with various infusions for sampling culicines (Burkett-Cadena and Mullen 2007; Irish *et al.*, 2012; Allan and Kline 2004; Muturi *et al.*, 2007). To my knowledge, no standard chemical attractant or infusion has been found for *An. gambiae s.l.* yet. The trap has worked well in the field system where there were competing natural aquatic habitats. However, an attractant chemical might further increase the performance of the trap.

The third limitation is associated with removal and identification of field collected mosquitoes from sticky surfaces and detergent treated ponds. Some mosquitoes got damaged and lost their important morphological features for identification during removal from insect glues and sticky films. Identification of anophelines into species level requires looking at detailed characters which were lost during removal. This was not a problem for distinguishing anophelines and culicines.

The fourth limitation is the season of the experiments. The open field and field experiments were conducted towards the end of the long rainy season (June) and during the long rainy season (May – June). The trap catches might reduce during rainy seasons as there could be high competition from other potential oviposition sites. However, the trap might be more effective during dry season for sampling the available gravid females.

The final point is that OviART gravid trap is not free of limitations like other surveillance tools. For instance the use of batteries that need recharging, liability of the batteries to theft and interference by animals or humans are potential challenges when the trap is used in the natural field system for surveillance or monitoring purposes.

7.7 Future work and recommendation

Malaria vector surveillance programmes focus on the use of sampling tools such as CDC light traps that are effective to collect mosquitoes indoors (Amusan *et al.,* 2005; Lines *et al.,* 1991; Mboera *et al.*, 1998). However, there have been growing recommendations on the necessity of outdoor sampling tools due to reduced house entry of mosquitoes (Russell *et al.,* 2011; Odiere 2007; Reddy *et al.,* 2011). The outdoor sampling tools developed so far such as resting boxes and clay pots are not as effective as the indoor sampling tools except the MM-X trap (Mukabana *et al.*, 2012a; Verhulst *et al.,* 2011; Mukabana *et al.*, 2012b). It is important to note the roles that could be played by gravid traps in malaria vector surveillance. They have advantages over the traps that collect mosquitoes searching for hosts or resting sites. Indoor traps collect endophagic/endophilic mosquitoes while the outdoor tools such as MM-X traps, resting boxes and pots collect exophagic/exophic mosquitoes. But gravid traps could collect mosquitoes with both behaviours at the same time during oviposition. The other advantage is that there is an increased chance of detecting parasites in gravid mosquitoes (Williams and Gingrich 2007). In addition, trapping a single gravid female could mean killing tens or hundreds of offspring.

However, further developments and adaptations are important for effective use of the trap in malaria vectors surveillance tools. One of the factors to be considered is cost. The OviART gravid trap was developed from cheap and locally available materials and it is cheaper than the gravid culicine traps. The construction and setting up this new trap is so simple that it can be done by any non-expert person. Except the battery and the motor fan the other materials such as plastic bottle, basin, collapsible pipe and netting are cheap and easily available. Altogether, the cost of the trap is about 150 USD while the Box gravid trap costs about 192 USD. Nevertheless, if monitoring at large scale is required or the trap is intended to be used as a control tool for mass trapping this is still expensive. Therefore, the next step should focus on a means of reducing the cost. The possibility of battery to being stolen is another issue. While trying to work for cost reduction, it is important to devise a mechanism of securing a battery.

The OviART gravid trap is worth use in surveillance programmes of malaria vectors. Adaptations may be needed on the trap based on the context of an ecological setting of a given sampling locality intended for sampling. For instance the trap could be evaluated in the regions with different soil and aquatic habitat types from the current study area. This study initiates future research on the use of gravid traps as surveillance tools and potentially as control tools.

7.8 Conclusion

- The design of gravid culicine traps resulted in reduced acceptance by *An. gambiae s.l.*
- The OviART gravid trap that provides free landing space for egg laying mosquitoes was effective both in semi-field and field systems. This trap is simple to construct and set up apart from being cheaper than the commercially available traps. It provides protection for trapped mosquitoes which makes it valuable tool to study several entomological parameters. The trap is valuable to be included in malaria vectors surveillance programmes.
- The OviART gravid trap provides new opportunities to study behaviour of gravid malaria vectors.
- A range of tools that can be used to study oviposition behaviour of malaria vectors were developed in this study. A square of e-nets is an effective tool to study behavioural orientation of mosquitoes. Detergent treated ponds and spray glue could be used to study oviposition behaviour of *An. gambiae* in a semi-field system.

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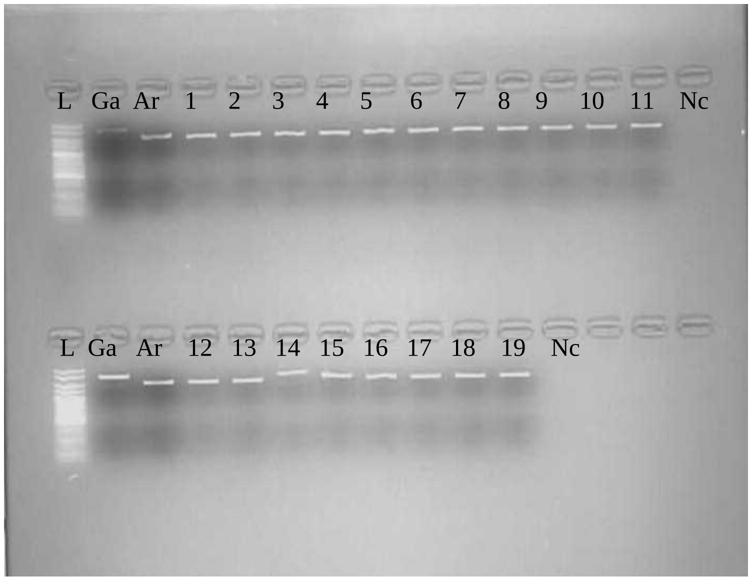
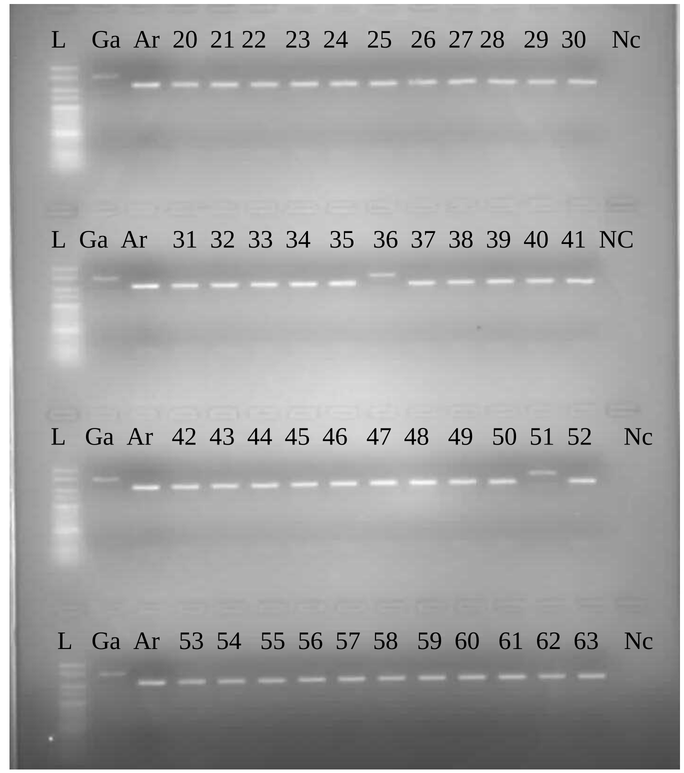
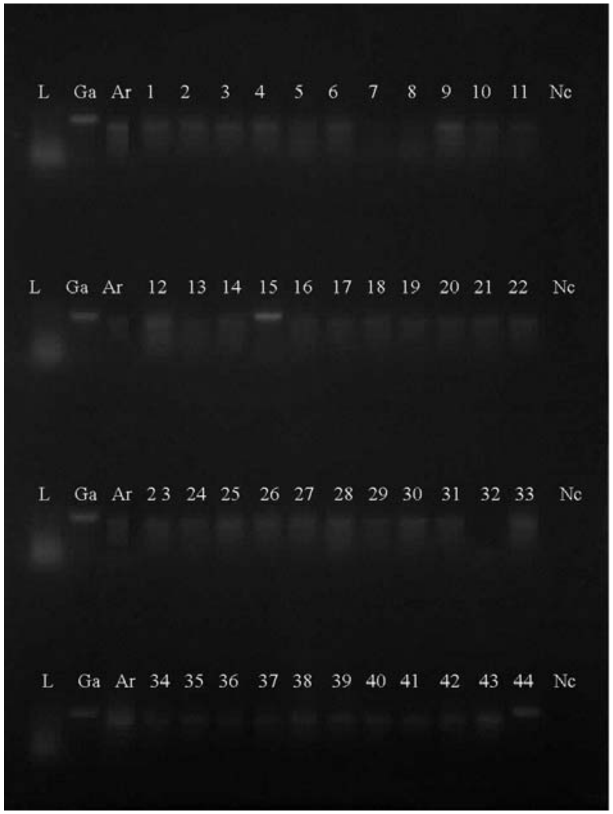
APPENDIX 1: PHOTOS TAKEN FROM GEL ELECTROPHORESIS OF THE PCR PRODUCTS FROM OPEN FIELD EXPERIMENT

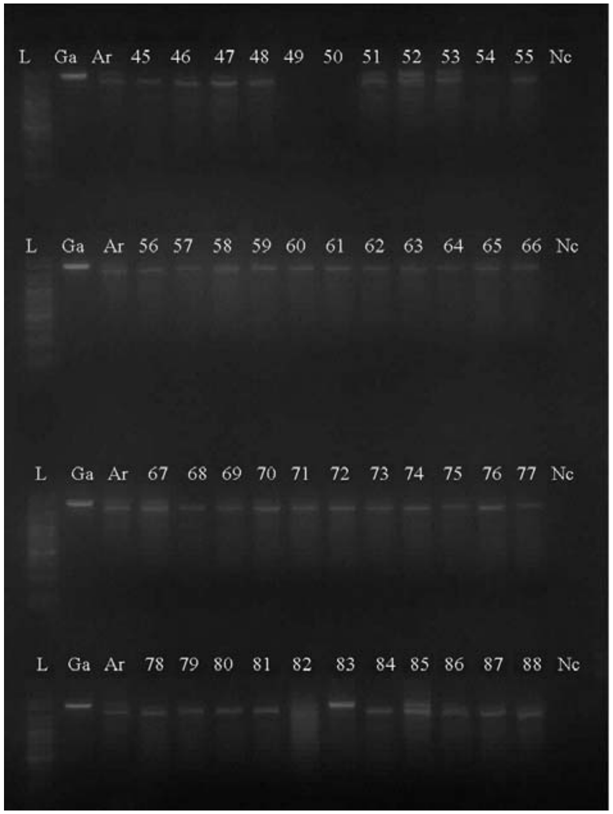




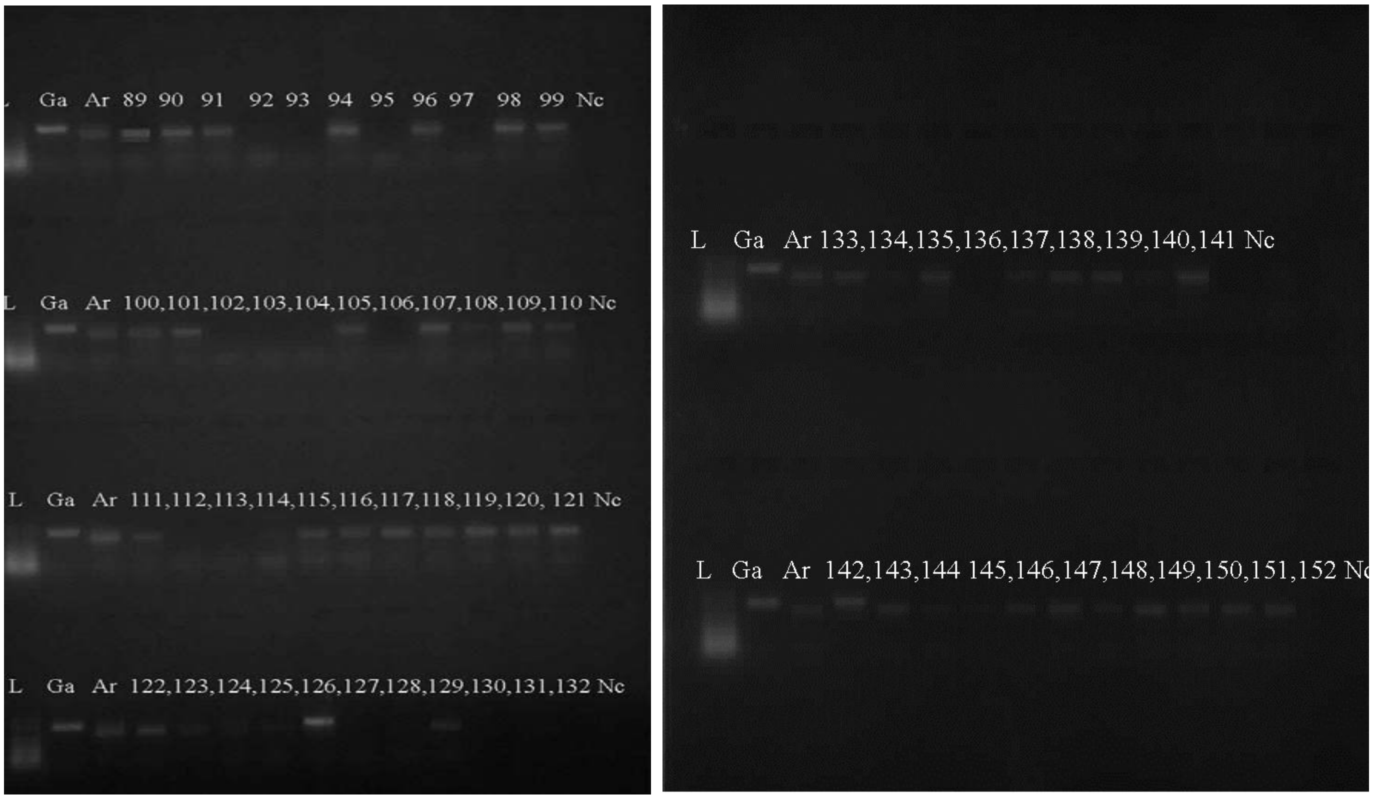
PCR result of the *An. gambiae s.l.* species identification L = HyperLadder V; Ga = positive control for *An. gambiae s.l.*, Ar = positive control for *An. arabiensis,* Nc = negative control; 14, 36 and 51 were *An. gambiae s.l.* and the rest were *An. arabiensis.*

APPENDIX 2: PHOTOS TAKEN FROM GEL ELECTROPHORESIS OF THE PCR PRODUCTS FROM FIELD EVALUATION OF THE OVIART GRAVID TRAP





PCR result of the *An. gambiae s.l.* species identification L = HyperLadder V; Ga = positive control for *An. gambiae s.l.*, Ar = positive control for *An. arabiensis,* Nc = negative control; 15, 44 and 83 were *An. gambiae s.l.* and the rest were *An. arabiensis.*



PCR result of the *An. gambiae s.l.* species identification L = HyperLadder V; Ga = positive control for *An. gambiae s.l.*, Ar = positive control for *An. arabiensis*, Nc = negative control; 126 and 142 were *An. gambiae s.l.* and the rest were *An. arabiensis.*

APPENDIX 3: AN EXAMPLE OF TABULATED RANDOMISED COMPLETE BLOCK DESIGN

Block 1

Day	**Site 1**	**Site 2**	**Site 3**	**Site 4**
1	Treatment A	Treatment C	Treatment B	Treatment D
2	Treatment B	Treatment D	Treatment A	Treatment C
3	Treatment D	Treatment A	Treatment C	Treatment B
4	Treatment C	Treatment B	Treatment D	Treatment A

Day	**Site 1**	**Site 2**	**Site 3**	**Site 4**
5	Treatment C	Treatment B	Treatment D	Treatment A
6	Treatment A	Treatment D	Treatment C	Treatment B
7	Treatment B	Treatment C	Treatment A	Treatment D
8	Treatment D	Treatment A	Treatment B	Treatment C

Block 2

Block 3

Day	**Site 1**	**Site 2**	**Site 3**	**Site 4**
9	Treatment D	Treatment A	Treatment C	Treatment B
10	Treatment A	Treatment B	Treatment D	Treatment C
11	Treatment C	Treatment D	Treatment B	Treatment A
12	Treatment B	Treatment C	Treatment A	Treatment D

APPENDIX 4: PUBLISHED PAPER 1

Dugassa et al. Malaria Journal 2012, 11:374
http://www.malariajournal.com/content/11/1/374

MALARIA JOURNAL

METHODOLOGY **Open Access**

Electric nets and sticky materials for analysing oviposition behaviour of gravid malaria vectors

Sisay Dugassa[1,2], Jenny M Lindh[3], Steve J Torr[4], Florence Oyieke[2], Steven W Lindsay[5,6] and Ulrike Fillinger[1,5*]

Abstract

Background: Little is known about how malaria mosquitoes locate oviposition sites in nature. Such knowledge is important to help devise monitoring and control measures that could be used to target gravid females. This study set out to develop a suite of tools that can be used to study the attraction of gravid *Anopheles gambiae s.s.* towards visual or olfactory cues associated with aquatic habitats.

Methods: Firstly, the study developed and assessed methods for using electrocuting nets to analyse the orientation of gravid females towards an aquatic habitat. Electric nets (1m high × 0.5m wide) were powered by a 12V battery via a spark box. High and low energy settings were compared for mosquito electrocution and a collection device developed to retain electrocuted mosquitoes when falling to the ground. Secondly, a range of sticky materials and a detergent were tested to quantify if and where gravid females land to lay their eggs, by treating the edge of the ponds and the water surface. A randomized complete block design was used for all experiments with 200 mosquitoes released each day. Experiments were conducted in screened semi-field systems using insectary-reared *An. gambiae s.s.* Data were analysed by generalized estimating equations.

Results: An electric net operated at the highest spark box energy of a 400 volt direct current made the net spark, creating a crackling sound, a burst of light and a burning smell. This setting caught 64% less mosquitoes than a net powered by reduced voltage output that could neither be heard nor seen (odds ratio (OR) 0.46; 95% confidence interval (CI) 0.40-0.53, $p < 0.001$). Three sticky boards (transparent film, glue coated black fly-screen and yellow film) were evaluated as catching devices under electric nets and the transparent and shiny black surfaces were found highly attractive (OR 41.6, 95% CI 19.8 – 87.3, $p < 0.001$ and OR 28.8, 95% CI 14.5 – 56.8, $p < 0.001$, respectively) for gravid mosquitoes to land on compared to a yellow sticky film board and therefore unsuitable as collection device under the e-nets. With a square of four e-nets around a pond combined with yellow sticky boards on average 33% (95% CI 28-38%) of mosquitoes released were collected. Sticky materials and detergent in the water worked well in collecting mosquitoes when landing on the edge of the pond or on the water surface. Over 80% of collected females were found on the water surface (mean 103, 95% CI 93–115) as compared to the edge of the artificial pond (mean 24, 95% CI 20–28).

Conclusion: A square of four e-nets with yellow sticky boards as a collection device can be used for quantifying the numbers of mosquitoes approaching a small oviposition site. Shiny sticky surfaces attract gravid females possibly because they are visually mistaken as aquatic habitats. These materials might be developed further as gravid traps. *Anopheles gambiae* s.s. primarily land on the water surface for oviposition. This behaviour can be exploited for the development of new trapping and control strategies.

Keywords: Malaria, *Anopheles gambiae*, Oviposition, Electric nets, Sticky film

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Background

Indoor-resting populations of malaria vectors declined in many African countries with the massive scale-up of long-lasting insecticidal nets and indoor residual spaying [1,2]. This is due not only to the mortality of mosquitoes that contact the insecticides but also due to their behavioural avoidance of contaminated surfaces [2-8]. In areas where malaria transmission occurs outdoors at low densities [9,10], light traps and other indoor surveillance tools, may underestimate transmission. Consequently, there is need to develop novel surveillance and control tools targeting vector populations outdoors [8,11-14]. Sampling of gravid females may provide better opportunities to quantify the size of the vector population, and may be an approach that is more acceptable to local communities since monitoring does not require entering a house.

The rational development of such tools is dependent on an understanding of the behaviour and ecology of vectors [15]. For instance, extensive studies of the processes involved in host seeking in *Anopheles gambiae s.l.* led to the development of a set of highly effective intervention strategies targeting indoor resting and feeding populations [15-17]. Similarly, an improved knowledge of how mosquitoes select an aquatic habitat in which to lay their eggs might provide the basis for new control strategies that exploit oviposition behaviour of *Anopheles.* For several culicine and aedine disease vectors, an understanding of oviposition behaviour has led to effective monitoring techniques and intervention strategies [18-22]. By contrast, surprisingly little is known about the oviposition behaviour in *An. gambiae* s.l., the major malaria vector in sub-Saharan Africa. As a consequence, methods for monitoring and control exploiting this behaviour are poorly developed.

To analyse oviposition behaviour, methods are needed to quantify the flight, landing and egg-laying behaviour of gravid mosquitoes in the wild. Two approaches offer the prospect of being suitable. First, electric nets (e-nets) have been used to study the orientation and landing responses of insects towards visual and chemical cues [23-28]. They were originally developed by Vale [23] to study the behaviour of tsetse flies and have been widely used to study odour- and trap-oriented behaviours of these flies [24-26,29,30]. Whilst e-nets have been used to study the behaviour of host-seeking mosquitoes [27,28], there is no report of them being used for studying the behaviour of gravid malaria vectors. Second, surfaces coated with adhesive have also been widely employed to sample insects as they land on a surface [31-39] and this approach might be used to sample mosquitoes as they land. These traps are cheap, work without a battery and, providing the adhesive is sufficiently strong, will prevent trapped insects from being eaten by most common predators. Third, adding surfactants (e.g. detergents) to the water to reduce surface tension, insects can be sampled as they land on water [40].

The present study was carried out to explore the use of electric nets and sticky materials for analysing oviposition behaviour of gravid *An. gambiae* s.s.. This study set out to develop a set of tools that can be used to study the attraction of gravid *An. gambiae* s.s. towards visual or olfactory cues associated with aquatic habitats. Specifically, the aim was to bisect the behaviour into two components: (1) approaching an aquatic habitat and (2) the actual process of egg-laying.

Methods

Study site

The study was carried out a semi-field system [41] located at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC), Mbita, on the shores of Lake Victoria, Kenya (geographic coordinates 0° 26’ 06.19” S, 34° 12’ 53.13”E; altitude 1,137m above sea level). This area is characterized by a very consistent tropical climate with an average minimum temperature of 16°C and an average maximum temperature of 28°C (based on data from icipe-TOC meteorological station for 2010–2011). The area experiences two rainy seasons, the long rainy season between March and June and the short, and less reliable rainy season between October and December. The average annual rainfall for 2010–2011 was 1,477mm.

Semi-field systems

The semi-field system was a screened greenhouse-like building (Figure 1) 7.1m wide, 11.4m long and 2.8m high at the wall and 4.0m high at the highest point of the roof [15]. The two opposite shorter walls and the roof were made of glass and the two longer walls were screened

Figure 1 **The semi-field system at icipe-Thomas Odhiambo Campus, Mbita, Kenya.**

with black fibreglass netting gauze (1.7×1.5mm). The floor was covered with sand to a depth of 30cm so that artificial aquatic habitats could be dug in to the ground to simulate a natural breeding site for the mosquitoes. To increase the relative humidity inside the semi-field system to 60-70% for experiments the sand floor was watered from 15:00–16:00h prior to the experiment. Care was taken to ensure that no pooling of water occurred on the floor and that the upper layer of sand was dry by the time mosquitoes were released into the system. When treatments were positioned in the corners of the semi-field system (Site 1–4) mosquitoes were released in the centre and when the treatment was positioned in the centre (Site 5) mosquitoes were released 1.5m from the wall at Site 6 (Figure 2). Treatments in the corners were always placed 1.5m from the two adjacent walls.

Artificial aquatic habitats

Two types of artificial habitats were used for experiments. For most experiments round ponds were constructed by positioning a black plastic bowl of 15L capacity (36cm diameter and 18 cm depth) into the ground so that the upper lip was at the same level as the sand floor. The pond was then filled with 9L of water originating from Lake Victoria and filtered through a charcoal-sand filter [42] henceforth called filtered lake water. Rectangular ponds were constructed by positioning black plastic containers of 17Lvolume (50cm long, 37cm wide and 18cm deep) into the ground and filled with 9L of filtered lake water.

Electric nets (e-nets)

E-nets consist of high-tension wires stretched in parallel, across an aluminium frame (1.0 m high×0.5 m wide) with aluminium rods fixed to the two shorter opposite sides of the frame (Figure 3A). Electricity flows between the two ends of each wire generating differentials of >2.5 kV between adjacent wires [23,27], which kills insects that touch the wires. The wires are invisible to flying insects and do not have significant impact on air movement [29].

The rods had holes at a distance of 8mm for fixing the wires into the rods, to enable the electric wires to be arranged in a vertical position. Small nylon loops (Damyl® fishing lines) were tied of the same size as Fabory® zinc-plated draw springs (0.5×3.5×20mm). Copper wires (diameter of 0.2mm) 1m in length were tied to the fish line loops (insulator) from one end and to the springs (conductors) on the other end (Figure 3B). The ends of the wires with the springs and with the fish lines were alternately fitted to the holes 8mm apart on rods to enable the flow of opposite charges in opposite directions (Figure 3A). Torr et al. [28] assessed different spacing of wires in the electric nets and observed no difference in mosquito catch size between 4, 6 and 8mm spacing. The e-nets were held upright by using clamps on metal stands with base (Figure 3A). Alternate wires in each row were charged by a 12V car battery via a transformer (spark box). In the spark box, the 12V direct current (DC) is first converted to an alternating current (AC) that is stepped up to 400 volts peak AC. It is then rectified and converted back to 400V DC. The 400V DC voltage is used to charge a bank of capacitors that are then discharged into the primary of the ignition coil. The voltage output to the e-nets can be reduced by an energy dial lowers the 12 DC input to the spark box, that in turn lowers the 400V output that charges the capacitors. The dial position roughly equates to the energy reduction not to a direct conversion of the voltage outputs to the nets. The output is 400V at 100% spark energy setting and approximately 300V at the 50% spark energy setting of the dial.

Sticky materials and detergent

A range of different materials were used as trapping devices for mosquitoes in the experiments. Throughout

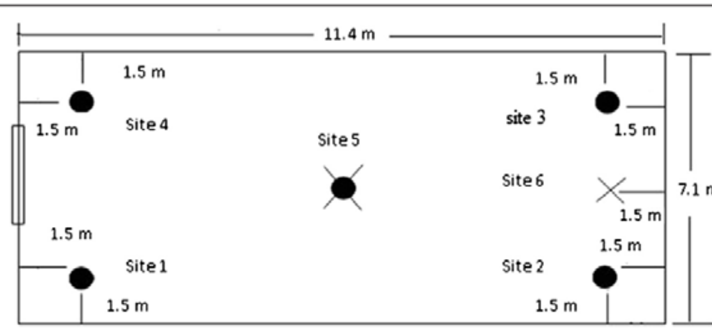

Figure 2 A schematic drawing of the semi-field system with the treatment sites and release points. X: mosquito release sites, round black circles: location of treatment

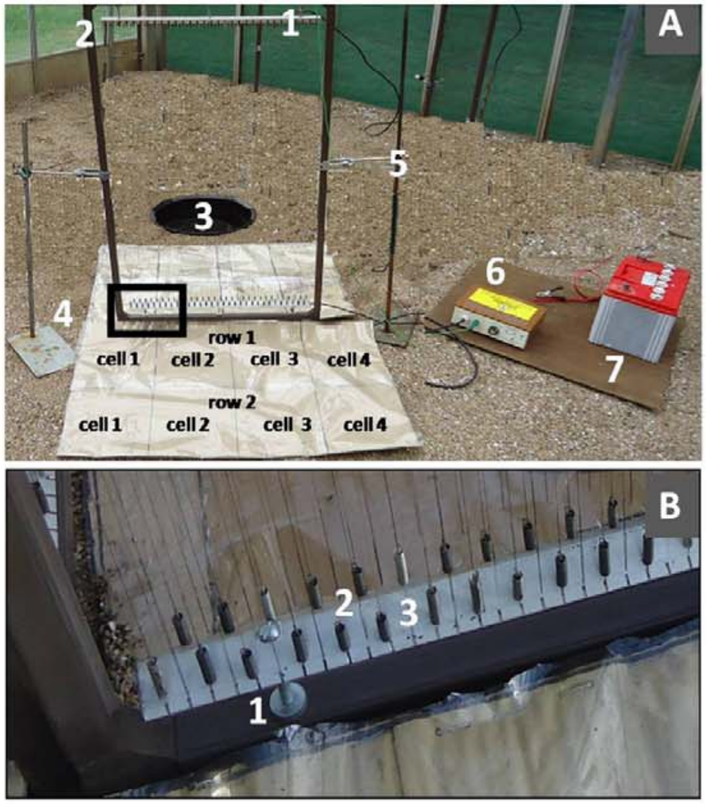

Figure 3 Electrocuting net with two mosquito collection boards made of transparent sticky film. (**A**) Overview of the set up: (1) aluminium rod, (2) aluminium frame, (3) artificial pond, (4) sticky boards on both sides of e-net, (5) stand and clamp to hold e-net, (6) spark box, (7) 12V battery. (**B**) Detail of wire connections: (1) bolt, (2) spring, (3) loop of fish line

the manuscript reference is made to the materials listed in Table 1.

Mosquito preparation

Insectary-reared *An. gambiae* s.s. mosquitoes were used throughout. Gravid mosquitoes were prepared as follows;300 female and 300 male mosquitoes, two to three days old, were kept in 30×30×30cm netting cages and provided with 6% glucose solution *ad libitum* at 25-28°C and a relative humidity from 68-75%. Saturated cotton towels (50x25cm) were folded and placed over the cages to avoid mosquito desiccation. Mosquitoes were starved from sugar for 7 h and allowed to feed on a human arm for 15 minutes at 19.00h on the same day the same procedure was repeated 24 hours later. After the first blood meal unfed mosquitoes were removed from the cages.

Table 1 Reference list of materials used in the experiments

Common name used	Product name	Manufacturer
Transparent double-sided sticky film	Clear rollertrap	Oecos, UK
Yellow sticky film	Yellow rollertrap	Oecos, UK
Transparent sticky film	FICSFIL	Barrettine, UK
Insect glue	OecoTak A5	Oecos, UK
Spray glue	Oecos spray	Oecos, UK
Detergent	Teepol	Chemical Industries, Nairobi
Black fly-screen	Polyester coated fibreglass mosquito netting (15×17 holes/ 2.54 cm^2)	Polytrex, China
Wire screen	Dark-green wire screen (9×11 holes/ 2.54 m^2)	Hebei Jimano, China
Transparency	A4 overhead projector transparency film (0.1 mm)	Ryman, UK

Fed mosquitoes were kept together with males for two more days after the second blood meal before they were utilized in an experiment (i.e. females aged 4–5 days after first blood meal). Host-seeking mosquitoes were prepared by selecting 300 two to three days old females on the day of experiment. Mosquitoes were starved for 6 h before the experiment commenced at 18.00h.

Experimental design

All experiments were implemented in a single semi-field system. Two hundred gravid female mosquitoes were selected from the holding cages based on their abdominal stages (whitish in colour and oval in shape) and were released into the semi-field system between 17.30h and 18.00h. Experiments were terminated at 08.00h the following morning. Experiments with more than one treatment followed a randomized complete block design. Treatments were assigned randomly (using a random number generator) to the corners of the semi-field system and rotated randomly across corners until all treatments were run once in each of the corners included in the respective experiment. This block of experiments was then repeated. Experiments were carried out for 8 or 12 nights.

Experiments

Evaluation of two spark box settings to optimize mosquito collections with e-nets

While e-nets hold promise for studying mosquito behaviour there are a few potential problems that needed to be investigated. High spark energy is used for collecting large insects like tsetse flies [27-29], however, such high energy makes the net spark, creating a crackling sound, a burst of light and a burning smell, that may affect mosquito movement or it may destroy them by burning. Therefore, a modified transformer was used which allowed the moderation of the voltage to eliminate the sparking. Nevertheless, reduced sparking might also allow mosquitoes to escape. Accordingly, an experiment was designed to compare the catches of e-nets powered by a low-power or standard transformer.

This experiment was done using unfed *An. gambiae* s.s. females since previous research using e-nets used mosquitoes of this physiological stage and therefore a reliable response towards the target was expected [27,28]. All consequent experiments were done with gravid females. Two e-nets were positioned in opposite corners of a semi-field system. E-nets were mounted over water-filled trays (45×85×6cm) that served to collect stunned mosquitoes that fell to the ground [27,28]. An odour source of carbon dioxide and a cotton sock worn for 8 hours was used as an attractant [43-46] and positioned on the opposite side of the e-net, 70cm from the e-nets and corner walls of the semi-field system. Two power settings on the spark boxes were compared: 100% spark energy which produced sparks and 50% spark energy which was the highest energy setting that did not produce sparks. The experiment was carried out for 8 nights.

Assessment of sticky boards as collection device under e-nets

A second problem associated with e-nets is how to collect the stunned mosquitoes. Insects killed or stunned after colliding with the e-net fall to the ground. For ease of collection and to prevent them from being eaten by ants and other predators a catching device on the ground was needed. Water-filled trays under the e-nets worked well in experiments with host-seeking mosquitoes [27,28], however when studying the behaviour of gravid mosquitoes, water-filled trays cannot be used since they might attract gravid mosquitoes in search of an oviposition site and divert them from the intended target. A series of experiments with e-nets positioned over sticky boards were carried out to find the most suitable material for collecting mosquitoes when stunned by the electric net.

Evaluation of cardboard mounted with transparent sticky films

One e-net was set up in a corner of a semi-field system (Sites 1–4) and a round pond placed 70 cm from the e-net, between the net and the corner to attract gravid females. Transparent sticky film was mounted on two 50x80cm cardboard rectangles. A grid of two rows, each row with 4 cells (20x25cm), was marked on the boards. One board was placed on each side of the e-net (Figure 3A). The e-net was charged using 50% spark energy and the experiment carried out for 8 nights. The number of mosquitoes that got stuck on the film was counted separately for each cell and direction towards the e-net.

Evaluation of potential attraction of gravid *An. gambiae* s.s. towards transparent sticky films

A collection device under an e-net should not attract gravid mosquitoes otherwise the number of mosquitoes approaching a target will be overestimated. Shiny sticky surfaces may, to a gravid mosquito, look like a water body. Accordingly, studies were undertaken to assess whether gravid mosquitoes landed on the shiny surfaces of the transparent sticky films. Four boards (50×80cm) were prepared with transparent sticky film. Two of the boards were placed on the ground in one corner and the other pair of boards in the opposite corner of the semi-field system. In order to test if landing on the sticky boards is associated with a resting behaviour close to a water source just prior or after egg-laying or if it is an actual attraction towards the surface an artificial pond was added to one of the two treatments. A round artificial pond was dug into the sand 20cm behind one of the pairs of the sticky boards. The experiment was carried out for 8 nights. The number of mosquitoes that landed on the boards was recorded.

Comparison of yellow, black and transparent film sticky boards for the collection of gravid *An. gambiae* s.s

To find a non-attractive device for the collection of electrocuted mosquitoes, three sticky surfaces different in texture and colour were compared. Three cardboard squares of 50x50cm were covered with one of the following treatments (Figure 4): (1) transparent sticky film; (2) black netting painted with 100 g insect glue dissolved in 25 ml hexane; and (3) yellow sticky film. Boards were positioned in three different corners of the semi-field system. One corner remained empty but was included in the random allocation of treatment location. Round artificial ponds were dug into the sand at a distance of 20cm behind each of the boards (Figure 4). The experiment was carried out for 12 nights. The number of mosquitoes that landed on the boards and the number of eggs laid in their respective ponds were recorded.

Collection efficacy of a square of e-nets surrounding an artificial oviposition site

A complete square of four e-nets was mounted around a rectangular pond set up in the centre of the semi-field system in order to estimate the number of gravid females approaching water. Adjacent e-nets were held together by clamps on stands and two of them shared one battery and a spark box (Figure 5). E-nets were charged with 50% spark energy. Four yellow sticky boards of 50×50cm were placed in front of each of the e-nets. Any open space inside the square of e-nets was also covered with yellow sticky board (Figure 5). Boards were divided into two horizontal rows (25×50cm) for further evaluation of the efficacy of the net and of the board as a collection device. The number of mosquitoes collected in the two rows of the board and inside the square of e-nets was recorded separately. Any eggs in the ponds were counted.

Figure 5 A complete square of four electrocuting nets surrounding an artificial aquatic habitat. Yellow sticky boards serve as collection device for stunned mosquitoes

The use of sticky materials and detergents to assess if and where *An. gambiae* s.s. land on aquatic habitats when laying eggs

A prerequisite for the development of new monitoring control tools targeting oviposition site seeking mosquitoes e.g. with gravid traps [18-20,38,47-53] is to know if and where gravid females land during oviposition. Very

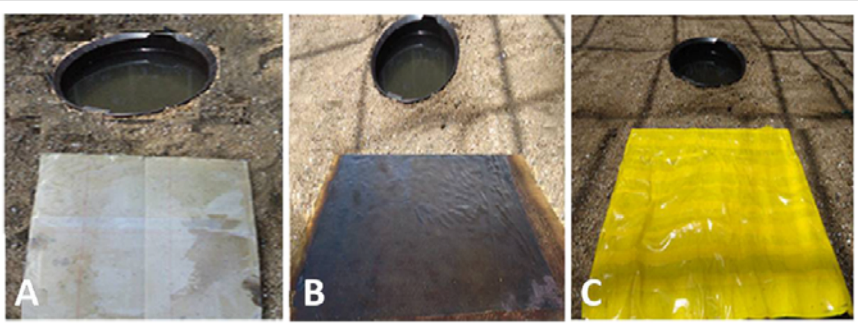

Figure 4 Three sticky boards evaluated in comparison to assess the attraction of gravid mosquitoes towards their surfaces: (A) transparent sticky film, (B) sticky black fly-screen, (C) yellow sticky film.

few studies have assessed this particular behaviour and a variety of different modes of oviposition have been described. Here the use of different sticky materials and a detergent are evaluated to analyse potential landing of gravid females on the water surface or habitat edge for laying eggs.

Assessment of landing on the habitat edge

The edges of three round artificial ponds were made sticky to trap any landing mosquito by applying one of the three treatments: (A) yellow sticky film, (B) spray glue or (C) transparent double-sided sticky film to their inner walls. The sticky edge was 7cm wide and bordered the water surface. The ponds were set up in three corners of a semi-field system. The empty corner was included in the randomization of the treatments. The experiment was run for 12 nights. The number of mosquitoes stuck to the sticky edges and the number of eggs laid in the ponds were recorded.

Assessment of landing on the water surface

Four round artificial ponds were prepared. One of the following four treatments were applied on the water surfaces: (1) two A4 overhead projection transparencies were overlain on each other with colourless adhesive tape to form a cross-shaped surface; the transparencies were coated on one side with 100 g insect glue dissolved in 30 ml hexane and placed on the water surface leaving 8areas of approximately 105cm^2 free water access at the edges (Figure 6A); (2) a circle of dark-green wire screen of the same area as the pond was prepared and coated with 100g insect glue dissolved in 30ml hexane; the wire screen was mounted on a square of wire and placed horizontally inside the pond 5cm below the edge of the pond and 2cm above the water surface (Figure 6B); (3) 225ml (2.5%) detergent was added to the water (Figure 6C); (4) insect spray glue was uniformly sprayed on the water surface (Figure 6D). Ponds were set up in the corners of the semi-field system and the experiment carried out on 12 nights. The number of mosquitoes caught and the number of eggs laid in each pond were recorded.

Evaluation of the landing behaviour using a combination of detergent in the water and spray glue on the edge of the pond

Finally the best catching tools from the previous two experiments were combined, to assess whether there is a sequence in the landing behaviour (e.g. landing on surface for egg-laying and then resting on the edge of the pond). One round artificial pond was prepared. On the edge spay glue was applied and 225ml detergent added to the water. The artificial pond was set up in the centre of a semi-field system. The experiment was carried out for 8 nights. The number of mosquitoes caught and the number of eggs in the pond were recorded.

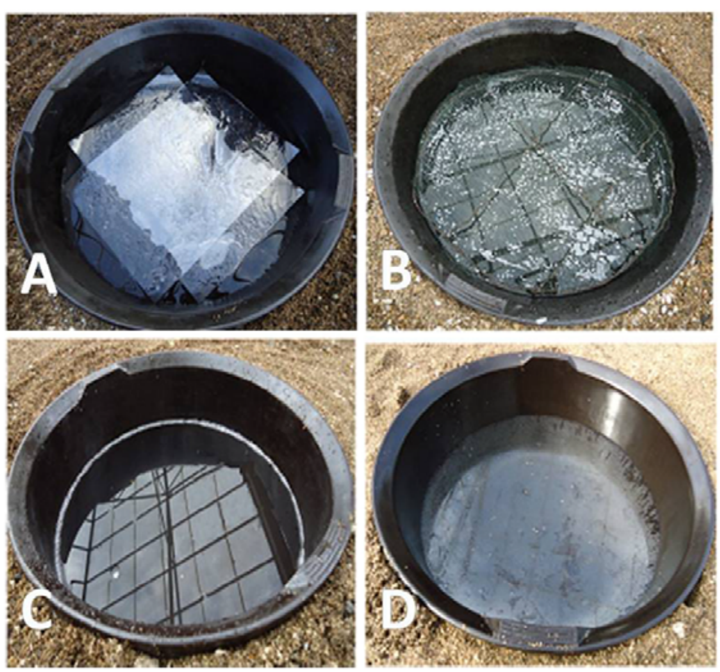

Figure 6 Surface treated artificial ponds with (A) sticky transparency, (B) sticky wire screen (C) detergent, (D) spray glue.

Data analysis

The data generated in this study were count data, i.e. either the number of gravid mosquitoes recollected or the number of eggs laid in artificial ponds, and were not normally distributed. Therefore, multivariable analyses were done with untransformed data using generalized linear models [54,55]. Data analyses were done with R statistical software version 2.13.1, including the contributing packages MASS, effects, epicalc, multcomp, lme4, gee, geepack, aod [56]. Experiments with one treatment tested in the semi-field system each day were analysed using generalized linear models with negative binomial data distribution using the glm.nb function and a log link function. Data collected for two or more treatments in the same semi-field system on the same day were not independent and were therefore analysed with generalized estimating equations using the gee or geepack function. In this case the repeated measure was the day of experiment. Here, a Poisson distribution of the data was used in the model and an exchangeable working correlation matrix. The fixed factor variables included in this model were the treatments of interest and the corner of the semi-field system (site) in which a treatment was placed. It was thought possible that the probability of catching mosquitoes might differ between the four corners (sites 1–4) of the greenhouse, independently from the test treatment, due to slightly different environmental factors such as light intensity, wind direction and microclimate. If the effect of site was insignificant this variable was removed from the final model. The output presented in the tables includes only significant factors from the final model.

The parameter estimates of the models were used to predicted the mean counts per treatment and their 95% confidence intervals (CIs) by removing the intercept from the models [54]. Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

Table 2 The development of electrocuting nets as a tool to study the orientation behaviour of oviposition site seeking *An. gambiae* s.s

Treatment	Mean no. of mosquitoes/eggs (95% CI)	OR (95% CI)	p
Experiment: Evaluation of high and low energy settings for electrocuting nets			
100% spark energy	9.0 (5.9 – 13.7)	1	
50% spark energy	19.9 (13.2 – 30.0)	2.2 (1.8 – 2.8)	<0.001
Experiment: Comparison of average mosquitocollections on transparent sticky film boards close and away from one e-net			
row 2 (>25 cm)	26.5 (16.15 – 43.49)	1	
row 1 (<25 cm)	77.63 (62.36 – 96.63)	2.93 (2.08 – 4.13)	< 0.001
Experiment: Evaluation of attraction of gravid *An. gambiae* s.s. to transparent sticky films			
without pond	29.4 (21.5 – 40.4)	1	
with pond	47.1 (37.4 – 59.3)	1.6 (1.1 – 2.3)	0.012
site 4	15.3 (11.2 – 20.7)	1	
site 2	38.4 (30.0 – 49.1)	2.5 (1.6 – 4.1)	<0.001
Experiment: Comparison of yellow, black and transparent sticky boards for the collection of gravid *An. gambiae* s.s.			
Mosquitoes*			
Transparent sticky film	24.6 (18.4 –32.9)	1 [a]	
Sticky black fly-screen	17.3 (12.0 –25.1)	0.71 (0.39 – 1.26) [a]	0.240
Yellow sticky film	0.58 (0.32 –1.09)	0.02 (0.01 – 0.05) [b]	<0.001
Eggs*			
Transparent sticky film	478 (356 – 643)	1 [a]	
Sticky black fly-screen	469 (326 – 469)	0.98 (0.80 – 1.20) [a]	0.841
Yellow sticky film	712 (525 – 712)	1.50 (1.18 – 1.92) [b]	0.001
Experiment: Comparison of average mosquito collections on yellow sticky film boards mounted under a square of e-nets			
row 2 (>25 cm)	5.1 (3.9 – 6.8)	1	
row 1 (<25 cm)	48.1 (40.7 – 56.9)	9.4 (7.7 –11.4)	<0.001
inside the square	12 (9.6 – 15.0)	2.3 (1.8 – 3.0)	<0.001

Results from generalized linear models for individual experiments.
CI=confidence interval.
OR=odds ratio.
*Multiple comparisons of treatments: treatments denoted with same letter are not significantly different.

Results

Evaluation of two spark box settings to optimize mosquito collections with e-nets

With the low energy setting, twice as many *An. gambiae s.s.* mosquitoes were collected than with the high energy setting (Table 2). Thus, the low energy setting was chosen for all subsequent experiments with gravid females.

Evaluation of cardboards mounted with transparent sticky films

In the first e-net experiment with gravid females, an average of 104.1 females (95% CI 78.0-138.9) were collected per night on the transparent film of the collection boards, representing around 50% of females released. Similar numbers were caught on both sides of the e-net, with greatest numbers close to the net in the centre (Table 2, Figure 7). This distribution indicated that most mosquitoes were electrocuted by the net but many females on the row furthest from the e-net appeared to 'sit' on the board rather than lay on the side as was the case when stunned, some were even still alive in the morning. This suggested that some females were not stunned by the net but had been attracted by the shiny film and landed on it. If this was true the number of mosquitoes on the collection board overestimated the number attracted by the water and stunned by the e-net. It was, therefore, necessary to evaluate the potential attractiveness of the collection device in the next experiments.

Evaluation of attraction of gravid *An. gambiae s.s.* to transparent sticky films

In this experiment mosquito collections were significantly affected by the corner in which the treatments were presented in the semi-field system. If any of the two treatments was set up in site 2 it was 2.5 times more likely to catch a mosquito than if it was set up in site 4 (Table 2). Adjusting for corner, the analyses showed that the sticky board alone caught approximately 15% of the released mosquitoes, while 24% were collected when the sticky board was placed next to water. These results suggest that the sticky board alone was attractive to gravid females and their landing on it was not associated with resting around a potential habitat otherwise females should not have been trapped by the boards without pond. This experiment also confirms that water vapour is a strong attractant for oviposition site seeking mosquitoes.

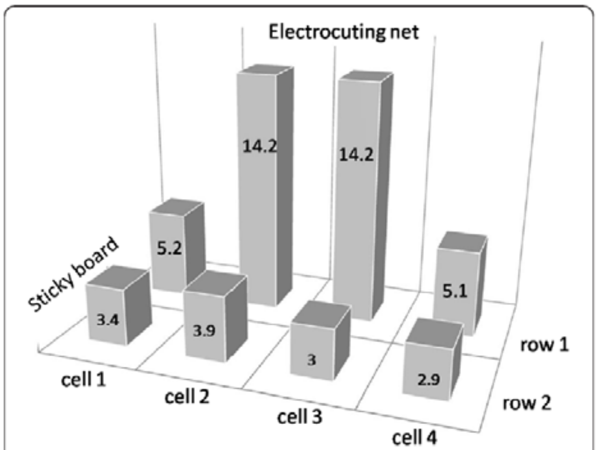

Figure 7 Distribution of electrocuted mosquitoes on the transparent sticky film collection board. The height of the columns show the average number of mosquitoes collected per cell of the grid drawn on the board

Comparison of yellow, black and transparent sticky boards for the collection of gravid *An. gambiae* s.s

Gravid females were equally attracted by the transparent sticky film and the sticky black fly-screen, yet few were collected on the yellow sticky film. Furthermore, a significantly higher number of eggs were laid in the pond behind the yellow boards than in the ponds behind the other sticky materials (Table 2, Figure 8). Yellow sticky boards did not interfere with the approach of the gravid female towards a pond and consequent egg-laying and were therefore chosen as routine collection device under e-nets.

Collection efficacy of a square of e-nets around an artificial pond

In order to estimate the number of gravid females approaching an aquatic habitat a complete square of e-nets was used that surrounded an artificial pond (Figure 5). On average one third (65.3 (95% CI 55.9 – 76.10)) of the 200 released mosquitoes were collected. Over 81% of these were found on the outside of the ring indicating that only few gravid females might have approached the oviposition site from a height above 1m

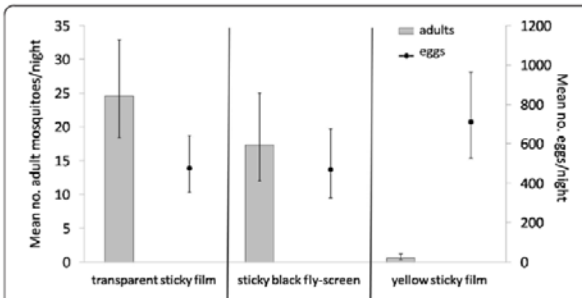

Figure 8 Mean number (error bars: 95% confidence intervals) of gravid females collected on three types of sticky boards and the mean number of eggs laid in the ponds associated with the boards.

from the ground or passed through the 8 mm gaps between the vertical aluminium frames and the wires. Low numbers of eggs were found on 6 out of 8 days. The average number of eggs was 80.3 (95% CI 43.6 – 147.8). On average over nine times as many mosquitoes were found on the sticky board close to the e-nets than further away suggesting that they were stunned by the electric nets and hence fell close to the base of the net (Table 2).

The use of sticky materials and detergents to assess if and where *An. gambiae* s.s. land on aquatic habitats when laying eggs

On average the number of females trapped on the water surfaces was over four times higher than on the edges (103.3, 95% CI 93.0-115 and 23.7, 95% CI 20–28.2, respectively) irrespective of collection device (Tables 3 and Figure 8). The detergent and the spray glue caught about

Table 3 The use of sticky materials and detergents to assess if and where *An. gambiae* s.s. land on aquatic habitats when laying eggs

Treatment	Mean no. of mosquitoes/eggs (95% CI)	OR (95% CI)	p
Experiment: Assessment of landing and egg-laying on the habitat surface			
Mosquitoes			
wire screen	11.9 (8.1 –17.6)	1[a]	
spray glue	35.2 (27.4 – 45.1)	3.0 (2.1 – 4.1) [b]	<0.001
detergent	41.7 (32.6 – 53.2)	3.5 (2.0 – 6.1) [b]	<0.001
transparency	14.6 (10.8 – 19.7)	1.2 (0.7 – 2.0) [a]	0.460
site 1	20.4 (14.3 – 29.2)	1[a]	
site 2	16.4 (12.5 – 21.6)	0.8 (0.6 – 1.0) [a]	0.070
site 3	37.5 (26.1 – 54.0)	1.8 (1.3 – 2.5)[b]	<0.001
site 4	29.0 (21.0 – 40.0)	1.4 (1.2 – 1.7) [b]	<0.001
Eggs			
wire screen	39 (23–65)	1[a]	
spray glue	464 (344 – 628)	11.9 (6.8 – 20.9) [b]	<0.001
detergent	12 (4 – 34)	0.3 (0.1 – 0.8) [c]	0.018
transparency	23 (10 – 52)	0.6 (0.3 – 1.1) [ac]	0.109
site 1	105 (42 – 259)	1[a]	
site 2	79 (29 – 219)	0.8 (0.4 – 1.6) [a]	0.546
site 3	173 (68 – 439)	1.8 (1.1 – 3.1) [b]	0.026
site 4	181 (79 – 419)	1.8 (1.3 – 2.5) [b]	0.001
Experiment: Assessment of landing on the habitat edge for egg-laying			
Mosquitoes			
spray glue	13 (9.4 – 18.0)	1[a]	
yellow sticky film	5.4 (3.0 – 10.0)	0.4 (0.2 – 0.8) [b]	0.012
transparent double-sided sticky film	5.3 (4.0 – 7.3)	0.4 (0.2 – 0.6) [b]	<0.001
site 1	4.9 (3.1 – 7.7)	1[a]	
site 2	10.8 (6.7 – 17.5)	2.5 (1.5 – 4.1) [b]	<0.001
site 3	9.0 (6.0 – 13.6)	1.9 (1.0 – 3.5)[b]	0.036
site 4	7.1 (4.1 – 12.2)	1.7 (0.8 – 2.9) [a]	0.062
Eggs			
spray glue	358 (232 – 552)	1[a]	
yellow sticky film	363 (240 – 549)	1.0 (0.6 – 1.7) [a]	0.930
transparent double-sided sticky film	297 (178 – 497)	0.8 (0.5 – 1.4) [a]	0.420
Experiment: Evaluation of sequence of landing on habitat during oviposition			
surface catch (detergent)	42.5 (37.7 – 47.9)	1	
edge catch (spray glue)	7.4 (5.1 – 10.8)	0.17 (0.12 – 0.25)	<0.001

Results from generalized linear models for individual experiments.
CI=confidence interval.
OR=odds ratio.
Multiple comparison of treatments: treatments denoted with same letter are not significantly different.

three times more mosquitoes than the sticky wire screen or transparencies (Table 3). The detergent lowered the water surface tension to such an extent that mosquitoes that landed on the water surface sunk, presenting little opportunity to lay eggs. On the other hand, a large proportion of the mosquitoes stuck on the surface with spray glue laid eggs, leading to more than 11 times higher mean egg numbers than other treatments (Table 3).

From those treatments applied to the edge of the pond, the yellow and transparent films trapped similar numbers of mosquitoes but less than half of the spray glue (Table 3). Similar egg numbers in all the treatments indicate that a similar number of gravid females approached these ponds and laid eggs. It is unlikely that all these eggs were laid by the few mosquitoes trapped on the edge. The mean number of eggs in these ponds is comparable with the mean number laid by mosquitoes stuck on the spray glue on the water surface (Table 3).

Finally, when detergent in the water was combined with spray glue on the edge of a pond, most mosquitoes were drowned in the water with only 15% stuck on the pond edge (Table 3). Notably, approximately a quarter of the released mosquitoes were collected with this method. This is only slightly less than the figures obtained from the square of e-nets where approximately one third of all released mosquitoes were collected. Eggs were not found in the pond throughout the test nights suggesting that oviposition did not take place in flight.

Discussion

Electric nets have been used successfully for the development of control tools for tsetse flies for nearly 40 years [23,29,57], yet have been used little for mosquito research [27,28]. Results presented here show that e-nets can be used to study the oviposition behaviour of malaria vectors. Importantly, it was found that reducing the voltage to prevent sparking doubled the catch, which confirms earlier findings by Torr and colleagues [28]. It is uncertain whether it is the visual, acoustic or chemical cues associated with the sparking that reduces the catch. When a single e-net was used next to an artificial pond, similar numbers of mosquitoes were collected on both sides of the net indicating that the mosquitoes approached the target from both directions. In order to quantify the total number approaching an attractive source, such as a water body, a complete square of e-nets surrounding the water was found useful. Field tests need to evaluate the performance of the e-nets for studying gravid mosquitoes under open field conditions, especially during rainy seasons the normal periods of maximum malaria transmission. Previous work on host-seeking *An. arabiensis* has shown that e-nets covered with a small roof work well even when it rains [28].

Sticky boards proved to be a simple method for collecting mosquitoes that were stunned after colliding with the net and fell to the ground since they effectively retained specimens and protected them from predation by ants. However, it was found that a transparent film was also attractive to gravid mosquitoes, even when used as sole collection device without any e-nets and without a water source nearby. Adding an artificial pond behind the transparent film sticky board increased the number of females trapped on the board confirming that water vapour is a strong attractant for oviposition site seeking mosquitoes [40,58,59].

In search of an alternative collection material under e-nets, the black fibreglass gauze coated with insect glue proved as attractive to gravid mosquitoes as transparent film. Both surfaces were conspicuously shiny for the human eye compared to the yellow film that appeared matt and might act as a visual cue for gravid females. Previously, black flies of all physiological stages have been successfully trapped with glue coated aluminium plates [60-62] and in a recent study, Harris and colleagues [63] utilized this principle to collect gravid mosquitoes from water surfaces using glue-coated transparencies. Many insects, including mosquitoes, respond to reflectance of water surfaces to locate water bodies to lay their eggs, often using horizontally polarized light reflected from the water surface as orientation cues [64-69]. Surfaces with high polarized light reflectance might be promising as trapping devices alone or in combination with a gravid trap for monitoring African malaria vectors. Nevertheless, it can not be excluded that a chemical cue associated with the insect glue attracted the gravid females and there is need to further investigate the properties of the glue-coated surfaces used in this study.

The low number of mosquitoes on the yellow sticky film and the high number of eggs laid in the adjacent pond suggest that this material does not have the same visual properties for a mosquito as the transparent film and black glue boards and does not attract mosquitoes. Oviposition site-seeking females fly straight to the pond to lay their eggs, and then fly off again, without landing close to the aquatic habitat before or after egg-laying. This might be due to the light colour [70] and the lack of reflectance. It is unlikely that it has to do with the actual colour of the board since mosquitoes have dichromatic vision, which results in good contrast sensitivity but poor colour resolution [71]. It is known that mosquitoes respond to contrasts [64,70] and gravid females are attracted by dark surfaces rather than light coloured ones [64].

The number of mosquitoes collected with transparent sticky boards was approximately twice the number collected with yellow sticky boards. It is likely that transparent films overestimated the number of mosquitoes that approached the pond when they were used in combination with e-nets but sticky boards made of the yellow film can serve as effective collection device. On the other hand, the attractiveness of the boards mounted with transparent sticky film might be exploited further in future for the development of new trapping devices for gravid malaria vectors.

For the development of new interventions (e.g. auto-dissemination of larvicides [72-74]) and monitoring tools (e.g. ovi-traps and gravid traps [18-20,38,47-53]) targeting gravid malaria vectors it is important to know if and where gravid females land during oviposition. Notably, very few studies have investigated this and all these studies used relatively small cages (less than $1m^3$) except one which was implemented under field conditions [63]. Gravid females were most commonly observed laying their eggs directly, either laying eggs when on the water surface or on the lip of the oviposition cup [59,64,75]. Occasionally, oviposition from flight has been described when the oviposition cup was placed over a black surface [64]. Here, for the first time, experiments in large semi-field systems are described that investigate if and where *An. gambiae* s.s. lands to lay her eggs. The results indicate that gravid females primarily land directly on the water surface to lay eggs. Since no eggs were found in ponds with both detergent and sticky sides, which prevents directly egg-laying on the water surface, there is no evidence for eggs being dropped in flight onto the water from these experiments. The relatively large number of eggs found associated with females caught on the spray glue applied on the water surface was probably due to stress induced oviposition on the surface [63].

Similar numbers of eggs were laid in ponds treated with different sticky materials at their edges, though the number of adults caught on the edges differed, the number of adults caught there was small. This suggests that even mosquitoes caught at the edge might have landed there to rest before or after laying eggs, rather than to lay whilst seated on the edge of the pond. In the case of the pond with spray glue at the edge attraction of female mosquitoes cannot be excluded since the numbers were significantly higher than for the other two treatments and the glue made the pond edge appear very shiny.

Some caution must be exercised when interpreting the data since the artificial ponds used in this study had a sharp vertical edge which was not utilized by gravid females to sit on and lay eggs. This might have been different if ponds with a slope would have been used. Previous cage experiments have shown that *An. gambiae* s.s. and *Anopheles arabiensis* laid a large proportion of eggs on water saturated slopes rather than the free-standing water when given a choice [58,75,76]. Nevertheless, even then it was observed that these eggs were laid whilst on the water surface rather than during flight [75].

The finding of this study that *An. gambiae* s.s. lays its eggs directly on the water surface supports the observations made on *An. arabiensis* by Harris and colleagues [63] in the field using transparencies floating on the water on the edge of natural habitats. The finding that gravid *An. gambiae s.s.* lay their eggs directly on the water surface is encouraging for two reasons. Firstly, it lends support to the principle that gravid females could be used to transfer larvicides from a resting site to a breeding site [72-74,77]. Secondly, it may lead to the development of a gravid trap where mosquitoes are attracted to a water source and trapped there [20].

Sticky materials and the detergent used in this study were shown to be useful methods for collecting mosquitoes when landing to lay eggs. Of all the tools tested the detergent and the spray glue directly applied to the water surface was most effective at collecting gravid females under semi-field conditions. Transparencies and sticky screens did not work as well which might be due to obstruction of water vapour coming off the pond by the transparency or due to visual obstruction of the water surface area. The latter two might have been useful tools for testing the attraction of female vectors towards a water source that was treated with putative oviposition semiochemicals or natural infusions [40] but due to their reduced trapping efficiency e-nets might be the best alternative for analysing such odour-oriented behaviour. Detergents and spray glue, though powerful in arresting approaching females, might interfere with the presented chemical or infusion. Therefore, further research would be required to present these in combination for attracting and trapping gravid female mosquitoes. The use of these tools under natural conditions also needs to be further evaluated.

Conclusion

This study demonstrated that electric grids are suitable devices for studying the egg-laying behaviour of *An. gambiae s.s.* when used in combination with yellow sticky boards for collecting stunned mosquitoes. Shiny sticky surfaces attract gravid females possibly because they are visually mistaken as breeding sites. These materials might be developed further as gravid traps. *Anopheles gambiae* s.s. primarily land on the water surface for oviposition. This behaviour can be exploited for the development of new trapping and control strategies.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UF, JL and ST conceived the idea for this research. UF, JL, SD and ST developed the experimental design and SD developed all protocols and implemented the experiments. SD and UF analysed the data and drafted the manuscript. SWL and FO contributed to the development of the protocols. All authors contributed to the final draft, read and approved the manuscript.

Acknowledgements
We would like to thank David Alila, Peter Ongele and Jackton Arija from the insectary at *icipe*-TOC, Mbita for providing mosquitoes for experiments and Benard Oyembe, Oscar Mbare, Mike Okal and Arthur Sune for technical assistance. We thank Richard Mukabana for comments on the study design. We extend our gratitude to Johan Esterhuizen, Kate Buck and Terri O'Halloran for helping with sourcing supplies. SD was supported by the African Regional Postgraduate Programme in Insect Sciences/International Centre of Insect Physiology and Ecology (ARPPIS/icipe) and DAAD. SWL was supported by the Research and Policy for Infectious Disease Dynamics (RAPIDD) Program of the Science and Technology Directory, Department of Homeland Security, and Fogarty International Center, National Institutes of Health. This project was funded through a National Institute of Health (NIH) grant no. R01AI082537.

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Received: 19 July 2012 Accepted: 31 October 2012
Published: 14 November 2012

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doi:10.1186/1475-2875-11-374
Cite this article as: Dugassa *et al.*: Electric nets and sticky materials for analysing oviposition behaviour of gravid malaria vectors. *Malaria Journal* 2012 **11**:374.

APPENDIX 5: PUBLISHED PAPER 2

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Development of a Gravid Trap for Collecting Live Malaria Vectors *Anopheles gambiae s.l.*

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Abstract

Background: Effective malaria vector control targeting indoor host-seeking mosquitoes has resulted in fewer vectors entering houses in many areas of sub-Saharan Africa, with the proportion of vectors outdoors becoming more important in the transmission of this disease. This study aimed to develop a gravid trap for the outdoor collection of the malaria vector *Anopheles gambiae* s.l. based on evaluation and modification of commercially available gravid traps.

Methods: Experiments were implemented in an 80 m^2 semi-field system where 200 gravid *Anopheles gambiae s.s.* were released nightly. The efficacy of the Box, CDC and Frommer updraft gravid traps was compared. The Box gravid trap was tested to determine if the presence of the trap over water and the trap's sound affected catch size. Mosquitoes approaching the treatment were evaluated using electrocuting nets or detergents added to the water in the trap. Based on the results, a new gravid trap (OviART trap) that provided an open, unobstructed oviposition site was developed and evaluated.

Results: Box and CDC gravid traps collected similar numbers (relative rate (RR) 0.8, 95% confidence interval (CI) 0.6–1.2; $p = 0.284$), whereas the Frommer trap caught 70% fewer mosquitoes (RR 0.3, 95% CI 0.2–0.5; $p < 0.001$). The number of mosquitoes approaching the Box trap was significantly reduced when the trap was positioned over a water-filled basin compared to an open pond (RR 0.7 95% CI 0.6–0.7; $p < 0.001$). This effect was not due to the sound of the trap. Catch size increased by 60% (RR 1.6, 1.2–2.2; $p = 0.001$) with the new OviART trap.

Conclusion: Gravid *An. Gambiae* s.s. females were visually deterred by the presence of the trapping device directly over the oviposition medium. Based on these investigations, an effective gravid trap was developed that provides open landing space for egg-laying *Anopheles*.

Citation: Dugassa S, Lindh JM, Oyieke F, Mukabana WR, Lindsay SW, et al. (2013) Development of a Gravid Trap for Collecting Live Malaria Vectors *Anopheles gambiae s.l.*. PLoS ONE 8(7): e68948. doi:10.1371/journal.pone.0068948

Editor: John Vontas, University of Crete, Greece

Received March 12, 2013; **Accepted** June 5, 2013; **Published** July 5, 2013

Funding: SD was funded by the African Regional Postgraduate Programme in Insect Sciences, International Centre of Insect Physiology and Ecology, Kenya (ARPPIS/icipe). Research funds were provided through a National Institute of Health (NIH) grant no. R01AI082537 supporting the OviART project (Oviposition of Anopheles gambiae: Attractants, Residual Larvicides and Traps). SWL received support from the Research and Policy for Infectious Disease Dynamics (RAPIDD) Program of the Science and Technology Directory, Department of Homeland Security, and Fogarty International Center, National Institutes of Health. The funders had no role in study design, data collection and analyses, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Vector control plays a central role in the prevention of malaria [1–5]. Monitoring vector populations and assessment of disease risk are among the key elements of vector management strategies [6–9]. So far various tools have been developed and utilized for sampling mosquito vectors and the pathogens they transmit [10–13]. In sub-Saharan Africa (SSA) the most commonly used sampling methods for malaria vectors are human landing catches, CDC light traps, and pyrethrum spray collections which are excellent for sampling host-seeking and indoor resting mosquitoes [14–16]. Effective vector control targeting host-seeking and resting mosquitoes indoors has resulted in a reduction in the number of mosquitoes entering and resting in houses [11,17–24] rendering some of these tools less effective for monitoring potential vector populations [25].

Collecting malaria vectors outdoors becomes increasingly important as surveillance tools; and effective traps might even be used for control purposes [26]. To date outdoor vector collections target either resting populations with pit trap [27,28], pot traps [13], resting boxes [29] and aspirator collections or host-seeking mosquitoes with animal-baited traps [13,30,31], human baited tent traps [32] or human landing catches [14,16] and, more recently, odour-baited MM-X traps [33]. In general, outdoor sampling is far more challenging than indoor sampling since the outdoor vector population is more dispersed over the landscape. Resting catches often underestimate actual vector densities [19] and odour-baited traps only target a proportion of the host-seeking population (either attracted to animals or to humans). Furthermore, animal or human-baited traps are complicated to organise and are inappropriate for using on a large scale.

Gravid traps would provide an important and novel sampling tool for sampling both endophilic and exophilic vector populations in search of an oviposition site, a grossly understudied phase in the life cycle of *Anopheles* mosquitoes. Gravid mosquito traps are designed to catch gravid females in search of an aquatic habitat. Such traps are used routinely for the surveillance of *Culex* and *Aedes* vectors [34,35] but not for gravid *Anopheles gambiae* s.l., the major malaria vector in SSA, Harris and collegues [36] have recently developed the first sampling tool for this species consisting of a transparent acetate sheet coated with insect glue that is placed on the water surface on the edge of a natural habitat. Their pilot study in an area of high vector densities showed that gravid culicines and anophelines in search of an oviposition site landed and got stuck on the transparencies [36]. Further studies need to confirm the effectiveness of this sticky transparency in areas of low mosquito and habitat densities. Here we were interested to evaluate suction traps that sample mosquitoes alive, that provide the opportunity to be moved around and do not depend on the presence of natural habitats. The first was developed by Reiter in 1983 [37] to collect gravid *Culex* and *Aedes* mosquitoes for West Nile virus isolation [34,38,39] and many gravid mosquito traps have been developed and modified since then [38,40,41]. Among these, the CDC gravid trap Model 1712, commonly referred to as CDC gravid trap, the CDC gravid trap Model 1719 commonly referred to as Frommer updraft gravid trap and the Box gravid trap (also referred to as Reiter-Cummings gravid trap) [38] are commercially available. These widely used traps are suction traps for the collection of live, gravid culicines [38,40]. However, to our knowledge, none of these have been purposely evaluated for collecting *An. gambiae* s.l. nor anophelines in general.

Here we aimed to investigate the factors that impact on the catching efficiency of these commercially available traps. Based on the results a new prototype gravid trap for the collection of malaria vectors was developed.

Materials and Methods

Study area

The study was carried out using a semi-field system [42] located at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (icipe-TOC), Mbita, on the shores of Lake Victoria, Kenya (0° 26′ 06.19″S, 34° 12′ 53.13″ E; altitude 1,137m above sea level). This area is characterised by a consistent tropical climate with an average minimum temperature of 18°C and an average maximum temperature of 28°C (based on data from icipe-TOC meteorological station for 2010-2012). 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 1,436mm.

The semi-field system and study design

The semi-field system was a screened building 7.1 m wide, 11.4 m long and 2.8 m high at the wall and 4.0 m high at the highest point of the roof. The two opposite shorter (7.1m wide) walls and roof were made of glass and the two long walls were screened with black fibre glass netting gauze (1.7 × 1.5 mm). The floor was covered with sand to a depth of 30 cm so that artificial aquatic habitats could be dug into the ground to simulate a natural larval habitat for the *An. gambiae s.s.* mosquitoes [43]. To increase the relative humidity inside the semi-field system to 60-70% the sand floor was watered from 15:00-16:00 h prior to the experiment. Care was taken to ensure that no pooling of water occurred on the floor and that the upper layer of sand was dry when the mosquitoes were released. Treatments were positioned in the corners of the semi-field system at a distance of 1.5 m from the two adjacent walls (site 1-4) and mosquitoes released from the centre (site 5; Figure 1). Experiments were conducted using randomized complete block designs (RCBD) and replicated for 12 nights. The number of replications was based on sample size considerations for comparing proportions of clustered data [44]. The preliminary data for this were generated by setting two identical artificial ponds in opposite corners of the semi-field system. The water in the ponds was treated with detergent as described in detail by Dugassa and colleagues [43]. Two hundred visually presumed gravid females (see details below) were released in the evening and the number of mosquitoes drowned in each pond was counted the next morning. This was done for 12 nights. The results showed that when presented with an identical treatment the gravid females approached both ponds in an equal proportion ($p1=0.5$). The variability of the nightly catches was used to calculate the coefficient of variation (ratio of standard deviation/mean) which was 0.26. At this ratio, replication of the experiment over 12 nights, assuming 100 responders out of 200 released mosquitoes per night, had 80% power to detect an increase or decrease in the catch rate of 20% ($p2=0.7$) at the 5% level of significance. This level of accuracy was appropriate for developing new traps for gravid *An. gambiae s.s.*. since we were looking to develop traps that were markedly better at collecting gravid mosquitoes than established traps, thus were interested in designs that increased trapping by at least 20%.

Mosquitoes

Insectary-reared *An. gambiae* s.s. mosquitoes were used throughout. Gravid mosquitoes were prepared as follows; 300 female and 300 male mosquitoes, two to three days old, were selected from the rearing cages at midday and kept in 30 cm × 30 cm × 30 cm netting cages at ambient conditions of 25-28°C and a relative humidity of 68-75%. Water saturated cotton

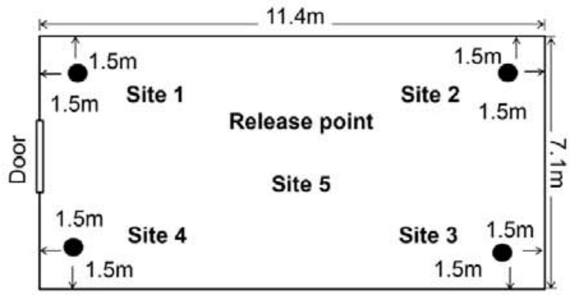

Figure 1. Schematic drawing of the dimension of the semi-field system, the treatment sites (1–4) and release point (site5).

doi: 10.1371/journal.pone.0068948.g001

towels (50 x 25cm) were folded and placed over the cages to avoid mosquito desiccation. Mosquitoes were starved of sugar for seven hours prior to blood feeding and allowed to feed on a human arm for 15 minutes at 19.00h on the same day. The same procedure was repeated 24 hours later. After the first blood meal unfed female mosquitoes were removed from the cages. After feeding mosquitoes were provided with 6% glucose solution *ad libitum*. Fed female mosquitoes were kept together with males for two days after the second blood meal before they were utilised in an experiment (i.e. females 4-5 days after first blood meal). At 16.30 h at the day of experiment 200 females with an enlarged, pale white abdomen were collected from the holding cage and visually presumed gravid (henceforth referred to as gravid females). A small proportion of these mosquitoes might not have been gravid because most females need two blood meals to reach full gravidity [45] and some never reach full gravidity even after three feeds [46]. Whilst we provided two meals we cannot guarantee that two meals were taken by all females.

Gravid Traps

The Box gravid trap (BioQuip, Rancho Dominguez, CA), CDC gravid trap (John W. Hock Company, Gainesville, FL)) and Frommer updraft gravid trap (John W. Hock Company, Gainesville, FL) were used in this study. These traps attract egg-laying females to a water-based oviposition medium added to bowls below the trapping device. The bowls' size varied slightly between different trap models. According to the manufacturers' recommendations oviposition medium was filled in the bowls to a level of about 3 cm below the opening of the intake ducts. This is equivalent to 8 L of oviposition medium in the Box gravid trap, 6 L in the CDC and 5 L in the Frommer trap. All traps operate by drawing air from the surface of the bowls and distributing any volatile chemicals associated with the oviposition medium, including water vapour, to the surrounding of the traps. Depending on the design of the trap and the location of the air intake duct, the air plume varies amongst the traps [40,47]. In all traps mosquitoes are sucked into a collection chamber while they evaluate the potential oviposition site and prepare to lay eggs. The collection chambers are found on top of a suction tube in the Box and Frommer updraft gravid traps to avoid exposure of mosquitoes to the aspiration fan. However, the collection chamber of the CDC gravid trap is placed above the aspiration fan [38,40] so that some mosquitoes are damaged by the rotating fan [48,49]. The water basins of the traps are usually placed on the ground for collection of *Aedes* and *Culex* mosquitoes (Figure 2A, B, C) which will readily lay eggs in containers. However, *An. gambiae* s.l. usually prefer natural habitats so here we aimed to reduce the container impression by sinking the bowls in to the ground for all experiments, where we refer to the water-filled bowls as ponds (Figure 2D).

Figure 2 shows the three traps. The Box gravid trap was set up by fitting the anti-spread bars that are found under the horizontal exhaust tube to the black bowl (16.5 L volume, 44 cm long, 34 cm wide and 12 cm deep). The black conducting duct with the large 'O' ring around it was placed into the hole at the bottom of the case. The wire screen of the collecting chamber was placed on the outside of the collecting duct with the intake screen facing the exhaust tube. The CDC gravid trap was set up by placing the aluminium supports of the trap on the rim of the pan (24 L volume, 44 cm long, 34 cm wide and 17 cm deep) and slipping the collection bag over the upright tube. The sleeve was slipped downwards towards the aluminium supports until the bottom of the bag rested on the top end of the trap. Setting up the Frommer updraft gravid trap involved fitting the rain shield with aspiration fan to the trap and setting it into the base stand. The parts were attached tightly. The base stand was placed in the black pan (24 L volume, 44 m long, 34 cm wide and 17 cm deep) so that the feet rested inside the tray.

For all experiments piped non-chlorinated water from Lake Victoria was filtered through a sand-charcoal filter and used in the containers of the traps as oviposition medium. All the gravid traps were operated by a fully charged 6 volt 12 Ah battery (Universal battery UB6120). Experiments were started at 17.30 h by releasing 200 gravid mosquitoes at the centre (site 5) of the semi-field system and stopped at 8.00 h the following day. Any live mosquitoes remaining in the semi-field system were removed and killed. The collection chambers from the traps were kept in a freezer for 30 minutes to kill the mosquitoes.

Trap comparison

In the first experiment the trapping efficacy of the Box gravid trap, CDC and Frommer updraft gravid trap was compared. In addition to the three traps an open pond made of a similar bowl but without a trapping device was set up in the semi-field system. The open pond was positioned in the same site each night (site 4). Traps were rotated over the 12 nights between sites 1, 2 and 3. The purpose of the open pond was to serve as a reference to compare mosquito responses from night to night and to compare the relative attractiveness of a 'natural' water body with ones that had gravid traps. The number of mosquitoes trapped in the collection chambers of the traps and the number of eggs laid in each pond was recorded nightly. To count the number of eggs the water from the bowls was filtered through a filter paper (Fisherbrand, QL 125) using a water suction vacuum pump. The bowls were rinsed with additional water and white filter paper passed slowly along the edges of the bowls to detect any more eggs that might have remained after rinsing.

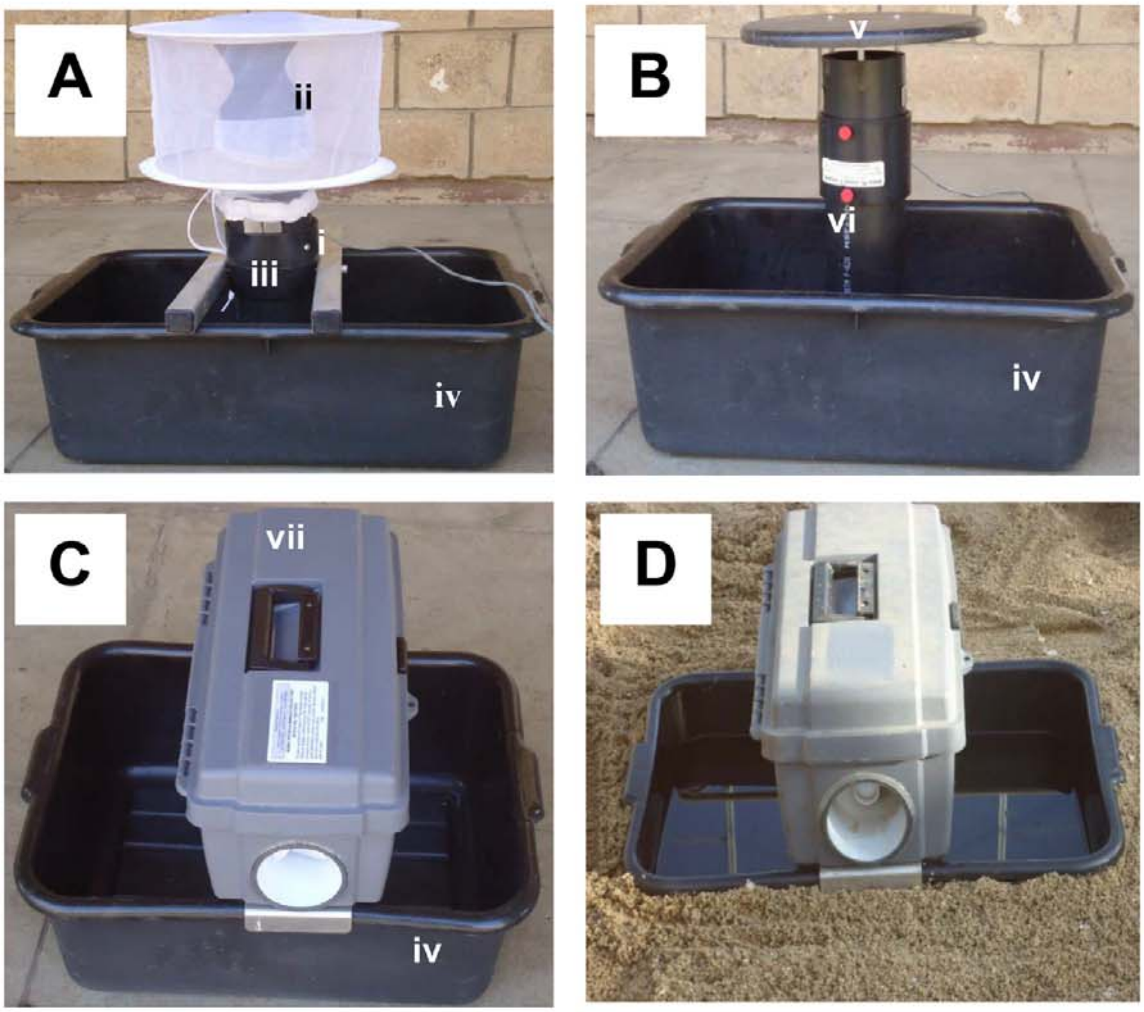

Figure 2. Set up of three gravid traps. (A) CDC gravid trap, (B) Frommer updraft gravid trap; (C) Box gravid trap. i) aluminium supports, ii) collection bag, iii) upright tube, iv) pan, v) rain shield, vi) base stand, vii) horizontal exhaust tube, viii) anti-spread bar; (D) Box gravid trap lowered into ground in semi-field system.
doi: 10.1371/journal.pone.0068948.g002

Trapping efficacy of the Box gravid trap

After the first experiment the Box gravid trap was chosen for further evaluation to investigate factors that might affect catch size. Experiments were designed to assess if the position of the Box trap on top of the pond or the sound of the fan affected the number of gravid females that approach the trap. In the following experiments, 8L filtered-lake water was used to prepare the ponds.

The first experiment had a fully-functional Box gravid trap in one corner of the semi-field system (Figure 3A) and an open pond (pond alone without trapping device) in the opposite corner (Figure 3B). In the second experiment the fan of the Box gravid trap was switched off to assess if the sound of the trap affected the number of gravid *Anopheles* mosquitoes approaching the pond. Here we compared a non-functional Box trap with an open pond in the opposite corner of the semi-field system. In both experiments treatments were rotated between all sites (site 1-4). To analyse the orientation of gravid females towards either of the ponds they were surrounded by a complete square of electrocuting nets (Figure 3). The adjacent wires of the nets were powered by a 12 V 50 Ah lead acid battery (Chloride Exide Ltd, Kenya) via a spark box (Alan Cullis, South Africa) adjusted to a low spark energy setting that did not produce any sound or spark but killed the mosquitoes that touched the net while approaching the pond. A detailed description of the electrocuting nets and spark box specifications can be found in a recent publication [43]. Yellow sticky films mounted on strips of cardboard served as collection

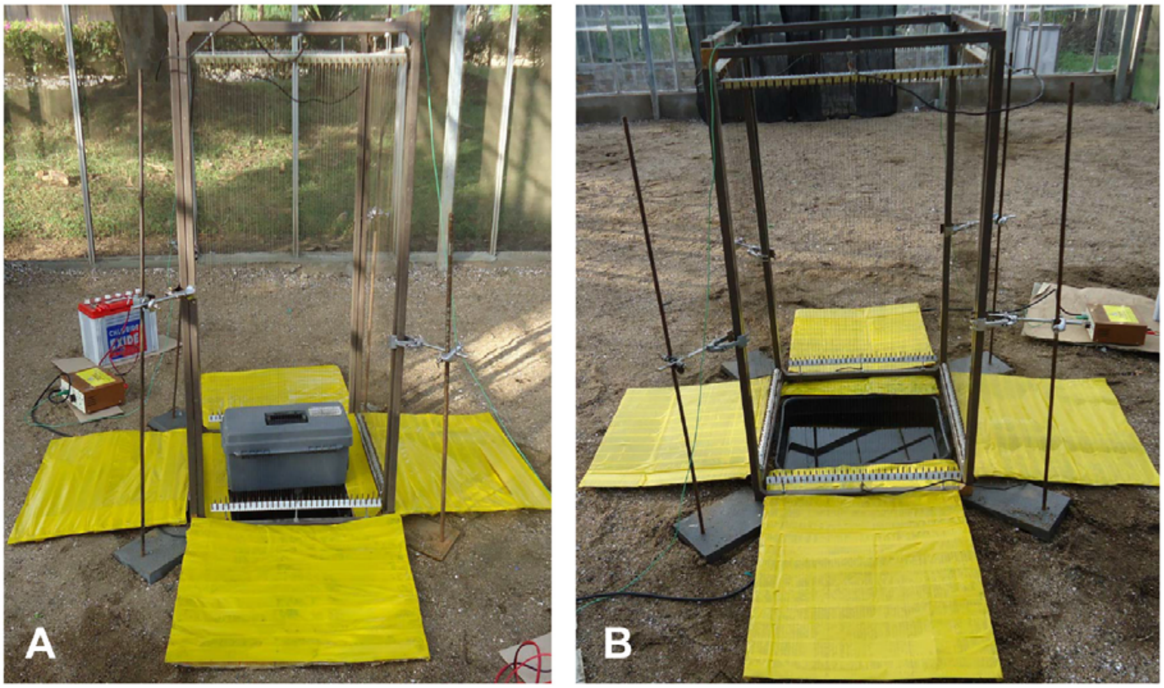

Figure 3. Box gravid trap (A) and open pond (B) surrounded by a square of electrocuting nets.
doi: 10.1371/journal.pone.0068948.g003

boards (Figure 3). These were placed under each net outside the closed square (50 x 60 cm) and in the gaps between the two longer sides of the bowls and the net (53 x 7 cm). The number of mosquitoes approaching either pond was estimated by counting the number on the net and on the collection boards.

Development of a new gravid trap (OviART gravid trap)

Results from previous experiments indicated that the presence of a Box on top of a pond affected the number of gravid females approaching this pond. Therefore we moved the Box trap from directly above the water of the pond to one side of the pond on the sand. The Box trap was positioned 50 cm from the edge of a pond and compared with an open artificial pond. The trap was not switched on as the aim was to test if the removal of the Box from the surface of the pond to the side would improve mosquitoes' approach to the pond or if the presence of a box close to the pond would also deter mosquitoes. To quantify the number of mosquitoes visiting the two ponds 200 ml detergent (Teepol Industries LTD, Nairobi) was added to the water of each pond [43].

Based on our findings from the above experiments we constructed a new prototype gravid trap ('OviART gravid trap' named after the research project funding this work on **O**viposition of *Anopheles gambiae*: **A**ttractants, **R**esidual larvicides and **T**raps) where the collection chamber and fan were positioned on one side of the pond (Figure 4). A black round bucket (20 cm high and 30 cm in diameter) filled with 8 L of filtered-lake water served as oviposition site. An oval slit (13 cm wide and 5 cm high) was cut 5 cm below the lip of the bucket into which a collapsible pipe (30 cm long and 10.2 cm in diameter) was inserted. This pipe was connected to a collection chamber made out of a water plastic bottle as described below. At the end of the collection chamber another 30 cm collapsible pipe and a fan of 12 V and 0.38 Ah current output (as opposed to 6 V and 0.1 Ah of the Box gravid trap) was fixed to create strong air suction. A strong suction of air from the entire water surface was needed to compensate for the reduction in airflow as a result of moving the suction point from above the pond to the side. The fan sucked air into a collection chamber (20 cm long and 10 cm in diameter) which was prepared from a plastic water bottle of 1 L volume and black fiber glass netting gauze (1.7 mm × 1.5 mm mesh size). A piece of netting gauze (15 cmx15 cm) was cut and prepared into a conical funnel with a 2.5 cm wide hole at the narrower end and 10 cm at the wider end. It was then fixed at the inlet side of the water bottle with the narrower opening of the funnel positioned inside the bottle. This narrow inlet minimized risk of escape of mosquitoes even when the power stopped due to battery failure. Another piece of the gauze (18 cm × 18 cm) was cut and tied to the opposite side of the bottle towards the fan (Figure 4).

The OviART gravid trap was set by sinking the water-filled bucket into the ground so that the lip of the bucket was flush with the sand surface. The suction tube was buried in the sand leaving only the end with the fan exposed above the soil in order to let airflow freely (Figure 5). Gravid mosquitoes passed through this tube into the collection chamber. The fan was

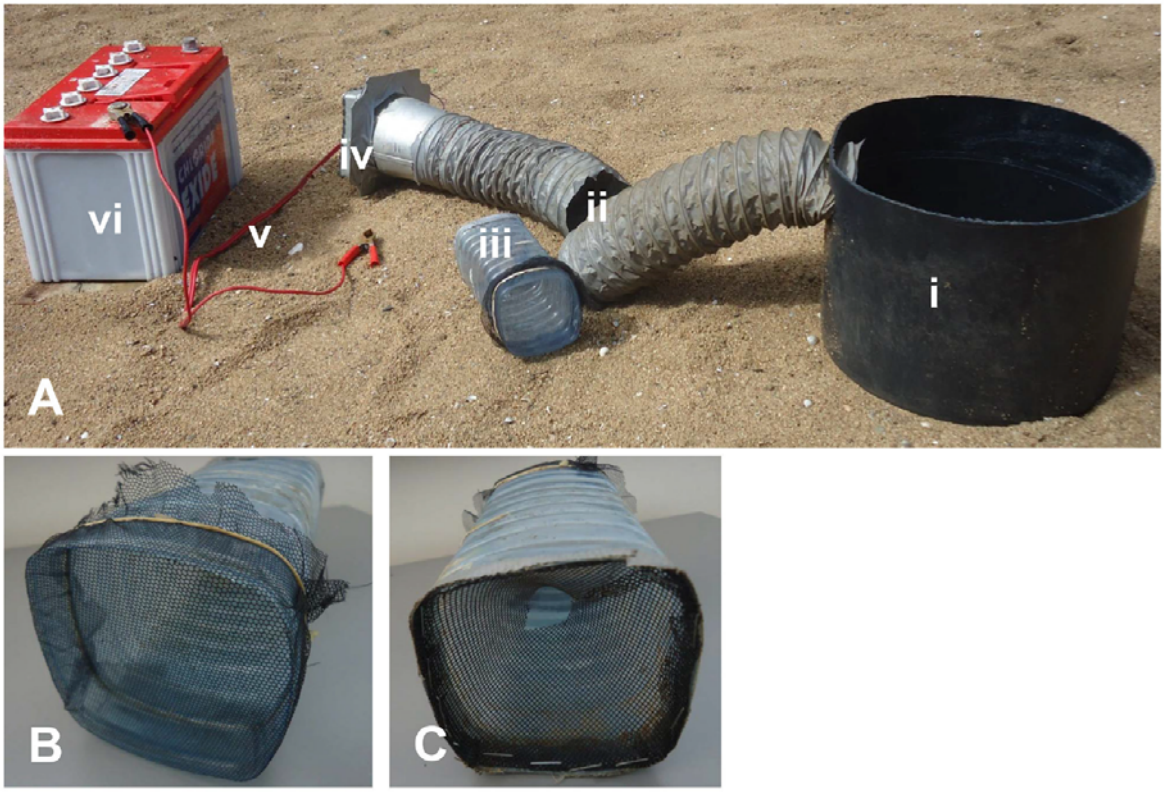

Figure 4. Ovi-ART gravid trap prototype. (A) Trap parts: i) round black bucket, ii) suction tube prepared from collapsible plastic pipe, iii) collection chamber prepared from plastic bottle and fiber glass netting gauze, iv) fan (12V, 0.38A), v) electric cable, vi) 12V battery. (B) Collection chamber backside towards fan. (C) Collection chamber entry funnel.

doi: 10.1371/journal.pone.0068948.g004

powered by a 12 V 50 Ah lead acid battery (Chloride Exide Ltd, Kenya). The trapping efficacy of the OviART gravid trap was compared with the Box gravid trap in two choice bioassays. The number of mosquitoes caught in the traps and the number of eggs in the ponds was recorded.

In a final step a single OviART prototype trap was tested nightly for 12 nights in a semi-field system. The intention was to determine the proportion of released *An. gambiae s.s.* that could be collected by the trap. The trap was rotated randomly between all four sites in the semi-field system. The number of mosquitoes trapped was recorded.

Data analysis

The data were analysed using generalized linear mixed effects models. The analyses were done with R statistical software version 2.14.2 including the contributing packages MASS, lme4, glht and multcomp [50]. Previous experiments [43] have shown that mosquito responses are highly variable between different batches of mosquitoes and between different sites in the greenhouse irrespective of the test treatments. Therefore, the night of experiment (same batch of mosquitoes) and location (site) where the traps were placed in the semi-field system were included in the models as random factors. To adjust for excess variation between rows (data points) recording the number of trapped mosquitoes (overdispersion) a factor was created with a different level for each row of the data set and also included as a random factor in the model. The experimental treatments were entered as fixed effects. A Poisson distribution of the data with a log link function were used. All mean counts per treatment and their 95% confidence intervals (CIs) were calculated as the exponential of the parameter estimates for models with no intercept included [51]. Similarly, multiple comparisons of treatments were calculated based on the model parameter estimates.

Results

Trap comparison

An. gambiae s.s. females were caught in all three traps in the semi-field system (Figure 6), but the total mean number trapped per night was low (59.3, 95% CI 50.3–70.0) i.e. <30% of released mosquitoes were recovered by the three traps. The

Figure 5. The OviART gravid trap set up. Trap was set by sinking the water-filled bucket into the ground so that the lip of the bucket was flush with the sand surface. The suction tube was buried in the sand leaving only the end with the fan exposed above the soil in order to let airflow freely.

doi: 10.1371/journal.pone.0068948.g005

Table 1. Results of the statistical analyses of the individual experiments implemented to develop a new gravid trap for *Anopheles gambiae s.s.*

Treatment	Mean (95% CI)*	RR (95% CI)	p
Response of gravid *An. gambiae s.s.* to a pond with an operating Box gravid trap			
Pond only	62.1 (40.2–95.8)	1	
Trap over pond	40.1 (25.9–62.1)	0.7 (0.6–0.7)	< 0.001
Response of gravid *An. gambiae s.s.* to a pond with a soundless Box gravid trap			
Pond only	51.2 (39.6–66.1)	1	
Soundless trap over pond	36.9 (28.4–47.9)	0.7 (0.6–0.8)	<0.001
Response of gravid *An. gambiae s.s.* to a pond with a Box gravid trap next to it			
Pond only	32.5 (22.8–46.5)	1	
Trap next to pond	33.4 (23.4–47.7)	1.0 (0.9–1.2)	0.693
Comparison of trapping efficacy of the prototype OviART gravid trap and the Box gravid trap			
Box gravid trap	25.2 (19.1–33.3)	1	-
OviART gravid trap	41.3 (31.6–53.9)	1.6 (1.2–2.2)	0.001

CI = confidence interval

RR = relative rate

* predicted by using the parameter estimates of the mixed effects model.

Box gravid trap and the CDC gravid trap collected similar numbers of mosquitoes (RR 0.8, 95% CI 0.6–1.2; p= 0.284). In contrast, it was 70% less likely to collect a mosquito with the Frommer updraft gravid trap (RR 0.3, 95% CI 0.2–0.5; p < 0.001) compared with the other two traps (Figure 6). On average, 858 (95% CI 570-1291) eggs were collected from the open pond per day, indicating that the low catch numbers in the traps were not because released mosquitoes did generally not respond due to environmental factors or because they were not gravid. Rather it appears that the females preferred to approach the open pond than a pond with a trap on top. Only few and similar numbers of eggs were found in ponds with traps (average for all three traps combined 125.1 (95% CI 82.8–189.0). This implies that most females that approached the pond were sucked into the trap before getting an opportunity to lay eggs, so low catch sizes are probably not due to weak suction of the fans.

Trapping efficacy of the Box gravid trap

Based on the trap comparisons the Box gravid trap was selected for further evaluation in the following series of experiments since it caught the greatest number of mosquitoes and provided protection for battery, cables and mosquitoes which would be an added advantage when used in the field during wet weather [38,40].

The number of mosquitoes approaching the Box gravid trap was reduced by 30% compared to the number that approached the open pond, irrespective of whether the trap was switched on, creating a distinct sound, or switched off and silent (Table 1). This suggested that the presence of the Box on top of the pond deterred mosquitoes from approaching the site and led to the next experiment.

Development of a new gravid trap

A test was designed where the number of mosquitoes that visited a pond with a Box gravid trap set next to it was

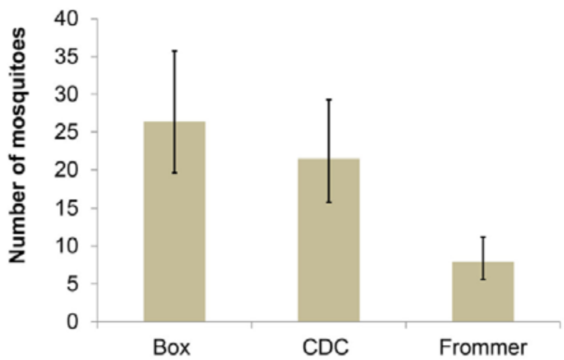

Figure 6. Mean *Anopheles gambiae s.s* catch sizes of CDC, Frommer updraft and Box gravid traps. Error bars equal the 95% confidence intervals; 200 gravid females were released in semi-field system per night for 12 nights.
doi: 10.1371/journal.pone.0068948.g006

compared with a pond without a trap. Similar numbers of females visited the two ponds (Table 1).

Based on the analyses of factors that affected the approach of gravid *An. gambiae s.s.* to a Box gravid trap a prototype of a new gravid trap (OviART gravid trap) was developed. Here the catching device was moved to the side of the pond; 60% more *An. gambiae s.s.* were collected by the OviART gravid trap prototype than the Box gravid trap (Table 1). A large difference was found in the egg numbers recovered from the ponds of the two traps. Eggs were only found on three out of the 12 collection nights in the pond of the new OviART trap (in total 87 eggs). In contrast, eggs were found nightly and nearly 19 times more eggs (total 1652) were laid in the pond of the Box gravid trap over the 12 nights period. When evaluated alone, the OviART prototype recollected approximately one third of the released mosquitoes in the single choice bioassay (31.9%, 95% CI 20.4-46.4%).

Discussion

In the situation where gravid *An. gambiae s.s.* females had a choice to oviposit in an open pond or in ponds with a trap on top <30% of the released mosquitoes were collected by the three commercially available gravid traps in the semi-field system. The Box and CDC gravid traps showed similar efficacy whilst the Frommer updraft gravid trap trapped relatively few mosquitoes. The extremely low efficacy of the Frommer updraft gravid trap may be due to its physical features since the base of this trap stands inside the water-filled basin. To be trapped, mosquitoes have to fly under the base of the trap that is only about 3 cm above the water surface. The lower volume of water as compared to the other traps might have also contributed to the lower catch size due to a lower release of water vapour. The low efficacy of this trap is consistent with recent observations by Irish et al. [47] for *Culex quinquefasciatus*.

It is interesting to note that the overall catching efficacy of the CDC and Box gravid trap under semi-field conditions falls into the same range as reported for gravid culicine mosquitoes where recollections were highly variable and between 22% and 63% of the released mosquitoes [38,52,53]. However, it is difficult to make direct comparisons between the efficacy of these traps for culicine and anopheline mosquitoes since (1) our semi-field system had a much greater volume than those used for culicines [38,40], (2) unlike the experiments for culicines which use attractive infusions and semiochemicals there are no confirmed attractants for *An. gambiae* s.s. available and (3) in our trap comparison the open pond competed with the traps and therefore might have diverted a proportion of the mosquitoes and reduced the number of mosquitoes approaching each trap.

There are very few reports of the trapping efficacy of commercially available gravid traps for collection of *An. gambiae* s.l., or any other anopheline species in the field and surprisingly none of these were set intending to collect malaria vectors. Nevertheless, there is a notion that these traps might be less suitable for anophelines than for culicines based on the actual trapping results [47,54]. This has to be cautiously interpreted because most gravid traps are used to collect culicine females and were baited with a range of fermented plant infusions which might repel malaria vectors which are generally associated with less strongly polluted water [55–57]. The only indication that it might be possible to sample malaria vectors with commercially available gravid traps comes from the work of Muturi and colleagues [54]. In outdoor collections in a rice agro-ecosystem they collected approximately 5-6 gravid anophelines each night with a grass-infusion baited CDC gravid trap compared with 18-20 host-seeking anophelines in a CO_2-baited CDC light trap [54]. Since host-seeking collections are usually higher than others [19], this ratio is encouraging for the development of a gravid trap for malaria vectors. Fresh water instead of grass-infusion might have increased the trapping result for anophelines in this study.

In our study the Box gravid trap was selected for further evaluation since its compact design meant that the internal parts were well protected from the elements, which would be an advantage during the rainy seasons, but it was found that the approach of gravid females was significantly reduced when the Box trap was positioned directly over a pond, compared to a pond alone. This effect was not due to the sound of the trap and the removal of the trapping device off the pond confirmed that the females were visually deterred by the presence of the trapping device directly on the oviposition medium.

Previous work has shown that *An. gambiae* mosquitoes first evaluate a potential larval habitat before making a decision to lay eggs [57–62]. The decision to lay eggs might be based on visual or chemical cues or a combination of both [63]. Mosquitoes in flight depend on optical inputs to orient themselves, identify and access a target [64–67]. Visual cues are believed to be long range cues important for gravid mosquitoes to identify different habitats and specific oviposition site characteristics before they evaluate the habitat using chemical signals received by olfactory receptors, hygroreceptors and contact chemoreceptors [63,68–71]. The visual parameters include shape, size, contrast, light quality and intensity, texture and colour of a pond [58,62,63,72–76]. *An. gambiae s.s.* prefers open sunlit habitats [59,77–79] and avoids habitats densely covered by vegetation that create obstacles to oviposition [55,62,80]. A recent study suggested that shiny sticky film attracted *An. gambiae s.s.* due to its close resemblance to water [43]. This phenomenon was also exploited by Harris and colleagues who developed a simple collection device for gravid mosquitoes using a transparent acetate sheet coated with insect glue placed to float on the edge of natural aquatic habitats [36].

The most likely reasons that the Box trap does not attract many gravid females are because the pond is too shady and the large trap over the water surface impedes their pre-oviposition flight. The new OviART gravid trap provided female *An. gambiae s.s.* with an open oviposition site which improved the catch size by 60% compared to the Box gravid trap. When the OviART trap was evaluated alone, approximately one third of all released mosquitoes were trapped. This corresponds well with observations we made previously when studying (1) gravid females' approach towards an artificial pond surrounded by complete square of electrocuting nets and (2) the numbers of mosquitoes drowned in an artificial pond treated with detergent [43]. The absence of eggs in the pond of the new trap during most of the nights also indicates that the great majority of the females that approach the ponds with the intention to lay eggs got sucked into the collection chamber.

The disadvantage of the OviART prototype trap is that the collection device and the battery are less protected from the elements compared to the Box gravid trap. Nevertheless, modifications might be possible to improve the design by providing a casing for both the collection device and battery. Furthermore, to power the stronger fan needed to suck mosquitoes from the entire water surface a larger battery was required that makes the trap more difficult to transport and increases the risk of theft when used outdoors. The strong suction in the trap, forcing mosquitoes into the netting at speed, probably contributed to some of the mosquitoes found dead in the trap. Since collection of undamaged gravid females is advantageous for the isolation of a number pathogens (other than malaria parasites that can be identified from dry specimen) [38,40,48,49] future modifications should aim to improve the survival of mosquitoes in the trap. Future work should also evaluate the airflow of the trap and its impact on attracting mosquitoes. Sucking the air from above the water surface through the collapsible pipe channels potential volatile chemicals from the water surface to the side of the pond, which might affect the response towards the trap especially if attractant semiochemicals were used [47,81]. To increase user safety and longevity a gel battery should be considered in future instead of a lead acid battery. Whilst the required battery is expensive (approximately \$100–120) all the other parts can be made from locally available plastic ware and electrical supplies. Costs for the entire trap are estimated to be less than \$150, which is still cheaper than the commercially available Box gravid trap (\$192) [82] but significantly more expensive than simple catching devices as the sticky transparency [36] or simply a pond treated with detergent which has shown to work well under semi-field conditions [43]. The advantages of the OviART gravid trap are: (1) that gravid females are caught alive which eases species identification and provides potential for pathogen identification other than malaria; (2) that the trap does not depend on the presence of natural habitats but can be moved around and located close to human habitation or close to aquatic habitats depending on the research question, and (3) that environmental conditions (e.g. rain, dust, predation) are less prone to affect the catch size and quality of specimen than with sticky material and detergents. The trap may be further improved should attractive semiochemicals be discovered for *Anopheles gambiae* s.l. that could be added to the water increasing the traps competitiveness with natural habitats.

Conclusion

The three commercially available gravid traps tested in this study were specifically developed for collecting culicine mosquitoes that differ greatly in their oviposition behaviour from the malaria vector *An. gambiae* s.s. [59,83]. Nevertheless, the Box and CDC gravid trap caught consistent numbers of this species under semi-field conditions but their performance was not considered satisfactory enough to evaluate them under field conditions. The present work revealed that gravid *An. gambiae* females were visually deterred by the presence of the trapping device directly on the oviposition medium. Based on these investigations, a gravid trap was developed that provides open landing space for egg-laying female mosquitoes which improved the catch size by 60% compared to the Box gravid trap. The efficacy of this prototype trap under semi-field conditions is promising and warrants further investigations to further improve the catch size by modifying the fan suction, the size of the oviposition bowl, and physical characteristics of the trap (e.g. include visual contrast), and to improve the physical structure of the trap and its components to reduce costs and increase durability.

Field evaluations under different ecological conditions have to be carried out to confirm the trapping efficacy since semi-field systems are limited by their size, which does not allow us to study the natural completion of long-range kinesis. Furthermore, field tests are needed to assess how competitive the OviART gravid trap will be in the field especially in areas with extensive aquatic habitats and during rainy seasons. Nevertheless, previous studies have shown that similar artificial habitats made of plastic tubs buried into the ground are well accepted by anophelines in areas with high habitat density and get colonised rapidly and densely during dry and rainy seasons [84–86]. An effective gravid trap for malaria vectors will not only be useful for monitoring population densities but also enables studies of vector dispersal in search of aquatic habitats, an area of research that has received very little attention to date [87,88].

Acknowledgements

We would like to thank David Alila, Peter Ongele and Jackton Arija from the insectary at icipe-TOC, Mbita for providing mosquitoes for experiments and Bernard Oyembe, Gregory Masinde, Elizabeth Masinde, Rose Emelda and Paul Ouma for technical assistance. We thank Steve Torr for valuable comments on the study design.

Author Contributions

Conceived and designed the experiments: UF JL SD SWL WRM FO. Performed the experiments: SD. Analyzed the data: SD UF. Wrote the manuscript: SD UF SWL JL.

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