ROLE OF GRASSES IN OVIPOSITION SITE SELECTION BY MALARIA VECTORS IN WESTERN KENYA

BY

GETACHEW ETICHA BOKORE (BED, MSC)

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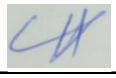
DECLARATION

I declare that this is my original work and it has never been presented for award of a degree in any University.

Getachew Eticha Bokore

PHD/SC/00102/2017

Signature  Date 21/01/2022

We confirm that the work reported in this thesis was carried out by the above candidate under our supervision as supervisors.

1. Dr Patrick Onyango
   Department of Zoology,
   School of Physical and Biological Sciences,
   Maseno University
   Signature _____ Date 21/01/2022
2. Dr Tullu Bukhari
   Human Health Theme,
   International Centre of Insect Physiology and Ecology
   Signature  Date 21/01/2022
3. Dr Ulrike Fillinger (Senior Scientist)
   Human Health Theme, International Centre of Insect Physiology and Ecology
   Signature  Date 21/01/2022

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ABSTRACT

Malaria continues to be among the top leading causes of illness and deaths in Africa. Its elimination is challenging in high transmission areas using insecticide-based intervention tools alone. Understanding the ecology and behaviour of disease vectors, including the olfactory cues, are essential for the development of novel, insecticide-free control tools. Recent work has highlighted that selected graminoid plants release volatile chemicals that attract malaria vectors. However, there is dearth of information on the type of dominant graminoid plants in habitats, nature of volatiles they emit and how volatiles affect behaviour of malaria vectors during oviposition sites selection. The objectives of this study include analysis of the association between graminoid plants and presence and abundance of early instar *Anopheles* larvae, evaluate response of gravid malaria vectors to the plant volatiles, characterize and identify attractive volatiles. A cross-sectional survey of aquatic habitats was done to sample mosquito larvae in Rusinga Island, western Kenya. The plant species were identified by an expert at East African Herbarium, National Museum of Kenya. Bioassays were implemented with live graminoid plants (*Cyperus rotundus, Cyperus exaltatus, Panicum repens, Cynodon dactylon* and *Cenchrus setaceus).* Olfactometers, WHO tubes, cages and BG-Sentinel traps were used in the experiments in Mbita and Ahero, western Kenya. The volatile compounds released in the headspace of these plants were identified using gas-chromatography/mass spectrometry and the synthetic chemicals were tested. The tests were conducted with *Anopheles gambiae* and *An. funestus*. No positive association was observed between graminoid plants and the presence and abundance of early instar *Anopheles* larvae. All the tested plants attracted gravid *An. gambiae* in an olfactometer and when tested with free-flying mosquitoes over a longer distance in large field cages. Limonene, β-pinene, β-elemene and β-caryophyllene were consistently detected in plant headspace. β-elemene (the first to be reported as being attractive to gravid malaria vector) and β-pinene elicited significant short-range attraction and egg-laying responses in gravid *An. gambiae* when tested individually. Addition of *C. rotundus* to the BG-Sentinel traps significantly increased the catches of gravid *An. funestus* than the control traps. This study confirms that gravid malaria vectors use chemical cues released from different graminoid plants to orientate. The potential utilization oviposition attraction of β-elemene and β-pinene should be further tested in the semi-field with free-flying mosquitoes for their utilization in attract-and-kill trapping strategies. Overall, there is need to invest more research into developing odour-blend formulations that can to improve surveillance and control of vectors.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACT: Artemisinin-based combination therapy drugs

ATSB: Attractive toxic sugar bait

BG-Sentinel: Biogents-Sentinel

CAR: Carboxen

CDC: Centres for Disease Control and Prevention

CI: Confidence interval

DEET- N,N-diethyl-3-methylbenz-amide

DHS: Dynamic headspace

DNA: Deoxyribonucleic Acid

DVB: Divinylbenzene

EAD/EAG: Electroantennographic detection/electroannographic

FID: Flame ionization detector

GC/MS: Gas chromatograph/Mass spectrometry

GEE: generalised estimating equations

icipe: International Centre of Insect Physiology and Ecology

IRS: Indoor residual spraying

ITNs: Insecticide treated nets

OR: Odds ratio

PCA: Principal component analysis

PCR: Polymerase chain reaction

PDMS: Polydimethylsiloxane

PET: Polyethylene terephthalate

PVC: Polyvinyl chloride

RR: Rate ratios

RTDs: Rapid diagnostic tests

SAGme: Strategic Advisory Group on Malaria Eradication

SHS: Static headspace

SMoTS: Solar-powered mosquito-trapping system

SPME: Solid-phase microextraction

SSA: Sub-Saharan Africa

TD: Thermal desorption

VOCs: Volatile organic compounds

WHO: World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is an infectious disease caused by protozoan parasites of genus *Plasmodium* transmitted to humans by the bites of infected female *Anopheles* mosquito species (Cox, 2010). The burden of the disease has declined remarkably because of concerted control efforts since 2000 (Bhatt et al., 2015). Globally, malaria cases declined from an estimate of 238 million cases in 2000 to 229 million cases in 2019 (WHO, 2020b). Similarly, malaria-related mortality has decreased from an estimate of 736,000 in 2000 to 409,000 in 2019 (WHO, 2020b). Consequently, between 2000 to 2019, 21 malaria-endemic countries had either eliminated the disease or interrupted indigenous transmission for three consecutive years (WHO, 2020b). Moreover, the disease was eliminated from all WHO European region in 2015 (Cibulskis et al., 2016).

Despite the gains, malaria continues to be among the top leading causes of illness and deaths in sub-Saharan Africa (SSA) (WHO, 2020b). The global malaria elimination goal by 2030 (WHO, 2015) appears currently less promising than when it was announced. The projection by Strategic Advisory Group on Malaria Eradication (SAGme) shows that by 2050 about 11 million cases will occur in Africa if vector control continue relying on the use of the currently available intervention tools alone (WHO, 2020a). Assessment has been made by SAGme to identify the potential factors attributed to this slowdown of the move to malaria eradication (WHO, 2020a). The major contributing factors identified to slow down malaria eradication are (1) biological factors such as development of resistance (insecticide and antimalarial drugs); (2) vector dynamics; and change in vector behaviour; (3) financial shortcoming due to the absence of consistency in commitment of countries and international donors; (4) lack of political will which is critically required for effective resource mobilization and action and; (5) lack of opinion leaders, political leaders and private sector engagement (WHO, 2020a).

Elimination is more challenging in high transmission areas using insecticide treated nets (ITNs) and indoor residual spray (IRS) alone (Lindsay et al., 2021; WHO, 2014). A “one-size-fits-all” strategy using these tools is not sufficient to solve the evolving and diverse problems of malaria vector control (Killeen et al., 2017; WHO, 2020b). Hence, ITNs and IRS cannot provide comprehensive protection from mosquito bites (Mafra-neto & Dekker, 2019; WHO, 2020b). A

major problem is the development of physiological resistance to the insecticides used in ITNs and IRS (Coleman et al., 2017). Resistance has been reported against the four major insecticide classes used for malaria vector control in all WHO regions apart from WHO European regions (WHO, 2020b). Another challenge is that these interventions are largely effective against mosquitoes biting indoors during nights and resting inside houses (Durnez & Coosemans, 2013; Govella et al., 2013; Killeen 2014; Reddy et al., 2011; Russell et al., 2011).

Malaria vectors use a range of strategies to evade contact with an insecticide. They feed indoors and exit immediately to rest outdoors, feed outdoors where people are not protected by ITNs and IRS and feed on alternative hosts outdoors (Killeen, 2014). Besides, a pre-existing behavioural resilience to avoid exposure to the higher doses of insecticides in some population of mosquito species enable them to survive and transmit malaria (Ferguson et al. 2010; Killeen et al. 2013; Killeen and Cis 2014). In Africa, *Anopheles arabiensis, An. coluzzi* and some secondary malaria vectors like *An. rivulorum* and *An. coustani* show such behaviour (Bayoh et al., 2010; Perugini et al., 2020; Tirados et al., 2006). Additionally, these vectors naturally have several alternative hosts including livestock (Degefa et al., 2017; Ogola et al., 2018; Tirados et al., 2006). Human behaviours such as living in a forest or visiting a forest, not sleeping in houses protected by ITNs or IRS, and spending more time outdoors during the evenings also makes prevention from bites difficult (WHO, 2019a).

In general, malaria elimination can be delayed due to these insecticide-resistant and/or non-targeted outdoor transmissions by a smaller proportion of vector populations (Feachem, et al., 2019; Govella & Ferguson, 2012). Malaria elimination strategies should integrate multiple and novel intervention tools (Mafra-neto & Dekker, 2019; Townson et al., 2005). Whilst the core intervention tools remain highly effective and indispensable, a wider arsenal of tools are required especially for achieving the last mile for elimination which needs to target all vector species, including those less abundant and less competent as well as those with varied level of resistance.

A better understanding of strategies that help malaria vectors to survive and reproduce during interactions with their environment can help in the invention of novel vector intervention tools (Ferguson et al., 2010; Sougoufara et al., 2020; Wooding et al., 2020). The survival of the mosquitoes is affected by how effectively they select appropriate shelters, mates, oviposition sites, blood and sugar meals, and reduce risks of predators and competitors (Hansson & Stensmyr, 2011; Wasserberg et al., 2014; Zweibel & Takken, 2004). Behaviours associated

with such activities are regulated by a range of factors such as temperature, moisture, and cues like visual, tactile and olfactory cues (Bentley & Day, 1989; Navarro-Silva et al., 2009; Zweibel & Takken, 2004). These behaviours can be exploited for the development of new vector control and surveillance tools (Brugman et al., 2018).

Selection of a suitable breeding site by gravid females is vital to the success of their progeny (Kershenbaum et al., 2012; Wasserberg et al., 2014; Yoshioka et al., 2012). This behaviour also determines their population size (Bentley & Day, 1989). Gravid females respond in their search for suitable egg-laying places to a range of cues, including volatile chemicals (Munga et al., 2006; Sumba et al., 2004; Wondwosen et al., 2016). Whilst not as well studied as the odour-orientation in host-seeking vectors (Mafra-neto & Dekker, 2019), there has been an increasing interest in the past two decades in finding odour cues that induce attraction or repellence in gravid malaria vectors. A range of sources of such odour cues have been implicated by the literature including cues produced by plants (Asmare et al., 2017; Wondwosen et al., 2016), immature conspecifics (Schoelitsz et al., 2020; Suh et al., 2016), predators (Kershenbaum et al., 2012), competitors (Kershenbaum et al., 2012; Wasserberg et al., 2014) and microbes (Eneh et al., 2016; Lindh et al., 2008). Additionally, water vapour serves as a general oviposition cue of malaria vectors (Okal et al., 2013). Such habitat cues might be responsible for the presence and abundance of larvae in the aquatic habitats. This is evident from previous ecological larval habitat risk factor surveys of aquatic habitats showing that productivity of the habitats is affected by a range of factors (Imbahale et al., 2011; Ndenga et al., 2011; Shililu et al., 2003; Wamae et al., 2010). Factors such as aquatic habitat vegetation cover determine the productivity of mosquitoes (Asmare et al., 2017; Fillinger et al., 2009).

However, the findings from different studies contradict regarding the role of plants in the presence and absence of immature malaria vector mosquitoes. For instance, it has been shown that the presence of *Anopheles* larvae was positively associated with the presence of emergent vegetations in the habitats (Fillinger et al., 2009). Similarly, in another study the occurrence of *Anopheles* larvae was highly associated with the presence of vegetations including grasses (Imbahale et al., 2011). On contrary, some studies have shown that *Anopheles* mosquito larvae were absent in aquatic habitats covered with grass-like plants. For example, *An. arabiensis* larvae were absent in habitats covered with *Typha domingensis* plants (Gouagna et al., 2012). A lower number of larvae were recorded in aquatic habitats covered with reeds (*Phragmites sp*.; Poaceae) and papyrus (*Cyperus papyrus*; Cyperaceae) (Asmare et al., 2017; Goma 1960;

Munga et al., 2006). These suggest a differential preference of mosquitoes for different plant species (Asmare et al., 2017). Graminoid plants include families of true grasses (Poaceae) and grass-like plants such as Cyperaceae, Juncuceae and Thyphaceae families. Additionally, some of the previous studies indicated the association of vegetations as a general or as family of the plants but not identifying into species of the plants. This makes it difficult to conclude whether the differences resulted due to differences in plant species or not. Therefore, a clear understanding about the association between specific graminoid plant species and malaria vector immature is important to select and evaluate the plants that might attract gravid females. However, no previous study has been conducted in Kenya to investigate the predominant graminoid plant species in potential aquatic habitats and their association with the presence and abundance of malaria vector progeny.

Recently, cedrol was identified as an oviposition attractant for gravid *An. gambiae s.l.* (Lindh et al., 2015). It was identified from headspace samples of soil infusions that were highly attractive for gravid malaria vectors. At closer investigation, it was found that the soil, which was taken from a natural *Anopheles* breeding site, included a large amount of root material from the swamp grass, *Cyperus rotundus* (Lindh et al., 2015). In another study cedrol has been identified directly from rhizome extracts of *Cyperus articulatus* (Olawore et al., 2006). Therefore, further evaluation of *Cyperus rotundus* one of the most predominant, a common graminoid plant in natural aquatic habitats, is warranted to identify the sources of cedrol. Other studies have identified attractive odour-blends from agricultural grasses, like rice, for gravid *An. arabiensis* (Wondwosen et al., 2016, 2017, 2018). It is however unclear if these odours are specific to such agricultural grasses and associated with a co-evolution of human land use and mosquito vectors as hypothesised by the authors (Wondwosen et al., 2016), or if most of grasses including the native (non-agricultural) grasses might induce such behavioural responses. It has also been suggested that true grasses in the Poaceae family are generally more attractive to gravid malaria vectors than grass-like plants in the Cyperacea family (Asmare et al., 2017). However, none of the previous studies on the chemical ecology of plant volatiles and Afro-tropical malaria vectors was done using intact live plants. Tests with intact live plants are more preferable than studying the extracts of dead plants (Tholl et al., 2006). This is mainly because of the release of volatiles from intact plants and extracts of plant materials vary and the natural release rates are more relevant to explore their ecological functions (Smith & Beck, 2015; Tholl et al., 2006). Gravid mosquito attractants originated from plants can be added into gravid traps and used in vector surveillance and control tools.

For the control and surveillance of *Culex* and *Aedes* mosquitoes, oviposition attractants have been widely deployed with different gravid traps (Baak-Baak et al., 2013; Mboera et al., 2000; Millar et al., 1992; Ponnusamy et al., 2015; Schorkopf et al., 2016). However, this strategy is still largely unexploited for malaria vectors (Hawkes et al., 2017). The reason for this is that *Culex* and *Aedes* react positively to microbial metabolites made from natural infusions and are attracted to container-type traps. *Anopheles* however avoids such microbial metabolites produced from fermentation processes, like hay infusion and also does not respond to containers traps (Eneh et al., 2016). *Anopheles* mosquitoes appear to respond to more natural scents – not surprisingly, since it has been shown from larval surveys that *Anopheles* prefers fresh and clean water over heavily organically polluted water (Akpodiete et al. 2019; Bøgh et al. 2003; Gillies & DeMeillon, 1968).

The major malaria vector species of Africa belong to *Anopheles gambiae* complex and *An. funestus* complex. *An. gambiae s.s., An. coluzzii* and *An. arabiensis* are sibling species of *An. gambiae s.l.* considered as primary malaria vectors (Coetzee et al., 2013; Gillies & DeMeillon, 1968; Sinka, et al., 2012). *Anopheles funestus s.s.* is the only primary malaria vector of *An. funestus* complex (Gillies & DeMeillon, 1968). These different species of mosquitoes prefer a wide range of aquatic habitats for oviposition and use the resources for food (Merritt et al., 1992). *Anopheles gambiae* complex prefer temporary, small, shallow habitats without vegetation or with short vegetation (Gillies & DeMeillon, 1968; Gimnig et al., 2001; Munga et al., 2006). In contrast, *An. funestus* complex prefer to oviposit in semi-permanent and permanent habitats covered with tall graminoid plants (Gillies & DeMeillon, 1968; Gimnig et al., 2001; Kweka et al., 2012). An oviposition substrate that is preferred by one species may not be chosen by another species (Afify & Galizia, 2015). Therefore, separate studies for each species type may be important.

Before the use of plant-based attractants in vector control and surveillance, they should be isolated and identified from the attractive plants (Ignell & Hill, 2020). The conventional approach for screening of attractants from plants involves a series of procedures such as evaluating and selecting attractive plants to gravid mosquitoes through bioassays; collecting volatile organic compounds (VOCs) from the attractive plants; isolating and identifying the collected compounds using analytical techniques; selecting bioactive compounds using antennal bioassays; and behavioural bioassays with the synthetic chemicals of the bioactive compounds in laboratory, semi-field and field settings (Barbosa-cornelio et al., 2019; Brugman

et al., 2018). If strong attractants become available, then novel attract-and-kill strategies might be developed for malaria vector surveillance and control.

1.2 Statement of the problem

Malaria-endemic countries aim to eliminate malaria or aim to reduce the disease's burden to move towards elimination by 2030 in accordance with the Global Technical Strategy for Malaria 2016-2030. To attain this, both indoor and outdoor malaria transmission must be interrupted. However, malaria vector populations with outdoor-biting, outdoor-resting behaviours and resistant to insecticide are currently not well controlled. This is because ITNs and IRS are only effective to control indoor mosquito populations that are susceptible to insecticide. These tools were designed considering the early characterization of only indoor feeding and indoor resting *An. gambiae s.s.* and *An. funestus s.s.*. Thus, there is no effective vector control tool that targets vector populations outdoors.

To develop such tools, a better knowledge of outdoor ecology and behaviour of malaria vectors is very important. In this context, targeting the oviposition site seeking female mosquitoes is promising since all vectors, no matter the feeding behaviour type or resistance to insecticides, search for a suitable breeding site to lay eggs outdoors. Previous studies have shown a positive association between vegetations and the presence of malaria vector larvae. However, there is a dearth of information on the type of dominant graminoid plants in breeding habitats, the nature of volatiles emitted from these graminoid plants and how they affect different malaria vector species' behaviour during oviposition sites selection. Additionally, previous studies are limited in number and not conducted with live plants, which are more appropriate during ecological studies. Therefore, here I sought to generate additional evidence with use of live plants in natural settings.

1.3 Study objectives

1.3.1 Main objective

To determine the role of graminoid plants in the chemical and behavioural ecology of gravid malaria vectors.

1.3.2 Specific objectives

1. To determine association between graminoid plant species and presence and abundance of immature malaria vector mosquitoes in natural habitats along the shore of Lake Victoria, western Kenya.
2. To assess the behavioural response of gravid *Anopheles gambiae s.s.* to volatile organic chemicals emitted from live graminoid plants of the Cyperaceae and Poaceae families under laboratory and semi-field conditions.
3. To characterize volatile headspaces of plants attracting gravid malaria vectors.
4. To explore the behavioural response of gravid vectors to putative oviposition semiochemicals identified from graminoid plant headspace samples.
5. To determine the odour-mediated response of wild gravid *Anopheles funestus s.s.* to selected plant species under field conditions.

1.4 Null hypotheses

1. There is no association between graminoid plant species and the presence and abundance of immature malaria vector mosquitoes in natural habitats at the shore of Lake Victoria, western Kenya.
2. Volatile organic compounds emitted from graminoid plants do not affect the orientation of the flight of gravid *Anopheles gambiae s.s.*.
3. There is no difference in the volatile organic compounds composition of the headspaces of the test plants.
4. Semiochemicals identified from headspace samples of graminoid plants do not serve as oviposition attractants.
5. Volatile organic compounds emitted from graminoid plant do not affect the orientation of the flight of gravid *Anopheles funests s.s.*.

1.5 Justification of the study

Malaria elimination can only be achieved if all vector species are targeted for control in an integrated approach. A mix of tools is needed to tailor programs to local ecological and epidemiological conditions. Gravid malaria vectors must search for aquatic breeding sites and lay their eggs outdoors and hence presents an excellent target for vector control. Trapping of gravid females can be more important in disease surveillance as they are more likely to be infected with pathogens following the take of human bloodmeal. Understanding how malaria

vectors find and select their egg-laying sites is an essential first step in developing strategies to manipulate this behaviour for surveillance and control. Malaria vectors breed in standing water.

A range of graminoid plant types are associated with wetlands and might serve as indicators of the presence of standing water. Hence it is plausible to hypothesize that gravid malaria vectors use volatile chemical cues associated with wetland grasses and grass-like plants, among other environmental cues, to guide them during their flight in search for suitable aquatic habitats. Knowledge about the role of graminoid plants in habitat selection is still very limited and further research is required to identify potential associations between these plants, natural habitat colonization and chemicals released to assess their potential for the development of attract and kill strategies for surveillance and control of gravid vectors.

1.6. significance of the study

Malaria vector control remains an important component of malaria control. The study about biotic factors which include gravid malaria vectors may provide a new approach in malaria vector control which is presently challenged due to the insecticide development and diversity in their behaviours. Oviposition site selection of gravid malaria vectors is important behaviour for the survival of their immature, their dynamics and distribution. Targeting gravid females is suggested to be an effective strategy for disease surveillance as well as control. This is because they become gravid after blood take and are more likely to be infected with malaria parasites. Additionally, a single female *Anopheles* mosquito deposits 50-150 eggs and killing one gravid female can be considered as killing several mosquitoes. Exploiting the ecological interactions of gravid *Anopheles* mosquitoes which uses oviposition olfactory cues during their search for suitable egg-laying sites play important role in the development of novel vector control and intervention tools. Graminoid frequently present in natural breeding sites and emit volatile chemicals which may orient gravid female malaria mosquitoes flight towards breeding sites. Identification of attractive volatiles from graminoid plants assists to incorporate them into gravid traps to increase their efficiency. Vector control strategies such as attract-and-kill strategy and mass trapping of oviposition site seeking females might be a potential control strategy that can complement the core vector control tools by deploying odour-based gravid traps. The present study investigates the behavioural influence of graminoid plants found in natural breeding sites in gravid *Anopheles gambiae* and *An. funestus* and identifies the attractive plant volatiles.

1.7. Limitations of the study

While this study clearly demonstrated that graminoid plants supported a large number of immature Anopheles mosquitoes, no preference for specific plant species. The timing of the aquatic habitats survey was not right as most of the potential breeding sites were vegetated with graminoid plants and oviposition cues were not limiting factors as gravid females can easily locate the habitats. This might have been different if the survey had been implemented during the dry season.

Additionally, electrophysiological bioassay was not conducted for this study. Electrophysiological bioassays of plant volatiles with antennae of gravid *Anopheles* mosquitoes assist to select plant volatiles used for attraction and egg count bioassays. It also helps to determine the right composition of blends evaluation which this study failed to identify. Plants release blends of volatiles that are discriminated and recognized by mosquitoes. The combinations and ratios of these volatiles affect the behavioural response of the mosquitoes. Studies need to be designed to determine the constituents, dases and consistent release of the blends.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria vectors and their life cycle

Malaria continues to be the most deadly vector-borne disease in high malaria burden countries (Derua *et al*., 2015). Malaria burden is the highest in SSA where the lives of children under the age of five years and pregnant women were most threatened (WHO, 2019b). About 94% of global malaria cases and malaria-associated deaths occurred in Africa in 2019 (WHO, 2020b). Additionally, it has enormously contributed to the deep-rooted poverty by decelerating economic growth of malaria-endemic countries (Ernst & Young, 2017; Gallup & Sachs, 2001; Sachs & Malaney, 2002; WHO, 2017). On the other hand, poverty also exacerbates the malaria burden by limiting the disease control capacity of poor countries (Sachs & Malaney, 2002). The disease is transmitted by an infected female *Anopheles* mosquito.

More than 100 *Anopheles* species are responsible for the transmission of malaria parasites though only a few transmit the largest proportion of the disease (Wiebe et al., 2017). About 40 species were reported to have major importance (WHO, 2019a). The major Afro-tropical malaria vectors belong to *An. gambiae* complex and *An. funestus* complex (Gillies & Coetzee, 1987; Sinka et al., 2012). A complex is a taxonomic rank consisting of closely related species of mosquitoes that cannot be identified morphologically (Wiebe et al., 2017). *Anopheles gambiae* complex consists of nine sibling species including *An. gambiae s.s.* (hereafter referred to as *An. gambiae), An. coluzzii, An. arabiensis, An. quadriannulatus, An. bwambae, An. merus, An. melas, An. amharicus* and *An. fontenillei* sp.n. (Barrón et al., 2019; Coetzee et al., 2013). Among these, *An. gambiae, An. coluzzii* and *An. arabiensis* are considered as primary malaria vectors with wide distribution in SSA (Coetzee et al., 2013; Gillies & DeMeillon, 1968; Sinka et al., 2012). *Anopheles gambiae* is the most effective vector in these complex as it feeds on humans indoors. *Anopheles merus* and *An. melas* have been reported as secondary malaria vectors in eastern and western Africa, respectively (Kipyab et al., 2013; Tsy et al., 2003). In many tropical African countries, *An. gambiae* and *An. arabiensis* share the same ecological zones including breeding sites (Coetzee, et al., 2000; Collins et al., 2019). Similarly, *An. gambiae* and *An. coluzzii* coexist over large areas in Africa (Fossog et al., 2014). *Anopheles funestus* complex consists of sibling species including *An. funestus s.s. (*hereafter called *An. funestus), An. funestus-like, An. rivulorum, An. rivulorum-like, An. parensis, An. vaneedeni,*

An. leesoni, An. confusus, An. fuscivenosus, An. longipalpis, An. brucei, and *An. aruni* (Coetzee, 2020; Cohuet et al., 2003; Gillies & Coetzee, 1987; Harbach, 2004; Spillings et al., 2009). *Anopheles funestus, An. vaneedeni, An. aruni* and *An. parensis* are known as *An. funestus* sub-group since they are morphologically similar at all developmental stages and their identification is only possible using molecular methods (Gillies & Coetzee, 1987; Gillies & DeMeillon, 1968). The eggs and larvae of *An. leesoni* and *An. confusus* are morphologically distinct from the rest (Coetzee & Fontenille, 2004). *Anopheles funestus* is the only major malaria vector of this group (Gillies & DeMeillon, 1968). Naturally, *An. funestus* is almost exclusively anthropophilic (it prefers to feed on human host rather than on animals) whereas the rest of the species are preferentially zoophilic (they prefer to feed on animal hosts rather on humans) feeding outdoors (Gillies & DeMeillon, 1968). *Anopheles rivulorum, An. parensis, An. vaneedeni, An. longipalpis* and *An. leesoni* were reported as secondary malaria vectors in many localities (Mulamba et al., 2014; Ogola et al., 2018; Temu et al., 2007; Afrane, 2016). In addition to the two main complexes, *An. nili* and *An. moucheti* groups are primary malaria vectors of forest and wet savannah regions of West and Central Africa (Antonio-Nkondjio et al., 2002; Fontenille, 2004). The other secondary malaria vectors in some localized areas in SSA include *An. pharoensis, An. coustani, An. rufipes,* and *An. ziemanni* (Degefa et al., 2017; Mukiama & Mwangi, 1989; Ogola et al., 2017; Tabue et al., 2014, 2017). *Anopheles stephensi* is another major malaria vector of Asia and recently detected in eastern African countries including Djibouti, Ethiopia and Sudan (Sinka et al., 2020). It is an urban malaria vector breeding in containers with a preference for clean water (Sinka et al., 2020; Sinka et al., 2011).

The females of these malaria vectors are anautogenous (require vertebrate host blood to produce eggs) and require vertebrate blood to obtain protein and energy required for the development of their eggs (Harrison et al., 2021). Following each blood-meal female mosquitoes produce and oviposit batches of eggs (Lardeux et al., 2008). The time between two consecutive egg-laying cycles is known as gonotrophic cycle (Lardeux et al., 2008; Santos et al., 2002). Two types of gonotrophic cycles are exhibited by mosquitoes. The first type is when a single blood-meal is needed to lay a batch of eggs and the time interval between two consecutive blood-meals (egg depositing) is called a gonotrophic concordance (Lardeux et al., 2008). This type of gonotrophic is very common in *Anopheles* mosquitoes in natural and normal conditions (Charlwood et al., 2016). The second type occurs in mosquito species which require multiple rounds of blood-meal to lay one batch of eggs and the time interval between two consecutive egg-laying is called gonotrophic discordance (Charlwood et al., 2016). This

type of gonotrophic occurs because of delay in oviposition caused by drought and high-temperature resulting in breeding sites dry up (Omer & Cloudsley-Thompson, 1970; Yaro et al., 2010) and such vectors are epidemiologically more important since they live longer (Charlwood et al., 2016). This cycle repeats in the life history of a mosquito and determines its vectorial capacity since it increases the vector-human contact and likelihood of getting infected with pathogenic agents and vectoring them (Charlwood et al., 2016; Lardeux et al., 2008). Gonotrophic cycle involves three main biological activities including (i) host-seeking and blood-feeding: identification of vertebrate host and blood-feeding; (ii) resting: digestion of the blood and egg maturation and; (iii) oviposition: identification of suitable breeding site and egg-laying as reviewed by (Lardeux et al., 2008).

Malaria vectors have four developmental stages including aquatic stages (egg, larva and pupa) and a terrestrial stage (adult) in their life cycle (WHO, 2005). The different species of *Anopheles* mosquitoes have different preferences for various breeding habitat types. *Anopheles gambiae s.l.*, for instance, prefer to oviposit in various aquatic habitat types ranging from small, shallow, temporary, open water bodies to vegetated habitats (Bøgh et al., 2003; Gillies & DeMeillon, 1968; Gimnig et al., 2001; Gouagna et al., 2012). *Anopheles funestus* prefer to breed in semi-permanent and permanent habitats covered with plants ranging from short grasses to tall and dense vegetations (Dia et al., 2013; Gimnig et al., 2001; Kweka et al., 2012; Munga et al., 2006; Takken & Knols, 2010). The immature stages have limited movement (Killeen et al., 2002) whereas the adults can fly usually in a range of 3 km distance from the breeding areas (WHO, 2005).

The knowledge about the life cycle, ecology and behaviour (Plate 2.1) of these vector species is essential to design control strategies. Factors such as indoor feeding/outdoor feeding behaviour, indoor resting/outdoor resting behaviour, early/late biting behaviour, nectar-feeding, oviposition site seeking behaviour and local vector species must be considered during vector control (Durnez & Coosemans, 2013; Govella & Ferguson, 2012; Muema et al., 2017; Nyasembe & Torto, 2014). The currently available vector control tools have been developed based on the earlier findings about the behaviours and life cycle of the vectors (Mwingira et al., 2021). For instance, ITNs are developed exploiting the knowledge of indoor blood-feeding behaviour of *An. gambiae s.s.* and *An. funestus s.s.* (Malima et al., 2009) whilst IRS was based on their resting behaviour on walls after bloodmeal (Curtis & Lines, 1985). Similarly, attractive toxic sugar bait (ATSB) is developed using the understanding of nectar-seeking behaviour of

male and female mosquitoes (Qualls et al., 2014; Revay et al., 2014). Similarly, larvicide and other source management rely on the knowledge that female mosquitoes lay eggs on water and their progeny develop in aquatic habitats (Curtis et al., 2002). But, the quantification of outdoor behaviours such as oviposition site selection of these mosquitoes are less explored for use in vector control (Hawkes et al., 2017).

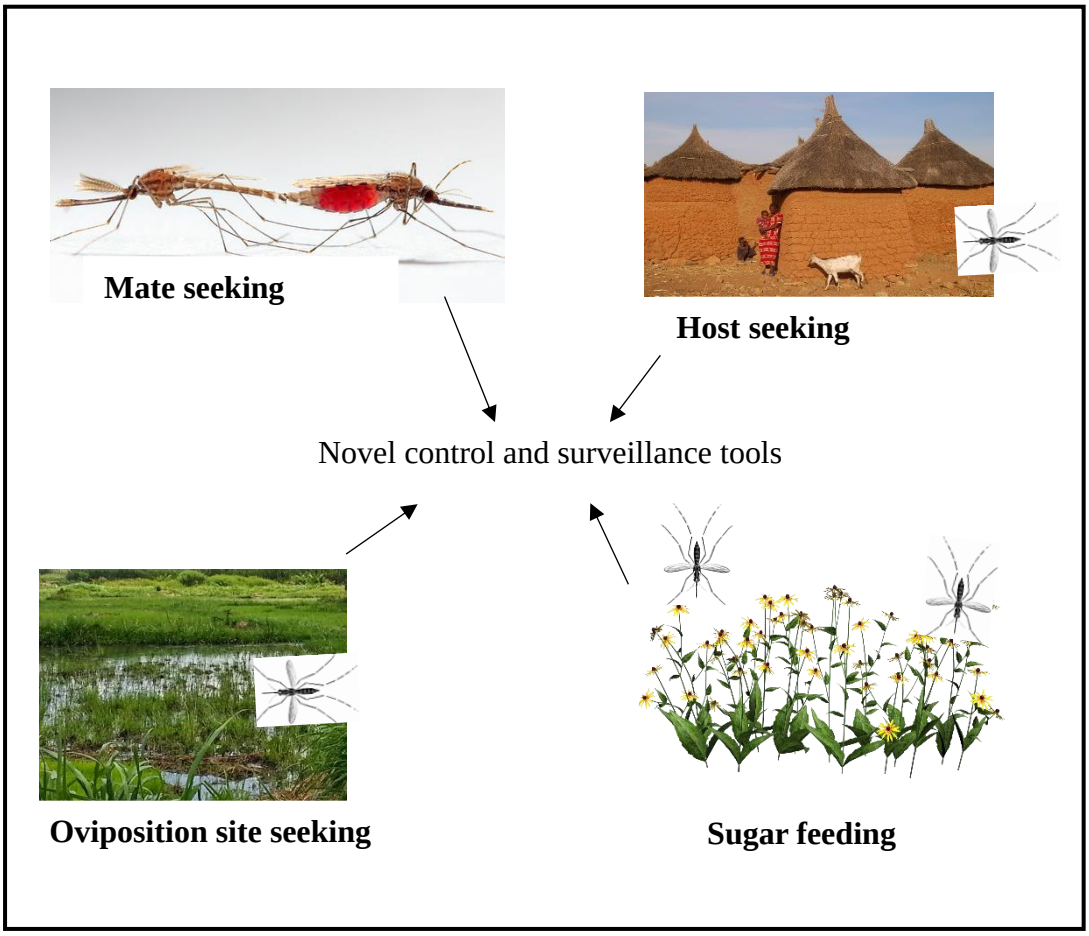

Plate 2. 1 Life history components of female malaria vector mosquitoes and the role of dour cues in various behaviours.

2.2 Association between aquatic habitat vegetations and the presence and abundance of immature malaria vector mosquitoes

Studies have shown a positive association between the presence of vegetations including grasses and the presence and abundance of *Anopheles* larvae in breeding sites. For example, it has been shown that there is a positive association between *Anopheles* larval density and the presence of vegetations (Fillinger et al., 2004). Similarly, it has been reported that many of the breeding sites (81.7%) of *An. gambiae* in Kenyan highlands were found to be covered with

short vegetations (Minakawa et al., 2004). *Anopheles gambiae* and *An. melas* larvae were mainly found in association with *Paspalum spp*. (Poaceae) and *Eleocharis spp*. (Cyperaceae) in the rural Gambia (Bøgh et al., 2003). The presence of late instar *Anopheles* larvae in aquatic habitats covered with grasses was significantly greater than in habitats without grasses in western Kenya (Imbahale et al., 2011). Similarly, in Brass-Panon, Madagascar, the presence of *An. arabiensis* larvae was highly associated with the presence of Cyperaceae (*Cypesrus haspan, Cyperus difformis, Fymbristilis glomerata*) plants (Gouagna et al., 2012). These vegetations protect mosquito progeny from predators, being washed off by river water, direct exposure to sunlight and serve as a source of larval food (Asmare et al., 2017; Dia et al., 2013; Foley et al., 2002; Merritt et al., 1992; Sinka et al., 2010; Ye-ebiyo et al., 2000).

Some contradicting results were also reported showing that gravid *Anopheles* mosquitoes avoid depositing their eggs in aquatic habitats covered with grasses and other vegetations. For instance, the presence of *An. arabiensis* larvae was negatively associated with the presence of *Typha domingensis* plant in the habitats (Gouagna et al., 2012). Small numbers of *Anopheles* mosquitoes were recorded in aquatic habitats covered with reeds (*Phragmites sp.*; Poaceae) and papyrus (*Cyperus papyrus*; Cyperaceae) (Asmare et al., 2017; Goma, 1960; Munga et al., 2006). Hence, there is a scarcity of information on the different graminoid plant species present in and around the breeding habitats and their role in oviposition site preference by gravid malaria vectors is also not clear.

2.3 Oviposition site selection behaviour of malaria vectors

After bloodmeal digestion, gravid female mosquitoes commence flights to search for oviposition sites (Day, 2016; Durnez & Coosemans, 2013). As there is no parental care and the immature have limited mobility, selection of oviposition sites is a key factor for their fitness, distribution and abundance (Bentley & Day, 1989; Rudolf et al., 2004; Thompson, 1988; Vonesh & Blaustein, 2010). Immature stages of mosquitoes live in aquatic habitats with limited resources. In such conditions specifically larvae may be more exposed to a shortage of food and predators attack (Lutz et al., 2017). Therefore, gravid mosquitoes should deposit their eggs in suitable breeding sites with ample food, few or no predators and competitors (Afify & Galizia, 2015). In addition, spatial limits can highly affect larvae via fluctuations in the physio-chemical factors such as temperature, acidity, plant compounds and salinity (Lutz et al., 2017).

Mosquito egg-laying is a combination of two different behaviours: pre-oviposition and oviposition. It involves several behaviours such as stimulation to take flight, oriented upwind flight towards attractants over a longer distance, arrestment and evaluation of a site, and acceptance and egg-laying behaviours (Isoe et al., 1995). Pre-oviposition is the selection of oviposition site while oviposition involves the process of egg-laying in preferred habitat (Bentley & Day, 1989). Pre-oviposition behaviours can be oriented by long-range cues such as oviposition odour cues and visual cues whilst oviposition behaviour can be oriented by short-range cues like contact cues (Isoe et al., 1995). Several environmental factors such as precipitation, moisture, temperature and wind speed influence the flight of gravid mosquitoes for egg-laying (Bentley & Day, 1989; Day, 2016). Mosquitoes prefer warm, humid, and stable wind flow for flight (Bidlingmayer, 1974). Visual, olfactory and tactile stimuli are used in the identification and discrimination of oviposition sites (Bentley & Day, 1989; Day, 2016). These cues affect the behaviour of gravid mosquitoes during the flight for breeding site search and after landing on aquatic habitats determining their preference and decision of egg-laying (Du & Millar, 1999).

2.3.1 Role of olfactory cues in oviposition site selection by gravid mosquitoes

The survival of mosquitoes relies on their responses to stimuli from different resources such as nectar sources, mates, resting sites, blood meal hosts and oviposition sites (Sutcliffe, 1994; Takken & Knols, 1999). They use their sensory system consisting of chemoreceptors, mechanoreceptors, hygroreceptors and thermoreceptors (Navarro-Silva et al., 2009) to discriminate the presence of a host, a mating partner, quality food and a suitable oviposition site (Luntz, 2003).

Olfaction is a vital sensory modality in insects life in a complex odour environment (Conchou et al., 2019). Olfactory cues also known as semiochemicals are chemical messengers which elicit behaviours associated with larval foraging, larval predator avoidance, vertebrate host-seeking, nectar-seeking, mating and oviposition site selection (Montell & Zwiebel, 2016; Zweibel & Takken, 2004). The semiochemicals which influence behaviour of gravid mosquitoes are called oviposition cues (Montell & Zwiebel, 2016).

Olfactory oviposition cues can be classified as attractants, repellents, stimulants and deterrents. The first two can be detected from a long distance whilst the last two can be detected from a shorter distance or direct contact (Afify & Galizia, 2015). These cues are released from microorganisms, predators, competitors, conspecific immature and vegetations (Afify &

Galizia, 2015). The understanding of the source, function and importance of volatile organic compounds (VOCs) or odour cues involve in mediating mosquitoes interaction with their environment and may help to develop novel odour based vector control and surveillance tools (Mwingira et al., 2020).

Volatile organic compounds are a type of organic compounds. They are characterized by having (i) low boiling points;(ii) high vapour pressure (≥0.01 kPa at 20°C; (Pagans et al., 2006; Qualley & Dudareva, 2009; Schulz-Bohm et al., 2017)); (iii) low molecular weight (Hung et al., 2015; Schulz-Bohm et al., 2017); and (iv) readily evaporates at room temperature and pressure (Hung et al., 2015; Schulz-Bohm et al., 2017). Most are soluble in lipids but less soluble in water (Herrmann, 2010; Morath et al., 2012; Pagans et al., 2006; Schulz-Bohm et al., 2017). They are relatively smaller hydrocarbons belonging to various groups of compounds including alkanes, alkenes, acids, alcohols, aldehydes, aromatics, ketones, terpenes, phenols, benzenoids, nitrogen and sulfur -containing compounds and fatty acid derivatives (Chowdhury et al., 2019; Dudareva et al., 2006; Materić et al., 2015; Pennerman et al., 2016). They do not include carbon dioxide, carbon monoxide, carbonic acid, metallic carbides/carbonates and ammonium carbonate (EPA, 2019). These compounds are emitted from all organisms and are involved in defence, attraction, response to stress, and mediate interactions between organisms (Herrmann, 2010; Pennerman et al., 2016). Plants are known to produce a diverse range of VOCs which consists of tens of thousands of compounds (Guenther et al., 2012). Table 2.1 shows a summary of olfactory substrates reported to influence oviposition behaviour of gravid mosquito species. Though the influence of VOC cues on the mosquitoes' behaviours is widely investigated, the underlying mechanism of how they function to elicit a particular behaviour and how insects use these cues to identify resources such as oviposition sites remain unclear (Conchou et al., 2019; Wooding et al., 2020).

Table 2. 1 Substrates reported as oviposition attractants/stimulants and repellents/deterrents of gravid malaria vector mosquitoes

Oviposition substrates	Source	Mosquito species	Assay	Response	Authors
Oviposition cues from plants					
Blends of β-Caryophyllene, Decanal, Sulcatone, Limonene, Nonanal, 3-Carene, α-Pinene & β-Pinene	Rice cultivars	*An. arabiensis*	Electrophysiological, olfactometer &	EAD active & Attraction	(Wondwose et al., 2016)

			BG-Sentinel traps		
Blends of Limonene, Nonanal, α-Pinene, Benzaldehyde & *p*-Cymene	Maize pollen	*An. arabiensis*	Electrophysiological & Olfactometer	EAD active & Attraction	(Wondwose et al., 2018)
Blends of o-Xylene, Styrene, α-Pinene, Benzaldhyde, 1,8-Cineole, Undecane, Nonanal, *p*-Cymene, N-Ethyl, Benzenamine, Dibuty phthate & Eicosane	Sugarcane pollen	*An. arabiensis*	Electrophysiological, cage & olfactometer	EAD active & Attraction	(Wondwose et al., 2018)
β-Caryophyllene, (*E*)-Caryophyllene, β-Elemene, ð-Elemene, α-Humulene, Terpinene-4-ol, γ-Muurolene & α-Selinene/Alaskene	*Commiphora leptophloeos* leaf oil	*Ae. aegypti*	Electrophysiological assay	EAD active	(da Silva et al., 2015)
(*E*)-Caryophyllene & α-Humulene	*Commiphora leptophloeos* leaf oil	*Ae. aegypti*	Cage bioassay	Deterrent	(da Silva et al., 2015)
Extracts of *Echinochloa pyramidalis* & *E. stagnina*		*An. arabiensis and An. coluzzii*	Wind tunnel, cage & tent	Attraction	(Asmare et al., 2017)
Organic extracts of *Cynodon dactylon, Jouvea straminea, Fimbristylis spadicea, Ceratophyllum demersum* & *Brachiaria mutica*		*An. albimanus*	Cage & wind tunnel	Attraction	(Torres-Estrada et al., 2005)
Plant essential oils	*Ocimum suave* & *O. kilimandscharicum*	*An. gambiae*	Cage	Repellency	(Kweka et al., 2010)
Oviposition from different plant matter infusions					
3-Methyl-1-butanol	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
Phenol	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
4-Methylphenol (*p*-cresol)	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
	Bermuda grass & wood infusions	*Cx. tarsalis, Aedes triseriatus*	Cage	Attraction	(Bentley et al., 1979)
	Bermuda grass infusion	*Cx. quinquefaciatus* & *Cx. tarsalis*	EAG, Cage & sticky screen	Attraction	(Du & Millar, 1999)
	Synthetic	*Ae. albopictus*	sticky screen	Repellency	(Trexler et al., 2003)
Indole	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
	Breeding habitat water with larvae	*An. gambiae*	EAG	EAG active	(Blackwell & Johnson, 2000)

	Bermuda grass hay infusion	*Cx. tarsalis* & *Cx. quinquefaciatus*	EAG, Cage & sticky screen	Attraction	(Du & Millar, 1999)
3-Methylindole (skatole)	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
	Bermuda grass hay infusion	*Cx. tarsalis* & *Cx. quinquefaciatus*	EAG, Cage & sticky screen	Attraction	(Du & Millar, 1999)
	Bermuda grass hay infusion	*Cx. quinquefasciatus, Cx. stigmatosoma* & *Cx. tarsalis*	gravid female trap (in the field)	Attraction	(Beehler et al., 1994)
Nonanal	Bermuda grass hay infusion	*An. gambiae*	Cage	Repellency	(Eneh et al., 2016)
	Bermuda grass hay infusion	*Cx. quinquefaciatus* & *Cx. tarsalis*	EAG, Cage & sticky screen	Attraction	(Du & Millar, 1999)
Dimethyl trisulfide	Bermuda grass infusion	*Cx. quinquefaciatus* & *Cx. tarsalis*	EAG, cage & sticky screen	Attraction	(Du & Millar, 1999)
2-Tridecanone	Bermuda grass infusion	*Cx. quinquefaciatus* & *Cx. tarsalis*	EAG, cage & sticky screen	Attraction	(Du & Millar, 1999)
Naphthalene	Bermuda grass infusion	*Cx. quinquefaciatus* & *Cx. tarsalis*	EAG, cage & sticky screen	Attraction	(Du & Millar, 1999)
Blends of Nonanal, 3-Methylindole, Dimethyl trisulfide, 2-Tridecanone, p-Cresol, Indole, 2-Undecanone, 4-Ethylphenol, Phenol & Naphthalene	Bermuda grass infusion	*Cx. tarsalis* & *Cx. quinquefaciatus*	Cage & sticky screen	Attraction	(Du & Millar, 1999)
Blends of Indole, Phenol, 4-Methylphenol (p-Cresol), 4-Ethylphenol & 3-Methylindole	Bermuda grass infusion	*Cx. quinquefaciatus*	Cage	Attraction	(Jocelyn et al.,1992)
Bermuda grass/hay infusions		*Ae. albopictus, Cx. quinquefaciatus, Cx. nigripalpus, Cx. erraticus, Cx. tarsalis*	Sticky screen, cage & field (CDC gravid trap)	Attraction	(Du & Millar, 1999; McPhatter & Debboun, 2009; Ponnusamy et al., 2010)
Acacia (*Acacia schaffneri*) infusion		*Cx. quin, Cx. nigripalpus* & *Cx. erraticus*	CDC gravid trap (field)	Attraction	(McPhatter & Debboun, 2009)
White oak (*Quercus alba*) infusion		*Ae. aegypti* & *Ae. albopictus*	Cage & sticky screen	Attraction	(Ponnusamy et al., 2008; Ponnusamy et al., 2010)
Bamboo (*Arundinaria gigantea*) leaf infusion		*Ae. aegypti* & *Ae. albopictus*	Cage & sticky screen	Attraction	(Ponnusamy et al., 2008; Ponnusamy et al., 2010)

Hyacinth infusion		*Ae. aegypti* & *Cx. quinquefasciatus*	Cage & olfactometer	Attraction	(Turnipseed et al., 2018)
Lettuce volatiles & hay infusion		*Ae. aegypti* & *Cx. quinquefasciatus*	Cage & olfactometer	Attraction	(Turnipseed et al., 2018)
Anacardium occidentale (non-fetid odour) infusion *Panicum maximum* (fetid odour) infusion		*Ae. aegypti*	Cage & field	Attraction	(Santos et al., 2010)
Panicum maximum infusion		*Aedes (Stegomyia)*	Traps (field)	Attraction	(Sant'ana et al., 2006)
Oviposition cues from water containing conspecific immature, predators and competitors					
Nonane 2,4-Pentanedione (2,4-PD)	Water containing first instar *An. coluzzii*	*An. gambiae*	Cage & plastic bowls (semi-field)	Attraction	(Schoelitsz et al., 2020)
Dimethyl disulfide	Water contained with first instar *An. coluzzii* larvae	*An. gambiae*	Cage & plastic bowls (semi-field)	Repellency	(Schoelitsz et al., 2020)
Dimethyl disulfide Trimethyl disulfide 6-Methyl-5-hepten- 2-one (Sulcatone)	Water contained overcrowded *An. coluzzii* larvae	*An. coluzzii*	Growth chamber	Repellency	(Suh et al., 2016)
(-)-(5R,6S)-6-acetoxy-5-hexadecanolide	Apical droplets on egg rafts of *Culex* & synthetic	*Culex*	Cage & traps	Attraction	(Bruno & Laurence, 1979; Mboera et al., 2000)
Dodecanoic acid (*Z*)-9-hexadecenoic acid	Extracts of *Ae. aegypti* eggs	*Ae. aegypti*	Cage	Attraction	(Ganesan et al., 2006)
6-Hexadecenoate Methyl dodecanoate, Methyl tetradecanoate Methyl (*Z*)-9-hexadecenoate	Extracts of *Ae. aegypti* eggs	*Ae. aegypti*	Cage	Repellency	(Ganesan et al., 2006)
Caproic acid	Eggs of *Ae. aegypti*	*Aedes aegypti*	Cage	Attraction	(Ong & Jaal, 2015)
n-Heneicosane	Larval cuticle of *Ae. aegypti*	*Ae. aegypti*	Cage & Y-maze olfactometer	Attraction	(Seenivasagan et al., 2009)
Phenol	Breeding habitat water with larvae	*An. gambiae*	EAG	EAG active	(Blackwell & Johnson, 2000)
o-Cresol	Breeding habitat water with larvae	*An. gambiae*	EAG	EAG active	(Blackwell & Johnson, 2000)
m-Cresol	Breeding habitat water with larvae	*An. gambiae*	EAG	EAG active	(Blackwell & Johnson, 2000)
	Synthetic	*Ae. albopictus*	Sticky screen bioassay	Repellency	(Trexler et al., 2003)

4-Methylcyclohexanol	Breeding habitat water with larvae	*An. gambiae*	EAG	EAG active	(Blackwell & Johnson, 2000)
Rainwater conditioned with backswimmer (Notonecta sp.)		*An. gambiae*	Cage bioassay	Repellency	(Munga et al., 2006)
Rainwater conditioned with tadpole		*An. gambiae*	Cage bioassay	Repellency	(Munga et al., 2006)
Oviposition cues from microbes					
Cedrol	Fungus (*Fusarium falciforme*) & soil infusions	*An. gambiae & An. arabiensis*	Cage & traps	Attraction	(Eneh et al., 2016; Lindh et al., 2015)
2-Methyl-3-decanol	*Vibrio metchnikovii*	*An. gambiae*	EAG	PCA	(Lindh et al., 2008)
2-Phenylethanol	*Bacillaus* sp.	*An. gambiae*	EAG	PCA	(Lindh et al., 2008)
3-Methylbutanoic acid	*Micrococcus* sp.	*An. gambiae*	EAG	PCA	(Lindh et al., 2008)
Phenylmethanol	*Exiguobacterium* sp.	*An. gambiae*	EAG	PCA	(Lindh et al., 2008)
2-Phenylethanol	*Proteus* sp.	*An. gambiae*	EAG	PCA	(Lindh et al., 2008)
Bacterial isolated denoted as DABH-1, DABH-5, DABH-6 and DABH-8	*Aedes* breeding habitat	*Aedes species*	Cage	Attraction	(Mondal et al., 2015)
Bacteria isolated from white oak & bamboo leaf infusion		*Ae. aegypti*	Cage	Stimulant	(Ponnusamy et al., 2008)
Nonanoic acid, tetradecanoic acid & methyl tetradecanoate		*Ae. aegypti*	Cage	Attraction/ stimulants	(Ponnusamy et al., 2008)
Proteus isolate L2 *Micrococcus* isolate L4 *Bacillus* isolate L6 *Exiguobacterium* isolate L9 *Comamonas* isolate L11 *Vibrio metschnikovii* isolate E2.5	Breeding sites *An. arabiensis* midgut	*An. gambiae*	Cage	Attraction	(Lindh et al., 2008)
Synthetic oviposition cues					
Blends of n-Heneicosane, 3-Methylindole (Skatole), 4-Methylphenol (*p*-Cresol) & Phenol	Synthetic	*Ae. aegypti*	Ovitraps	Attraction	(Baak-Baak et al., 2013)
Eugenol, Citronellal, Thymol, Pulegone, Linalool, Rosemary oil & p-Cymene	Synthetic	*Ae. aegypti*	Cage bioassay	Deterrence/ repellency	(Waliwitiya et al., 2009)
Borneol, Camphor, Borneol acetate & β-Pinene	Synthetic	*Ae. aegypti*	Cage bioassay	Stimulant	(Waliwitiya et al., 2009)
Blends of Nonanal & Trimethylamine	Synthetic	*Cx. P. quinquefaciatus*	Traps (field)	Attraction	(Barbosa et al., 2008)

Blends Skatole & Nonanal	Synthetic	*Cx. P. quinquefaciatus*	Traps (field)	Attraction	(Barbosa et al., 2008)
Blends of Skatole & Trimethylamine	Synthetic	*Cx. P. quinquefaciatus*	Traps (field)	Attraction	(Barbosa et al., 2008)
Skatole (3-Methylindole)	Synthetic	*Cx. quinquefasciatus, Cx. stigmatosoma & Cx. tarsalis*	Traps (field)	Attraction	(Barbosa et al., 2008)
Dodecyl nonanoate Decyl undecanoate Nonyl dodecanoate Pentyl hexadecanoate Propyl octadecanoate	Synthetic	*An. stephensi*	Cage	Attraction	(Sharma et al., 2009)
Hexadecyl pentadecanoate Pentadecyl heptanoate Hexyl pentadecanoate Octadecyl propanoate Tridecyl octanoate Pentadecyl hexanoate Undecyl decanoate	Synthetic	*An. stephensi*	Cage	Repellency	(Sharma et al., 2009)
Hexadecyl pentanoate Tetradecyl heptanoate Tridecyl octanoate	Synthetic	*Ae. aegypti & Ae. alpopictus*	Cage	Repellency	(Sharma et al., 2008)
Propyl octadecanoate	Synthetic	*Ae. aegypti & Ae. alpopictus*	Cage	Attraction	(Sharma et al., 2008)
	Synthetic	*An. stephensi*	EAG, Y-tube Olfactometer & cage	Attraction	(Seenivasagan et al., 2012)
Carboxylic acids isobutyric, Butyric, Isovaleric, & Hexanoic (individually & blend)	Animal feed (Purina) infusion	*Cx. p. quinquefasciatus & Cx. tarsalis*	Plastic container	Repellency	(Hwang et al., 1980)
***Oviposition cues from other resources**					
Fresh cow urine Old cow urine		*An. gambiae s.l.* *Cx. quinquefasciatus*	Cage & artificial habitats	Attraction	(Kweka et al., 2011)
Water vapour	water	*An. gambiae & An. arabiensis*	WHO test tubes		(Okal et al., 2013)
Soil infusions		*An. gambiae s.l.*	Cage, artificial ponds & BG-Sentinel traps	Attraction	(Herrera-Varela et al., 2014; Okal et al., 2015)
Pellet infusions		*An. gambiae s.l.*	Artificial ponds	Repellency	(Herrera-Varela et al., 2014)
Purina Laboratory Chow infusion		*Cx. p. quinquefasciatus &Cx. tarsalis*	Plastic container	Repellency	(Hwang et al., 1980)

CDC- Centres for Disease Control and Prevention; PCA-principal component analysis; EAD-electroantennographic detection; EAG – electroantennography; WHO- World Health Organization; *The oviposition cues different from plants, plant infusions, microbes, predators, conspecific immature and competitors.

2.3.2 Role of plant odour cues on the oviposition behaviour of gravid malaria vectors

The greatest olfactory environment of insects is made of plant VOCs (Conchou et al., 2019). It has been shown that more than 1700 VOCs are known to be produced by plants (Dicke & Loreto, 2010; Knudsen et al., 2006). These volatile chemicals are produced by all types of plants and all parts of plant tissues (roots, leaves and flowers) with variations in composition and amount (Conchou et al., 2019). Certain VOCs are produced commonly by several plant taxa whereas others are produced only by specific plant taxa (Conchou et al., 2019). High chemical profile variability has also been found between plants of the same species (Conchou et al., 2019). Plants use VOCs for various ecological services such as defence against herbivores and pathogens attack, to attract insect pollinators and predators, interact with neighbouring plants, interact with pathogens, thermo-tolerance, and adapt to environmental stress (Bruce et al., 2005; Dudareva & Pichersky, 2008; Mccormick et al., 2012; Spinelli et al., 2016).

Cedrol is the first oviposition attractant of *An. gambiae s.l.* and identified from headspace sample of soil infusions (Lindh et al., 2015). The soil was collected from natural mosquito breeding sites which contained a large amount of the root materials of *Cyperus rotundus* (Lindh et al., 2015). In another study it was shown that cedrol is produced by two fungal species, *Fusarium fujikuroi* and *F. falciforme*, isolated from the rhizomes of *C. rotundus* (Eneh et al., 2016). Cedrol has also been identified from different plants such as sorghum (Khwatenge, 1999), *Artemisia annua* L. (Mercke et al., 1999) and *Cyperus articulatus* (wetland grass) (Olawore et al., 2006). This suggests the need for evaluation of influence VOCs released from *Cyperus rotundus* in gravid malaria vector mosquitoes as it may release cedrol. Other studies have shown that gravid malaria vectors attracted to VOCs of domesticated agricultural grasses. For instance, blends of odour cues from wetland rice plants (*Oryza sp*.) and pollens of maize (*Zea mays*) and sugarcane (*Saccharum officinarum*) increased egg-laying response of gravid *An. arabiensis* (Wondwosen et al., 2016, 2017, 2018). The authors of that work suggest that mosquitoes have selectively adapted to habitats dominated by agricultural grasses of the Poaceae family which in turn would suggest that these grasses release a unique odour profile that separates them from wild grasses. Additionally, a study by Asmare et al. shows that gravid *An. arabiensis* and *An. coluzzi* attracted to the extracts of plants of Poaceae family (Asmare et

al., 2017). The same study has shown that the attraction and egg-laying response of the gravid mosquitoes were highly reduced when the mosquitoes were provided with the plant extracts of Typhaceae and Cyperaceae family. However, none of these studies tested the response of gravid malaria vectors to the live plants. Evaluation of the intact live plants for the preference of gravid malaria vectors is ecologically more important than the extracts or dead plants. This is because the rate and diversity of the VOCs released by plants increase when they are injured or exposed to stresses (Portillo-Estrada et al., 2021; Smith & Beck, 2015; Tholl et al., 2006).

Insects perceive odour stimuli differently when they are in a blend or individual compound (Wright & Smith, 2004b, 2004a). Insects' behavioural responses are usually elicited by the integration of certain compounds which operate in synergism or antagonism (Conchou et al., 2019). For instance, studies have illustrated that different malaria vectors were attracted to or repelled by different plant VOCs during a search for oviposition sites. Laboratory and semi-field studies have shown that gravid *An. arabiensis* were attracted to blends of chemical volatiles from rice plants and maize pollens (Wondwosen et al., 2016, 2017). Similarly, gravid *An. coluzzii* and *An. arabiensis* were attracted to chemical cues released from Poaceae (*Echinochloa pyramidalis and Echinochloa stagnina*) grasses (Asmare et al., 2017). A three-component attractive blend consisting of (*E*)-linalool oxide, β-pinene and β-ocimene became non-attractive to non-blood fed *An. gambiae* when limonene was added to the blend (Jacob et al., 2018).

Studies have also highlighted that the preference of gravid mosquitoes for plant extracts varies based on the concentrations. Torres-Estrada et al., for instance, investigated that *Anopheles albimanus* was attracted to 0.1%, 0.01%, and 0.001% of *Brachia riamutica, Cynodon dactylon, Jouveas traminea, Fimbristy lisspadicea,* and *Ceratophyllum demersum* extracts (Torres-Estrada et al., 2005). The same study has revealed that higher concentrations (1%, 10%, and 100%) of these plants extracts repelled gravid *An. albimanus*. 3-metylindole, identified from grass infusion, was strongly attractive to *Culex quinquefasciatus* at concentrations between 1 and 10 ng/L (Millar et al., 1992).

2.4 Volatile organic compounds collection and analysis

Various techniques have been developed for sampling volatiles from different sources. Solvent extraction and headspace sampling methods are commonly used for the sampling of plant volatiles (Agelopoulos & Pickett, 1998; Fäldt et al., 2000). Before choosing a particular VOCs

collection technique different factors such as the objective of the collection and the substrate from which volatiles are collected should be considered (Tholl et al., 2006). For example, analysis of plant VOCs under controlled laboratory settings may be implemented using computer-assisted equipment whereas the study of the ecological role of volatiles in the natural ecological system needs a portable device to use in the field (Tholl et al., 2006).

2.4.1 Solvent extraction

Solvent extraction is among the oldest and commonly used methods (Kloskowski, 2003; Ormeño et al., 2011; Tholl et al., 2006) for the collection of VOCs from plants using one solvent (Otienoburu et al., 2012) or more than one solvents (Vargo & Foster, 1982). The features of the targeted VOCs to be sampled determines the choice of the solvents based on solubility, volatility and polarity of the compounds: polar solvents e.g. dichloromethane, methylene chloride, methanol, ethanol, acetonitrile diethylether, ethylacetate, acetone are used to extract polar compounds while non-polar solvents e.g. hexane, benzene, light petroleum, toluene, chloroform are for the extraction of non-polar compounds (Abarca-Vargas et al., 2016; Kloskowski, 2003; Wells, 2003; Yalavarthi & Thiruvengadarajan, 2013). Extraction is only possible if the VOCs dissolve in the organic solvent used for the extraction (Wells, 2003) and a complete profile of organic compounds could be obtained by diversifying the type of solvents (Nyasembe & Torto, 2014; Yalavarthi & Thiruvengadarajan, 2013). This method is advantageous as it provides a complete profile of VOCs when more solvents with different properties are used (Nyasembe & Torto, 2014). The extraction method has some disadvantages. It can be contaminated by the impurities in the solvent used for extraction (Nyasembe & Torto, 2014). Also, some of the VOCs identified using this method may not be released in natural settings and the method is not suitable for determining the ecological role of the volatiles (Knudsen et al., 2006; Nyasembe & Torto, 2014). The other limitations are that the method is time-consuming and labour intensive (Vas & Vékey, 2004).

2.4.2 Headspace volatile collection techniques

Headspace volatile collection is widely used in VOC sampling. It gives a better overview of the profiles of compounds that are actually released by plants and used in plant-insect interaction is more preferable for ecological studies (Jele et al., 2017; Tholl et al., 2006). This technique is non-destructive and ideal when plant volatile sampling needs to be done from live plants (Knudsen et al., 2006; Tholl et al., 2006). It does not require the use of solvents and not

prone to contamination (Jele et al., 2017). Equipment selection for headspace sampling should be done carefully to avoid materials that can hold or emit volatiles and contaminate the samples (Tholl et al., 2006). The often preferred materials include those made from glass, metal and some plastic types such as Teflon and polyethylene terephthalate (PET) (McCormick et al., 2014). Headspace volatile sampling can be static headspace (SHS) and dynamic headspace (DHS) based on the presence or absence of airflow in the collection chamber (Bylaite & Meyer, 2006; Cavalli et al., 2003).

Static headspace volatile collection is applied without air circulation in the collection chamber. This ensures the collection of VOCs reducing the chance of contaminations of the samples by the airflow from the surrounding (Tholl et al., 2006). SHS increases the likelihood of capturing the less abundantly available volatile compounds (Tholl et al., 2006). Therefore, this method is more reliable to collect volatiles from low releasing plants (Nyasembe & Torto, 2014; Tholl et al., 2006). This sampling method has some limitations including the collected samples cannot be reused and may accumulate humidity and heat that affects physiology of organisms (Tholl et al., 2006). Improvement has been made to SHS by developing solid-phase microextraction (SPME) which is a simple and fast method (Nyasembe & Torto, 2014; Tholl et al., 2006). The SPME method uses various fibres with different affinity for the volatile types. These fibres include (i) polydimethylsiloxane (PDMS): effectively to collects non-polar VOCs; (ii) polydimethylsiloxane (PDMS)/divinylbenzene (PDMS/DVB): effectively collects VOCs with medium polarity; (iii) carboxen/ polydimethylsioxane (CAR/PDMS): effectively collects non-polar VOCs; (iv) poly(butyl acrylate (PA): effectively collects polar VOCs; (v) carbowax/ divinylbenzene (CB/DVB): effectively collects polar VOCs; (vi) divinylbenzene/carboxen/ polydimethylsiloxane (DVB/CAR/PDMS): effectively collects a variety of VOCs (Piotrowicz, 2016). A single fibre can be used repeatedly up to about 100 times (Tholl et al., 2006).

Dynamic headspace sampling is the most repeatedly used method to collect plant VOCs (Agelopoulos & Pickett, 1998; Jhumur et al., 2007, 2008; Nyasembe et al., 2012; Tholl et al., 2006; Tolosa et al., 2019). This technique uses purified air (carrier gas) which circulates over the samples enclosed in a container (Tholl et al., 2006). Volatile organic compounds emanated from plants are transported by a carrier gas to a solid porous adsorbent such as Tenax TA, Porapak Q, Carbotrap, Carboxen and Super Q (Agelopoulos & Pickett, 1998; McCormick et al., 2014). The efficiency of these adsorbents relies on their polarity and affinity (McCormick et al., 2014; Wondwosen et al., 2016). An adsorbent which is efficient for the collection of

VOCs of low polarity and low molecular weight is not efficient for the collection of VOCs of high polarity and high molecular weight (Tholl et al., 2006). Tenax TA is used to collect compounds with lipophilic to medium polarity (Nyasembe & Torto, 2014) and Carbotrap is used to trap a wide range of compounds but terpenes decompose rapidly (Nyasembe & Torto, 2014). This method has several benefits over the SHS as it ensures the collection of VOCs that are adequate for detection and structure elucidation, avoids temperature and humidity increase and low retention of contaminant compounds in the headspace (Tholl et al., 2006). The main drawback of this technique is collection of incomplete profile of VOCs due to the specific affinity of the adsorbents for VOCs (Nyasembe & Torto, 2014). Therefore, to obtain a complete profile of VOCs, the use of more than one type of adsorbent matrices is advisable (Nyasembe & Torto, 2014).

2.4.3 Separation and analysis of plant organic chemical compounds

Plants release various VOCs which mix in the atmosphere. Isolation, identification and quantification of these VOCs from plants are very important steps during the study of their ecological functions. The analysis of these compounds for the exploration of their ecological function need special equipment and techniques with high resolution and sensitivity (Barbosa-cornelio et al., 2019; Tholl et al., 2021). Some of the most frequently applied plant volatiles analysis (quantitative and qualitative techniques) include gas chromatography (GC) combined with mass spectrometry (GC-MS), GC coupled to flame ionization detector (GC-FID) and thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) (Materić et al., 2015).

The GC-MS is a standard and most efficient analytic instrument for the separation, identification and quantification (qualitative and quantitative) of plant volatile compounds (Materić et al., 2015; Tholl et al., 2021). The collected VOCs can be eluted from the adsorbents into glass vials using organic solvents or can be directly inserted into thermal desorber (Materić et al., 2015; Tholl et al., 2006). Eluted samples are injected into the column (Pichersky & Dudareva, 2020). Injection of the samples can be done either in split or splitless mode. Less concentrated samples are injected in splitless mode (Pichersky & Dudareva, 2020). The eluted samples can be reanalysed and used in behavioural bioassays but affect the sensitivity because of dilution (Tholl et al., 2006).

The GC is used to separate organic volatile compounds but cannot identify them (Hussain & Maqbool, 2014; Tholl et al., 2006). Identification and quantification of the separated compounds are performed with detectors such as MS, FID and electroantennography detector (EAD) (Barbosa-cornelio et al., 2019; Materić et al., 2015). The MS is highly sensitive and commonly used to identify (based on their mass-to-charge ratio) and quantify volatiles (Hussain & Maqbool, 2014; Materić et al., 2015). Wiley and NIST MS databases are used for the identification of VOCs (Barbosa-cornelio et al., 2019; Tholl et al., 2006). The GC-MS is highly effective to analyse low-molecular mass and low-to-mid polarity compounds (Pocsfalvi et al., 2016). The FID detectors are used for quantitative analysis (Tholl et al., 2006). The FID can only identify compounds accurately with the use of standards, comparison with linear retention indices in databases, or use of well-characterized profile samples (Barbosa-cornelio et al., 2019). Electroantennography and single sensillum recordings are used to identify bioactive compounds (Barbosa-cornelio et al., 2019; Qiu et al., 2004). But, they do not demonstrate the type of behavioural influence the compound elicits and require behavioural bioassays to determine the type of response they elicit (Barbosa-cornelio et al., 2019; Nyasembe et al., 2012; Wondwosen et al., 2016).

Thermal desorption is important to detect less concentrated samples and for the identification, separation and quantification of isomers (Materić et al., 2015). It has some disadvantages such as the samples cannot be reused, high molecular compounds may not be fully desorbed, degradation of thermally unstable compounds and artefact formation from the adsorbent used (Materić et al., 2015; Tholl et al., 2006).

2.5 Tools used for behavioural screening of plant-based organic chemical compounds

Several tools have been designed for the study of odour mediated behaviours of mosquitoes in laboratory, semi-field and field settings. Some of the tools frequently used in mosquito behavioural bioassays to test oviposition cues are mosquito cages, olfactometers and wind tunnels (Haynes & Millar, 1998; Herrera-Varela et al., 2014; Okal et al., 2015; Wondwosen et al., 2016). Additionally, oviposition bioassays have been done using WHO test tubes, gravid traps, and ovitraps (Baak-Baak et al., 2013; Du & Millar, 1999; Logan et al., 2008; Okal et al., 2015; Okal et al., 2013). These devices are categorized as still air and moving-air bioassays based on whether they use active airflow or not. The behaviusof mosquitoes can be recorded either by direct observation (Dekker et al., 1998) or video tracking (Beeuwkes et al., 2008) during the oviposition bioassays.

In still-air bioassays, there is no air flow and the VOCs are delivered using a dispenser from which they spread by simple diffusion (Baker & Carde, 1984; Haynes & Millar, 1998). The test insects are released into the odour arena and they respond by moving towards or away from the VOCs source (Baker & Carde, 1984). Only simple equipment such as petri dishes or small cages are used in this type of bioassays (Haynes & Millar, 1998). Cage and two-port olfactometer are most commonly used in still-air bioassay (Kweka et al., 2011).

Cage bioassays are the commonly used still-air bioassays important to study oviposition behaviour. It can be choice or no-choice egg-count bioassays. Choice egg-count bioassays in mosquito cages are mostly used to evaluate attraction or repellence of olfactory cues to gravid mosquitoes (Okal et al., 2015). It is a simple and fast step, and very useful to identify short-range chemical cues involved in oviposition site selection (Isoe et al., 1995). The main limitation of choice egg count bioassay is that it does not reveal detailed information about the behavioural influence of test compounds as it only reflects the final result of all the egg-laying processes (Isoe et al., 1995). Mosquitoes respond by laying eggs in the preferred oviposition substrates. There is a wealth of literature showing study of stimulation and deterrence response of gravid *Aedes, Cuqlex* and *Anopheles* mosquitoes to different oviposition substrates (Eneh et al., 2016; Herrera-Varela et al., 2014; Munga et al., 2006; Ponnusamy et al., 2008; Turnipseed et al., 2018). The response of mosquitoes is determined by counting the number of eggs deposited (Isoe et al., 1995; Sumba et al., 2004b) or comparing the proportion of gravid females that responded by ovipositing in reference or test compound as used in the present study and elsewhere (Eneh et al., 2016; Herrera-Varela et al., 2014).

The interpretation of the effect of oviposition odorants on oviposition behaviour using the results of egg-count bioassays is often challenging due to the difficulty of distinguishing between attractant or stimulant and repellents or deterrents (reviewed by Isoe et al. 1995). Clear differentiation between stimulants and attractants is very vital to develop long-range attractants which can be used in mosquito surveillance and control (Isoe et al., 1995). Sticky screen and detergent bioassays have been developed to solve these problems. These bioassays are useful to estimate the number and proportion of gravid mosquitoes which show a preference for the oviposition cue by attempting to oviposit. Sticky screen bioassays use glue to catch the landing gravid mosquitoes (Isoe et al., 1995). These bioassays have been widely used to evaluate the oviposition response of gravid *Aedes* mosquitoes to various oviposition substrates (Ponnusamy et al., 2015; Ponnusamy et al., 2010; Roslan et al., 2017; Trexler et al., 2003; Trexler et al.,

1998). Similarly, detergent bioassays use laboratory-grade surfactants to break the surface tensions drowning the landing mosquitoes (Isoe et al., 1995).

Two-port olfactometer has two arms to carry test substrates (Wondwosen et al., 2016). It is used to test short-range effect of volatile chemical cues (Haynes & Millar, 1998).

In moving-air bioassays, mosquitoes respond by oriented upwind flight to locate the source of the odour cues or downwind flight to avoid the odour cue (Isoe et al., 1995). Such types of bioassays require equipment that generates a constant rate of airstream with uniform power and direction (Haynes & Millar, 1998). Filtered and humidified air passes through plume in a downwind direction to carry VOCs (Haynes & Millar, 1998; Knols et al., 1994). Several dispensers such as nylon strips, glass rods or beads, metal surfaces, filter paper, cotton balls/wicks, rubber septa, polyethylene vials, and glass capillary tubes are used (Haynes & Millar, 1998; Okumu et al., 2010). The behavioural responses are recorded by observing the movement of the released test insects into the plume or leaving the plume (Haynes & Millar, 1998). The success of this bioassay depends on the ability of the test insects to detect the chemical compounds and the direction of air flow through a laminar air stream (Millar, 1998). The consistent release rate of the VOCs, their composition and dose determine the behavioural response of organisms (Millar, 1998). The commonly used moving air bioassay tools are olfactometers and wind tunnels. Olfactometers can have one or many arms and each arm has different sources of VOCs (Millar, 1998). Wind tunnels have the same source of odour cues and are used to test short-range odour cues.

2.6 Novel odour-based strategies for gravid mosquito surveillance and control

The use of non-chemical strategies associated with mosquito behaviour for malaria vectors intervention and monitoring has been neglected for many years (Hawkes et al., 2017). This is mainly attributed to the lack of effective synthetic attractants (Mweresa et al., 2016). The use of odour-cues as a component of integrated vector control is advantageous as they (i) work at very low concentrations; (ii) help for communication over a relatively long distance than other types of insect communication; (iii) are species-specific (specially the pheromones); (iv) less likely to cause resistance development and relatively safe to non-target organisms and environment; (v) can be deployed both indoors and outdoors and; (vi) can influence the behaviour of one or both sexes based on their physiological state (Kline, 2007; Maia et al., 2018; Mweresa et al., 2016; Nyasembe et al., 2018). Trapping of gravid females can be more

important as they are more likely to be infected with pathogens following the take of human bloodmeal (Maciel-de-freitas, 2008).

The main strategies used for the application of odour baited tools include mass trapping, attract-and-kill, mating disruption and push-pull (Cook et al., 2007; Homan et al., 2016; Kline, 2007; Mafra-neto & Dekker, 2019). Mass trapping is a strategy to trap a large number of insects using visual and olfactory cues to lure them into the trapping device and then kill them (Homan et al., 2016). Attract-and-kill is designed to lure the targeted insects to bring into contact with killing agents such as insecticides, sterilizing, pathogen, or desiccant (Brugman et al., 2018; Mafra-neto & Dekker, 2019; Mweresa et al., 2020). Mate disruption is interference of mate searching behaviour of males using pheromones to prevent the occurrence of mating. This method works by preventing pheromone emission or release of pheromones to saturate the environment (Mweresa et al., 2020). Push-pull strategies deploy repellents that avert mosquitoes from the treated material (the 'push') and orient them to the traps baited with attractants (the 'pull') so that the mosquitoes are collected to reduce their densities (Kitau et al., 2010; Menger et al., 2014; Paz-soldan et al., 2011). The most successful odour-based tools use was applied for the control of tsetse flies. For example, beta traps and black cloth target traps baited with odour cues such as acetone, octanol and insecticides effectively resulted in the elimination of tsetse flies in Antelope island, Zimbabwe (Vale et al., 1986, 1988).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

Laboratory and semi-field behavioural bioassay experiments with *An. gambiae* were conducted at the International Centre of Insect Physiology and Ecology, Thomas Odhiambo Campus (*icipe*-TOC), Mbita (00° 26’ 06.19’’ S; 34° 12’ 53.13’’ E and altitude of 1137 m above sea level), western Kenya. *Anopheles* mosquitoes breeding sites surveys were conducted on Rusinga Island (00° 24' 41.76"S and 34° 9' 51.48"E, altitude ranging from 1100 m to 1300 m above sea level) along the shore of Lake Victoria. Rusinga Island is located 100 m away from the mainland (Mbita) and connected via a bridge (Figure 3.1). Mbita is characterized by equatorial tropical climate with daily average minimum and maximum temperatures ranging from 16°C to 28°C. Fieldwork to investigate the behavioural response of *An. funestus* to *C. rotundus* were implemented in the Ahero rice irrigation area (00° 10' 27.84"S and 34° 55' 13.08"E, altitude 1162 m), Kisumu County, western Kenya. Western Kenya has a long rainy season between March to June and a short rainy season between October to December.

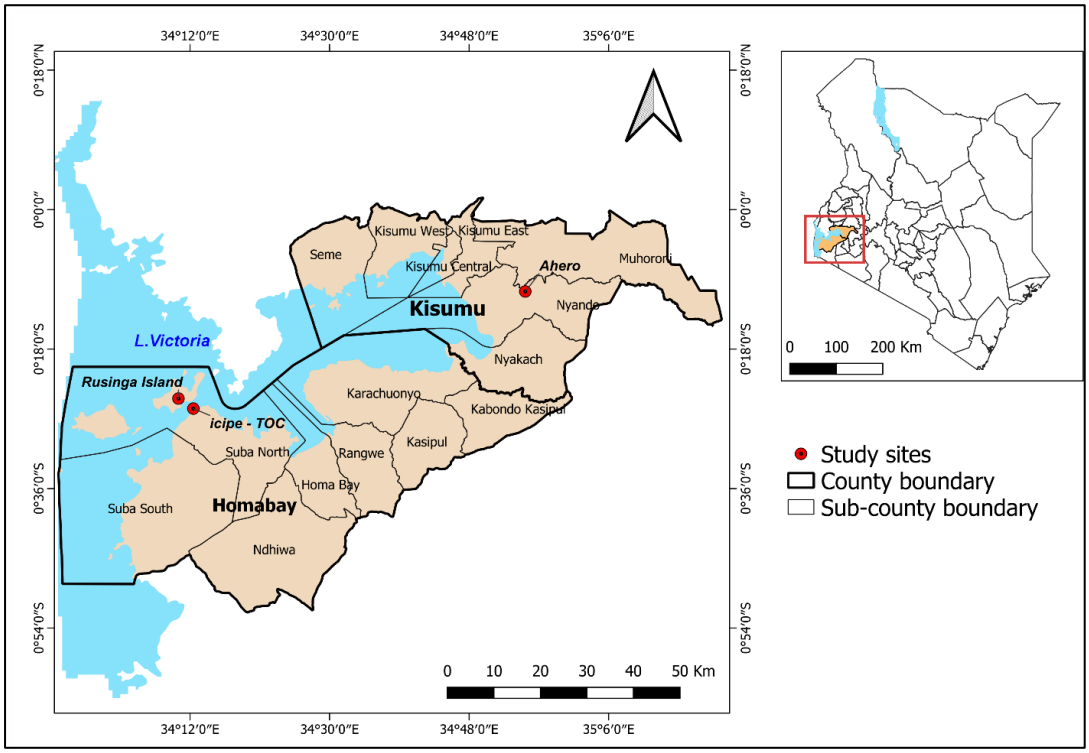

Figure 3. 1 Map of Kenya (A) and the study sites (B) in Mbita (*icipe*-TOC), Rusinga Island and Ahero (Source: Google Earth, (Google Earth, n.d.)).

3.2 Aquatic habitat surveys

In order to investigate association between graminoid plant species and presence and abundance of immature malaria vector mosquitoes in natural habitats the potential aquatic habitat types, the species of mosquitoes and type of graminoid types in the habitats were determined. A cross-sectional habitat survey was done along stretches of 700 m long and 300 m wide (clusters of approx. 0.2 km^2). A total of 13 sampling clusters were selected around the lake shores of Rusinga Island. (Figure 3.2). The areas were selected with the help of Google Earth, aiming at a similar distribution around the island. Inaccessible areas with steep rocks at the shoreline were excluded. Within each sampling cluster, location of all aquatic habitats was recorded using OPPO A37 smartphone with GPS application (GPS coordinates, version 4.68), a unique identifier was allocated and sampled as outlined below.

Figure 3. 2 Map of the study clusters (rectangles in red) along the shores of Lake Victoria in Rusinga Island (Source: Google Earth (Google Earth, n.d.)).

The aquatic habitat types were categorized as either swamp, puddle, fishpond, drainage or trench or artificial pit. Every aquatic habitat was inspected for the presence of larvae using the sweep-net method as described by (Ndenga et al., 2011). The sweep-net (40 cm × 15 cm × 30 cm) was made from fine cotton cloth with a 150 cm long handle. It was chosen for sampling due to its better efficiency in sampling the diverse aquatic fauna including freshly hatched mosquito larvae and mosquito pupae than the standard dipper (Harrera-Varela, 2015; Ndenga et al., 2011). A dipper (350 ml; BioQuip products, Rancho Dominguez, USA) was used for sampling when the habitat was too small to be sampled by a sweep-net. Sampling of mosquito larvae using either sampling tools was randomly done at different points of the habitats. The duration of sweeping was dependent on the perimeter of the habitat. About 10 minutes were taken to sweep habitats with perimeters exceeding 10 m, while 5 minutes were taken to sweep habitats <10 m in perimeter. All sweeps were emptied into white trays and mosquito immature stages were counted separately for the two encountered genera, *Anopheles* and *Culex*. *Culex* and *Anopheles* larvae were identified morphologically. *Culex* larvae possess a siphon on the posterior part of their abdomen for breathing whereas *Anopheles* larvae have no siphon and rest horizontal to the water surface (de Klerk & Wepener, 2011). The larvae were grouped as early (1^{st} and 2^{nd} instar) and late (3^{rd} and 4^{th} instar) instars based on their body size. Larval instars are stages between moults that vary in size. The late instar *Anopheles* larvae were classified using identification key (Gillies & Coetzee, 1987).

All late instar *Anopheles* larvae and mosquito pupae were transferred to water bottles (1 L) containing habitat water and taken to the *icipe*-TOC for rearing to adults. Rearing of the field collected larvae was done in 1 L plastic containers. Larvae were fed with a pinch of ground dry cat food (Nestlé Purina PetCare Company, Nairobi, Kenya) once daily. The emerged adults were killed in a -20°C refrigerator, sorted by genera and all *Anopheles* adults stored in Eppendorf tubes (1.5 ml) at -71°C until they were identified morphologically using printed keys (Gillies & Coetzee, 1987) and molecularly using polymerase chain reaction (PCR) followed by gel-electrophoresis (Scott et al., 1993). Morphological features such as wing, legs and abdominal segments were used (Gillies & Coetzee, 1987).

Vegetation coverage, vegetation types and the dominant vegetation type were recorded separately for habitat edge and water surface. Habitat edge was defined as the area along the waterline, approximately 10 cm inside and/or outside the water. Vegetation coverage and the dominant vegetation type were estimated visually, always by the same field worker to reduce

bias, as the proportion of the habitats covered with vegetations and categorized as (1) 1–25% (2) 26–50% (3) 51–75% (4) 76–100%. Graminoid plants across the edge and inside water were recorded as Poaceae, Cyperaceae, Juncaceae and Typhaceae. The graminoid plants were identified to family using the morphology of their leaves (two or three-ranked; open or closed sheaths), and their stem type (three-sided or round; hollow or solid) using identification key (Revuelta, 2019). Furthermore, herbaceous (not woody and non-graminoid plants) were collectively recorded as forbs. For each habitat, the dominant type of vegetation was identified and recorded. Full specimens of all dominant graminoid plants found in the aquatic habitats were collected and planted at *icipe*-TOC for further identification (Fish et al., 2015; Revuelta, 2019). The plants were labelled with the date of collection, cluster name, unique habitat identifier number and plant family. The plant specimens were identified to species level by an expert at East African Herbarium, National Museum of Kenya.

3.3 Gravid mosquito preparation

Anopheles gambiae s.s., Mbita strain insectary-reared mosquitoes, were used for all experiments conducted at *icipe*-TOC. Mosquitoes were reared under ambient conditions following the protocol described by (Okal et al., 2015). Adult mosquitoes were held in 30 × 30 × 30 cm netting-covered cages (Plate 3.1) at 25-28°C temperature and 68-75% relative humidity in a 12 h: 12 h light: dark photoperiod. Equal numbers of 2-3 days old adult female and male mosquitoes were transferred into a clean cage and starved for six hours starting at 13:00 h before allowed to feed on human arm at 19:00 h for 15 minutes. Blood feeding was done by inserting an exposed arm into a cage containing the starved mosquitoes. Blood feeding was repeated the next day at 19:00 h using the same procedure. After each blood-meal, the mosquitoes were provided with 6% glucose solution *ad libitum*. A wet towel was placed on top of the cages to provide water and additional humidity. After the second blood-meal, the mosquitoes were kept for another two days with access to glucose solution. On the third day, gravid females were selected and used in bioassays.

Plate 3. 1 Mosquito preparation for bioassays. Cages containing male and female mosquitoes were arranged on a shelf. Wet towels were placed on the top of each cage to maintain humidity and temperature in the cages between 68-75%, and 25-28°C.

For the choice experiments using olfactometer with *An. funestus* conducted in Ahero wild mosquitoes were used. More than 300 wild blood-fed *An. funestus* mosquitoes resting inside houses were collected every morning by aspirating them into standard cages for each round of the experiment. *Anopheles funestus* was distinguished from other anophelines based on their morphological appearance by an experienced field technical assistant before use in the experiments using identification key (Maureen Coetzee, 2020). The collected females were maintained in an experimental hut as stated above for the insectary colony. The collected mosquitoes were kept in the cages for two to three days until they became gravid.

3.4 Preparation of test substrates from breeding sites

Four graminoid plant species, naturally occurring frequently in malaria vector breeding sites in western Kenya (Bokore et al., 2020), namely the grass-like sedges (Cyperaceae), *Cyperus rotundus* (nut grass), and *Cyperus exaltatus* (giant sedge), as well as the true grasses (Poaceae), *Panicum repens* (torpedo grass) and *Cynodon dactylon* (Bermuda grass) were collected from

wetlands along the shores of Lake Victoria, around Mbita and Rusinga towns, western Kenya. The plants were carefully uprooted and the plants with soil were transported to *icipe*-TOC for bioassays in olfactometers and large field cages, and for volatile collections. A drought-tolerant grass, not native to wetlands and frequently used as ornamental grass in gardens, *Cenchrus setaceus* (purple fountain grass; Poaceae) was obtained from plant nurseries in Kisumu town and maintained at *icipe*-TOC. *Cenchrus setaceus* was selected for this study to check if all graminoid species or only the plants associated with water in natural breeding sites are attractive to the mosquitoes. The plants were used only in their non-flowering stage (roots, stems and leaves only) to standardise the experiments (flowering plants likely release different odours than non-flowering) and be in the position to have sufficient plant material at any time. In preparation for bioassays, the plants were washed thoroughly using lake water to remove the soil. Fresh plant samples were used for every round of bioassays. A bunch of several individual plants, weighing approximately 350 g, was used for every replicate bioassay.

Soil collected from the habitat where *Cyperus rotundus* was uprooted, was used for a preliminary bioassay. The soil was taken from the upper 10 cm of the habitat and plant material sieved out before use. For each replicate bioassay, 4 kg of fresh soil was used.

Water was used in all bioassays, acknowledging that water vapour is a major oviposition attractant (Okal et al., 2013). The water originated from Lake Victoria and sediments allowed to settle before the clear supernatant was used for experiments done *icipe*-TOC with graminoid plants and soil. For the experiments conducted with synthetic chemicals distilled water was used. In all the experiments implemented in Ahero river water from rice irrigation canal was used.

A hay-infusion previously shown to be repellent for gravid *An. gambiae* (Eneh et al., 2016) was prepared for the initial calibration of the olfactometer bioassays. The infusion was prepared by mixing 24 L of lake water and 90 g of hay in a bucket and kept in a dark place with the temperature ranging from 18°C to 29°C for three days before use for the bioassays. Before use, buckets were thoroughly cleaned with odourless soap and allowed to dry under the sun.

3.5 Two-port airflow olfactometer bioassays

Four two-port olfactometers were constructed from galvanised iron sheets (Figure 3.3) to test the odour-orientation of gravid *An. gambiae s.s.* in response to test substrates. The olfactometers were placed in a netting-screened makeshift shed where experiments were run

overnight under ambient conditions. The olfactometers had two large substrate holding chambers (1 × 0.9 × 1 m), two trapping chambers made of polyvinyl chloride (PVC) pipes (0.3 m long and 0.1 m diameter), a fan and mosquito release chamber (0.5 × 0.2 × 0.3 m). The size of substrate holding chambers was sufficient to carry whole live plants. Mosquitoes were introduced into the release chamber through an opening at the bottom. The electricity-powered fan drew air from the two substrate holding chambers through the holding chamber to the outside. Funnels inserted into the trapping chamber prevented mosquitoes from returning to the release chamber.

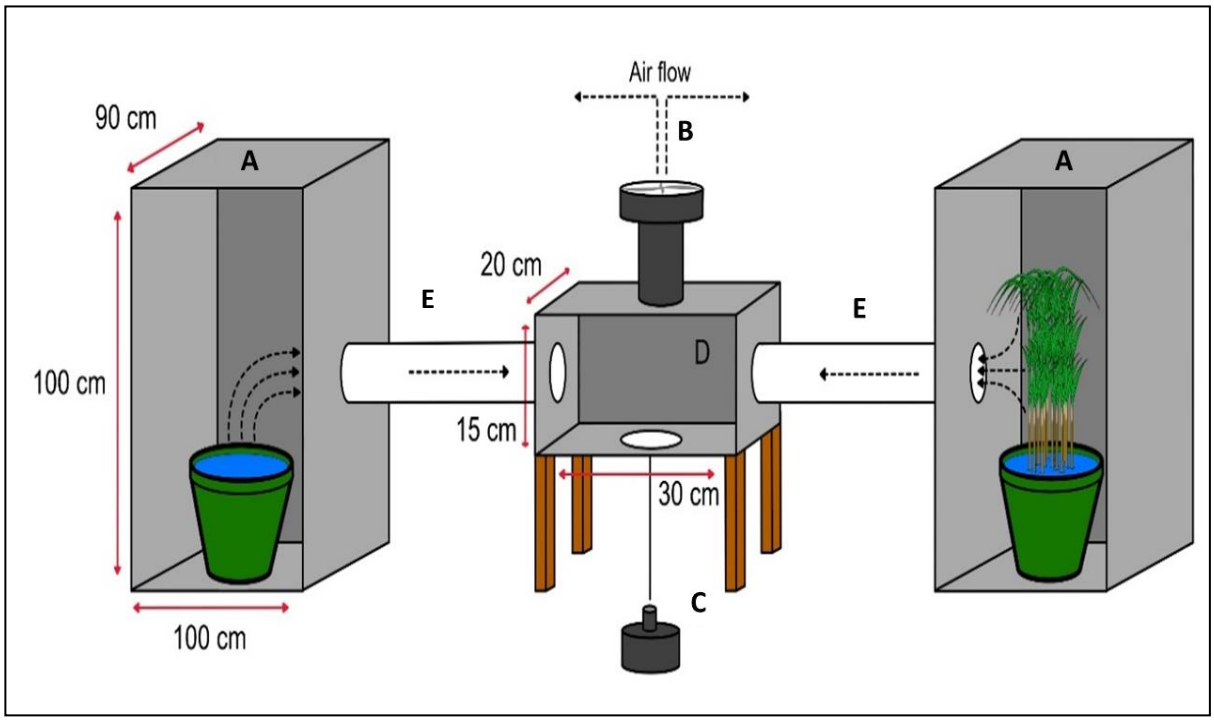

Figure 3. 3 The olfactometer bioassay experimental setup. The substrates were placed in the two large (1 × 0.9 × 1 m) chambers (**A**) from which 12-volt electric fan (**B**) drew air to the outside. The fan pipe (**C**) was fitted on the top side and the mosquito release cup at bottom side of the release chamber (**D**). The mosquitoes that made a directional choice were trapped in either of the two trapping chambers (**E**) and data were recorded every morning by removing the fan pipe and the trapping chambers.

Test substrates were placed in both holding chambers. The fan was switched on five minutes before releasing 100 gravid *An. gambiae* to the choice chamber at 18:00 h. The choice made by mosquitoes was recorded the following morning at 8:00 h by counting the number of mosquitoes trapped in each trapping chamber. The positions of the two test substrates were

randomly rotated between chambers so that each substrate spent the same number of nights in each location.

All choice experiments conducted in the two-port airflow olfactometers and in the large field cages are listed in Table 3.1. After calibration of the olfactometers (Appendix 1), a series of choice tests were done with intact plant materials (Table 3.1). Each comparison was replicated over 16 nights using a new batch of mosquitoes and fresh test substrates for every replicate. The replicate was discarded and repeated when mortality was ≥20% in the release/choice chamber or when less than 50% of the released mosquitoes responded (meaning majority remained in the central release chamber for the night).

Table 3. 1 O**viposition substrates used in behavioural bioassays with gravid *An. gambiae s.s.* in two-port airflow olfactometers and in large field cages.**

Treatment 1 ('control')	**Treatment 2 ('test')**	**No of replications**	**Total no of gravid *An. gambiae* recollected (out of total released)**
Two-port airflow olfactometer bioassays			
Choice between wet soil vs. wet soil + graminoid plant from			
Based on previous work on soil infusions (Herrera-Varela et al., 2014), is the associated sedge, *Cyperus rotundus*, attractive to gravid mosquitoes or is attraction based on soil alone?			
Water	Water	16	1060 (1600)
Soil	*Cyperus rotundus*	16	875 (1600)
Choice between water vs. water + graminoid plants			
Do intact graminoid plants from natural aquatic habitats attract gravid *Anopheles gambiae s.s.*? Is *Cyperus rotundus* more attractive than other graminoid plants? Is there a difference in behavioural response to a grass not naturally associated with breeding sites?			
Water	*Cyperus rotundus*	16	1245 (1600)
Water	*Cyperus exaltatus*	16	1204 (1600)
Water	*Panicum repens*	16	1194 (1600)
Water	*Cynodon dactylon*	16	1016 (1600)
Water	*Cenchrus setaceus*	16	1064 (1600)
Choice between two graminoid plant species			
Panicum repens	*Cyperus rotundus*	16	1224 (1600)
Cynodon dactylon	*Cyperus rotundus*	16	1179 (1600)
Large-cage choice bioassays with free-flying mosquitoes			

Do gravid *Anopheles gambiae s.s.* show similar behavioural response to the plant volatiles at longer-range?			
Water	Water	16	1431 (3200)
Water	*Cyperus rotundus*	16	2125 (3200)
Water	*Cyperus exaltatus*	16	2075 (3200)
Water	*Panicum repens*	16	1858 (3200)
Water	*Cynodon dactylon*	16	1988 (3200)
Water	*Cenchrus setaceus*	16	1478 (3200)
Panicum repens	*Cyperus rotundus*	16	2234 (3200)

*Two-equal choices bioassays using lake water were used as reference experiments. Modified BG-Sentinel mosquito traps were used in large-cage experiments.

Similar bioassays were implemented with wild gravid *Anopheles funestus s.s.*. Wild *Anopheles funestus s.s*. were used since their colonization in laboratory conditions is challenging. The experiments were conducted in experimental huts. Attraction response of gravid mosquitoes to river water which passes through rice fields and *C. rotundus* was investigated. The experiments were replicated over 11 nights using fresh test substrates and new batches of gravid mosquitoes. The mosquitoes used for the experiments were killed using chloroform and preserved in 1.5 ml microcentrifuge tubes using silica gel for molecular analysis using polymerase chain reaction (PCR) followed by gel-electrophoresis (Scott et al., 1993).

3.6 Large field-cage experiments with free-flying mosquitoes

Test treatments that elicited a positive response in olfactometer bioassays were then further evaluated with free-flying gravid *An. gambiae s.s.* in large field cages (11.8 m long × 6.8 m wide × 2.4 m high; Figure 3.4A) under ambient environmental conditions to mimic a more natural setting and test for longer-range attraction (Okal et al., 2015). The test substrates were placed inside the modified BG-Sentinel traps (Biogents AG, Regensburg, Germany). This trap was originally developed for mass trapping of host-seeking mosquitoes such as *Aedes aegypti* and *Ae. albopictus* using human odours (Maciel-de-freitas et al., 2006). Gravid *Anopheles* mosquitoes oviposit in aquatic habitats or at least on moist surfaces and recent study has shown that water vapour is an oviposition an attractant of *An. gambiae s.s*. (Okal et al., 2013). Therefore, this trap was modified to hold 4 L of water and other oviposition substrates by inserting a tightly fitting black plastic bucket (Pride, Mombasa, Kenya), 34 cm high and 30 cm in diameter, inside white fabric container (Okal et al., 2015). Since *An. gambiae s.l.* naturally

do not oviposit in container type of habitats, the traps were buried into the ground so that only the netting top of the trap and collection funnel containing the fan, was visible (Figure 3.4C). Two traps with either equal or different test substrates included were set up per field cage (Table 3.1). The two traps were placed 4 m apart and 1.4 m away from the nearest wall. Mosquitoes were released from the opposite side, 9 m away from the traps (Figure 3.4B). The two test substrates were allocated to the location randomly and the positions of the two traps were exchanged between the two shorter walls of the cage in consecutive nights. Every experimental night, 200 gravid *An. gambiae s.s.* were released in the field cage at 18.00 h. The next morning at 08.00 h the traps were collected, and the number of mosquitoes recaptured in the traps' catch bags counted. Every experiment was repeated over 16 nights.

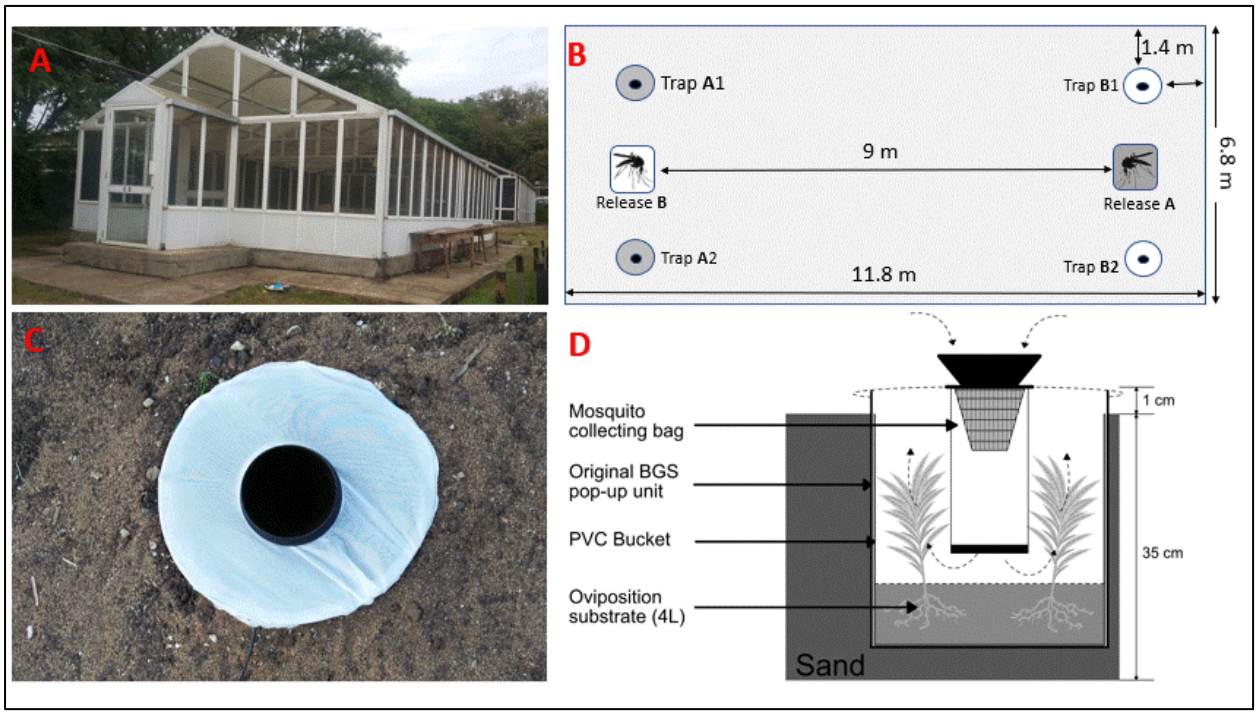

Figure 3. 4 Experimental set up in the large field cages (A) with schematic overview of mosquito release points and trap positions (B). The blue (trap A1 & trap A2) and white (trap B1 & trap B2) colours show the trap locations and their respective mosquito release points. Test substrates were provided in modified BG-Sentinel traps buried in the ground (**C**). The cross-section through the modified BG-Sentinel gravid trap (**D**) shows the location of the plants and the airflow generated by the trap.

3.7 Sample size considerations for bioassays

Sample sizes of the bioassay experiments in these studies were calculated based on previous simple size considerations calculated for similar studies (Herrera-Varela et al., 2014; Okal et al., 2013; Okal et al., 2015). The sample size for replication was estimated using the formula developed by Hayes and Bennett (Hayes & Bennett, 1999) for comparing proportions of clustered data. For equal choices an equal proportion responding to either choice was assumed for the reference (p1=0.5). Based on previous work (Herrera-Varela et al., 2014), I aimed to be able to detect an increase in attraction by 16% (p2=0.66). Assuming a coefficient of variation (k) of 0.25 based on preliminary nightly test runs, and assuming at least 50 responding mosquitoes per night (n in each group), 16 replicates would be required for both treatment arms (p1 – equal choices; p2 – two choices) to detect the effect with 80% of power at a 5% significant level. This level of accuracy was determined to be adequate for identifying significant behavioural stimulants influencing oviposition choice (Herrera-Varela et al., 2014). The sample of the replication of WHO tube experiments and cage bioassays were determined based on previous works (Okal et al., 2013) and (Okal et al., 2015), respectively.

3.8 Sampling of headspace from intact plants

Volatile chemicals released from test plants were trapped from intact live plants using dynamic headspace sampling. For this, several non-flowing plants (approximately 350 g) were placed with some soil in a bucket with water, similar to the experimental conditions (Plate 3.2). The sampling was done for 48 hours under ambient conditions in the field cage. The aerial parts of the intact plants were enclosed into heat-resistant roasting bags (Sainsbury's Supermarkets Ltd, London EC1N 2HT) which were kept in an oven at 200°C for two hours prior to use. Porapak Q (50 mg, 50/80 mesh; Supelco) sorbent material was packed in a glass liner with glass wool on both ends to hold the sorbent in place. The Porapak Q traps were washed using 4 ml of hexane and kept in an oven for 2 hours at 50°C before use. Headspace collection was done by pumping 500 ml/min charcoal-filtered air into the bags through the inlet port and drawing the air out at a rate of 300 ml/min through the outlet port (Raguso & Pellmyr, 1998). Headspace collections were done on two different dates, sampling four replicates of every plant species per date (total 8 headspace samples per plant species). Collections were also done from three replicates of empty cooking bags to account for the background chemicals concurrently for the two dates. After sampling, the traps were sealed with polytetrafluoroethylene (PTFE) tape and kept in a freezer at -71°C. The filters were shipped to KTH Royal Institute of Technology,

Stockholm, Sweden, where they were first eluted using 3 ml hexane to decrease the likelihood of chemicals remaining in the trap and then concentrated to 250 µl using a desiccator connected to a duo rotary vane pump before chemical analysis.

Plate 3. 2 Plant preparation (A) for dynamic headspace sampling of volatile chemical compounds (B).

3.9 Chemical analysis based on gas chromatography coupled with mass spectrometry

The headspace samples were analyzed using a Trace 1300 gas-chromatograph (GC) coupled to an ISQ LT mass-spectrometer (MS; Thermo Fisher Waltham, MA, USA). For each analysis, 1 µL of sample was injected in splitless mode. The temperature program started at 40 °C and was held for 1.8 min, after which the temperature was ramped to 200°C at 20 °C/min. After reaching 200°C, the ramp was changed to 50 °C/min until the temperature reached 240°C, at which the temperature was held for 3 minutes. A 15 m × 0.25 mm × 0.25 µm (5% phenyl)-methylpolysiloxane column (Thermo Fisher) was used for all analyses. The carrier gas was helium and had a constant volumetric flow of 1 ml/min or a linear flow rate of 34 cm/s. The temperature of the transfer line between the GC and MS was set to 250°C. The ionization source was an electron impact with ionization energy of 70 eV. Heptyl acetate was used as an internal standard to evaluate any instrumental variations for a selection of the replicate analyses. All GC-MS data was handled with Thermo Scientific™ Xcalibur™ software. Results from the mass spectrometry were submitted to the NIST MS Search 2.0 program for the

NIST/EPA/NIH Mass Spectral Library version 2.0 g. The volatile organic compounds of the plants were identified using mass spectrometry (MS), retention time index (RI) and external standards (Ext Std). For each plant type, a minimum of 2 replicates from 2 different rounds were analysed to identify consistent compounds. For each plant type, one sample was also analysed three times to evaluate the variations in the same sample due to any possible instrumental drifts. For the calculation of the linear retention time index, the 49452-U C7-C40 alkane standard (Supelco, Bellefonte, PA, USA) was used as a reference. The cannabis terpene mix CRM 40755 (Sigma Aldrich, St. Louis, MO, USA) was used as external standards. The mix contained the following 20 terpenes α-pinene, β-pinene, camphene, 3-carene, α-terpenine, R-(+)-limonene, γ-terpinene, L-(-)-fenchone, fenchol, (1R)-(+)-camphor, isoborneol, menthol, citronellol, (+)-pulegone, geranyl acetate, α-cedrene, α-humulene, nerolidol, (+)-cedrol and (-)-α-bisabolol. This standard was complemented with the β-caryophyllene standard 22075 (Sigma-Aldrich) and the (–)-caryophyllene oxide 91034 (Sigma-Aldrich), to confirm the identified compounds. The area percentage was determined as the quotient between the area of compound peak as the numerator and the sum of all peaks detected in the corresponding chromatogram as the denominator. The mean area percentage was then calculated from all the DHS samples analysed and reported in the results. The peak areas were determined using the ICIS peak detection method in the Xcalibur™ software.

3.10 Oviposition behavioural bioassays with synthetic chemicals

3.10.1 Preparation of test chemicals

The synthetic chemicals used in the bioassay experiments include limonene (Sigma-Aldrich, >99%), ß-pinene (Sigma-Aldrich, >99%), (-)-α-cedrene (Sigma-Aldrich, >=99%), (-)-ß-elemene (BOC Sciences, 98%), (-)-trans-caryophyllene (Sigma-Aldrich, >=98.5%), α-humulene (Sigma-Aldrich, >=96.0%) and (-)-caryophyllene oxide (Sigma-Aldrich, >=99.0%,).

These chemicals were selected out of the 43 plant volatiles identified from the five graminoid plants examined for gravid mosquitoes attraction. The chemicals were selected based on: (1) previous reports regarding their bioactivity with mosquito antennal bioassays and behaviour influence on mosquitoes (Wondwosen et al., 2016, 2017); (2) consistent presence in replicates of the headspace samples; (3) presence in the headspace of the most attractive plant tested (*C. rotundus*); (4) presence in more than one plant species headspace and (5) commercial

availability for purchase. For the WHO tube bioassays, two stock solutions (1000 ppm and 10 ppm) of each chemical were first prepared.

The first stock solution of 1000 ppm was prepared by adding 1 µl or 1 mg of the specified chemical to 1000 µl of hexane (absolute, ≥99.8% (GC), Sigma-Aldrich) following (Lindh et al., 2015). The second stock solution of 10 ppm was prepared by adding 10 µl of the first stock solution into 1000 µl of hexane. Different working concentrations: 0.05 ppm, 0.1 ppm, 0.2 ppm, 0.4 ppm, 0.8 ppm, and 1.6 ppm were prepared by adding appropriate amounts of the second stock solution to hexane. For instance, to make a 0.1 ppm solution of a chemical in hexane, 20 µl of the second stock solution was added to 2000 µl of hexane.

For two choices egg count bioassays, the test chemicals were prepared as described in earlier study (Lindh et al., 2015). To make the desired dose of each chemical, a specific volume of 10,000 ppm of the chemical prepared in a similar way described for the WHO tube was added to 1 L of distilled water which was enough for ten replications. For example, 0.2 ppm of β-elemene was prepared by mixing 20 µl of 10,000 ppm from stock solution with 1 L of distilled water. Similarly, to prepare a blend consisting of 0.2 ppm of β-elemene and 1 ppm of β-pinene, 20 µl of 1000 ppm of β-elemene solution and 100 µl of 10,000 ppm β-pinene solutions were added to 1 L of distilled water. Accordingly, various doses of β-elemene and β-pinene were prepared. An equal volume of hexane was added to the l L distilled water for the controls. Blends comprising 2-5 chemicals were prepared and tested (Table 3.2).

Table 3. 2 Blends tested in egg-count bioassays and their compositions.

Treatment 1 ('control')	**Treatment 2 ('test')**	**Total no of gravid *An. gambiae s.s.* recollected/laid eggs (out of total released)**
WHO tube bioassays		
Calibration experiments		
Do the WHO tube bioassays result in reproduceable outcomes? What is the response rate that can be expected from released gravid mosquitoes?		
Water	Water	52 (90)
Water	Hay infusion	59 (129)
Preliminary egg count bioassays of the synthetic chemicals singly and in blends		

Do gravid *Anopheles gambiae s.s.* show similar behavioural response to the synthetic chemicals as in the WHO tubes?		
Water	ß-Elemene 0.1 ppm	46 (50)
Water	ß-Elemene 0.2 ppm	44 (50)
Water	ß-Elemene 0.4 ppm	19 (20)
Water	ß-Elemene 1 ppm	17 (20)
Water	ß-Elemene 5 ppm	17 (20)
Water	ß-Pinene 0.8 ppm	47 (50)
Water	ß-Pinene 1 ppm	45 (50)
Water	ß-Pinene 3 ppm	19 (20)
Water	ß-Pinene 5 ppm	35 (40)
Water	ß-Pinene 20 ppm	14 (20)
Water	Blend 1 (ß-Elemene 0.05 ppm + ß-Pinene 0.4 ppm)	59 (70)
Water	Blend 2 (ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm)	46 (50)
Water	Blend 3 (ß-Elemene 0.2 ppm + ß-Pinene 1 ppm)	44 (50)
Water	Blend 4 (ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm + Limonene 1.6 ppm + ß-Caryophyllene 0.8 ppm + ß-Caryophyllene oxide 0.2 ppm)	37 (40)
Water	Blend 5 (ß-Elemene 0.2 ppm + ß-Pinene 1 ppm + Limonene 2 ppm + ß-Caryophyllene 1 ppm + ß-Caryophyllene oxide 0.4 ppm)	39 (40)
Water	Blend 6 (ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm + Limonene 1.6 ppm + ß-Caryophyllene 0.8 ppm + ß-Caryophyllene oxide 0.2 ppm)	18 (20)
Water	Blend 7 (ß-Elemene 0.2 ppm + ß-Pinene 1 ppm + ß-Caryophyllene 1ppm + ß-Caryophyllene oxide 0.4 ppm)	17 (20)
Water	Blend 8 (ß-Elemene 0.05 ppm + ß-Pinene 0.4 ppm + ß-Caryophyllene 0.4 ppm)	27 (30)

3.10.2 WHO-tube bioassays

The experiments were implemented in laboratory conditions to assess the attraction response of gravid *An. gambiae s.s.*. Standard WHO test tubes (125 mm long and 44 mm diameter) (WHO, 2018) were used for the bioassays as described by (Okal et al., 2013) with modifications. In this experiment, WHO tubes alone were used instead of mosquito cages. A complete setup was made by fitting three WHO tubes using two screw caps having sliding unit as gates. Two locally prepared funnels were fixed between the inner/middle and outer/side of

the tubes. The outer tubes closed using mesh gauze wrapped in kitchen cling-film to reduce the diffusion of the putative substrates to the outside of the setup (Plate 3.3). The funnels were used to prevent the responded mosquitoes in the outer tubes from flying back. The middle tube served as a release point for a single gravid mosquito while the two outer tubes held oviposition putative substrates; distilled water soaked in 100 g of cotton wool and 200 µl of the test chemical applied on 10 g of cotton wool. All the WHO tubes were cleaned using ethanol before the start of each experiment. 200 µl of hexane were applied on the 10 g cotton wool in control. The applied hexane was evaporated by keeping the treated cotton wool at room temperature for 30 minutes after which they were placed in the WHO tubes.

Two sets of experiments were done (1) equal choice experiment (a mosquito was presented with an equal choice: distilled water in both tubes) and (2) different choices experiments (a mosquito was provided with two different choices: distilled water in one tube and distilled water with the test chemical in the other tube). The positions of test and control treatments were changed from the front side to the backside between rounds. Two rounds of experiments were set each night with different batches of fully gravid mosquitoes. Single mosquito was introduced in each set at 17:30 and 20:00 and left to acclimatize for 10 minutes before opening the sliding units at 17:45 and 20:15, respectively. The experiments were run for two hours. The number of rounds and the time of the experiments were determined based on preliminary experiments conducted. Majority of the mosquitoes responded within two hours after being introduced to the tubes and this was in agreement with the previous report (Okal et al., 2013; Sumba et al., 2004a). Mosquitoes were considered responded when they entered one of the two outer tubes whilst mosquitoes remaining in the middle tube were considered non-responders.

The presence of eggs in the tubes was also recorded. The experiments were replicated six times and repeated until the total number of the responded mosquitoes reached 65 or more. A minimum of 65 total responses was set for each dose based on the previously calculated sample size by (Okal et al., 2013). With this sample size, a 34% increase in attractiveness of the synthetic chemicals could be detected when it was compared to the equal choice (water versus water) experiment at 5% level of significance and 80% power (Brant, n.d.).

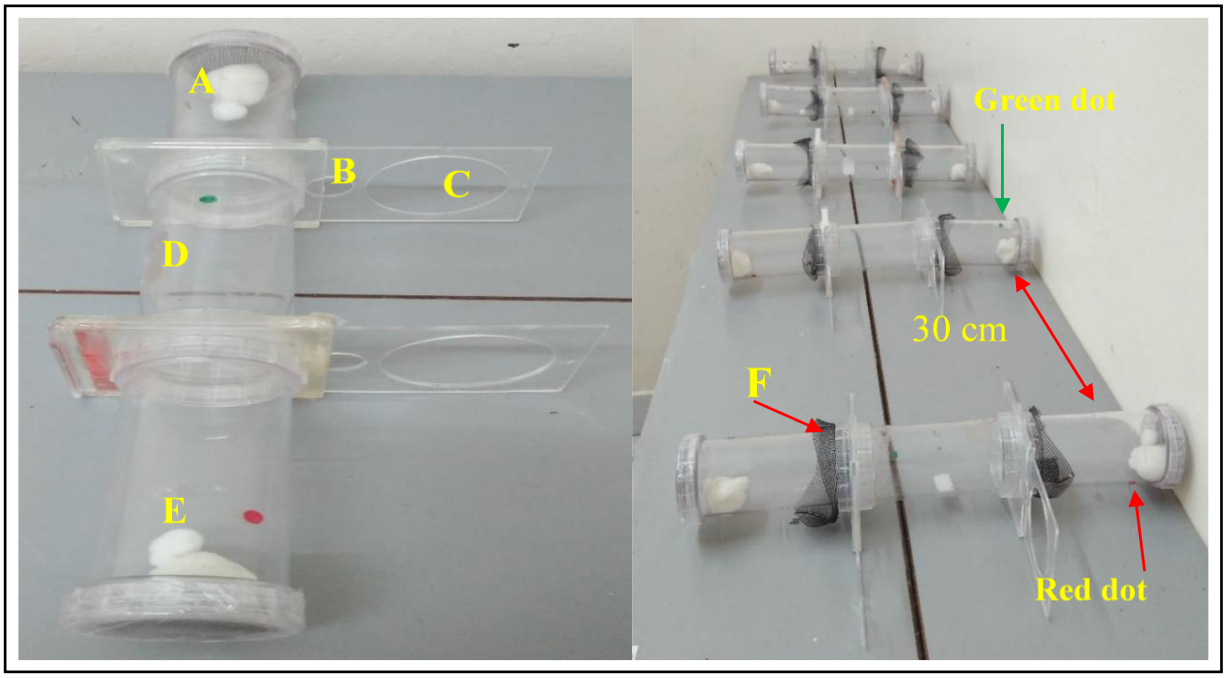

Plate 3. 3 Experimental set up for WHO-tube bioassays. The control and test substrates were placed in the outer tubes (**A**) and (**E**), respectively. A single mosquito was introduced into the middle tube (**D**) through a small hole (**B**) on a slide unit. The test treatments (200 µl of hexane for control and 200 µl of the synthetic chemical dissolved in hexane) were applied on the small rolls of cotton wool whereas the bigger cotton wool was sunk in distilled water (found in **A** & **E**). Big holes (**C**) on the outer sides of the middle tubes were opened to allow the mosquitoes to choose between the control and test treatments. Two funnels were used to prevent the mosquito from flying back once it made a choice (**F**). Greed dot (for the placement of control treatment). Red dot (for the placement of test treatment).

3.10.3 Two-choice egg-count bioassays of synthetic chemicals

Egg-laying bioassay experiments were conducted in previously described make-shift sheds at *icipe*-TOC (Okal et al., 2015). These experiments were conducted to evaluate the short-range effect of synthetic chemicals on the egg-laying response of *An. gambiae s.s.*. The experiments were implemented under natural ambient temperature, humidity and light. Standard cages (30 x 30 x 30 cm) were used for the bioassays. The cages were made of steel metallic frames and galvanized metallic base covered with mosquito nettings. A sleeve on the cage net was used to introduce and remove the substrates containing glass oviposition cups (Pyrex, 100 ml, 70 mm diameter) and gravid mosquito. The oviposition cups were cleaned and autoclaved at 121°C for 20 minutes daily before use in the experiments. Two sets of experiments were conducted. The first set was a two-equal choice experiment in which the mosquito was presented with

distilled water-filled in two oviposition cups and placed in a cage at the corners diagonally. The second set of experiment was two different choices bioassay in which one cup filled with distilled water (control) and the second cup filled with the desired dose of single chemical/blends of the chemicals (test) in distilled water place diagonally in two corners of the cage. In two equal-choices experiment, one oviposition cup was designated randomly as a control and the second as a test. The positions of the test and control cups were assigned systematically starting from front left (FL) corner to back left (BL) to back right (BR) and front right (FR) in a clockwise direction between the preceding cages to minimize bias due to position of the cups (Figure 3.5). The cage corner was given a name based on the front side of the cage and the treatments were randomly assigned to the front left and then arranged systematically in the preceding cages. The cages were placed 30 cm apart. Initially, 12 different concentrations of β-elemene and β-pinene and eight blends comprising 2-5 chemicals with different concentrations were tested for certain nights (Table 3.2). Out of these, six treatments (two blends and two chemicals with two different doses) were selected based on the laboratory results, initial assessment responses of the gravid mosquitoes towards the chemical composition and doses in cages, and complete sets of rounds were conducted. Full rounds of tests were conducted for doses and blends which did not show repellence. Bioassays of all the treatments were implemented concurrently replicating each treatment using ten cages. In each cage, a single gravid mosquito was introduced at 18:00 hr and the experiment was stopped the following morning starting from 08:00 hr by recording the presence/absence and the number of eggs using maginifying lens at 10x maginification. The experiments were repeated until the minimum target (165 responders) was achieved using new batches of females (Okal et al., 2015).

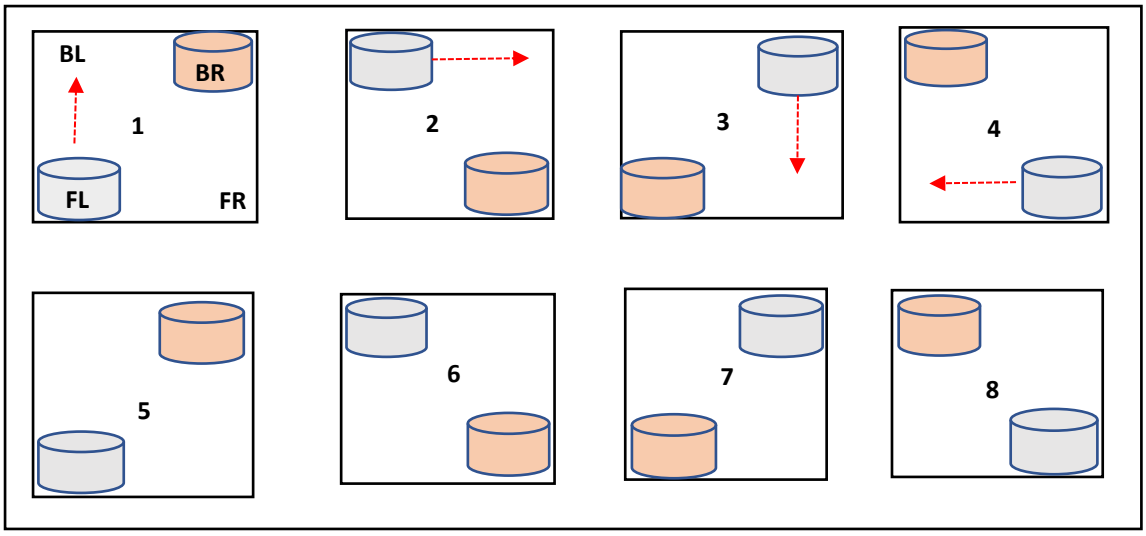

Figure 3. 5 A schematic of the arrangement of cages and oviposition cups inside the cages for egg-count bioassays. Different colours of the cups show the arrangement of the control and test substrates in a clockwise direction. **FL=**front left, **BL**=back left, **FR**=front right and **BR**=back right. The difference in the colour of the cups indicates the control and test substrates.

3.11 Standardized field bioassays to measure attraction of wild gravid *Anopheles funestus* mosquitoes

Six houses in rice irrigation area where *An. funestus* dominates were selected in Ahero. In this experiment modified BG-Sentinel gravid traps were deployed to collect gravid *An. funestus* from inside of inhabited houses. The preference of gravid *An. funestus* to river water and *C. rotundus* was investigated. In these experiments, the traps were placed on the floor inside rooms to trap indoor resting blood-fed and gravid mosquitoes since blood-fed mosquitoes stay inside the houses after blood meal for a few days and commence oviposition sites search flight once they became gravid. Studies have shown that majority of *An. funestus* rest indoors in western Kenya (Degefa et al., 2017; Machani et al., 2020). Six similar houses (mud walls and corregated roofs) were selected for this study. Three of the houses were randomly assigned to the test traps and the other three houses were assigned to put the control traps. A single trap was placed in each selected house. The placement of control and test traps were altered between the houses on daily basis for 12 nights to reduce the bias of mosquito preferences of houses i.e. one house received control traps for six nights and test traps for the other six nights. Collections of mosquitoes were done from 18.00 to 07.00 h. The collected mosquito samples were identified morphologically (Coetzee, 2020) and their physiological stages were determined as unfed, blood-fed, and gravid by looking at their abdomen (WHO, 2013). The number of males and females were also recorded. *Anopheles* mosquitoes were knocked down using chloroform, sorted, placed in 1.5 ml Eppendorf tubes, preserved using silica gel and transported to *icipe*-TOC for molecular analysis.

3.12 DNA extraction and identification of *Anopheles funestus s.l.* and *Anopheles gambiae s.l.* collected from field

Individual whole mosquito specimen extraction technique was implemented to extract genomic DNA using Quigen DNeasy Blood and Tissue Kit (Qiagen, GmbH Hilden, Germany) following the manufacturer's instructions. Using the extracted DNA, identification of specimen species was implemented. Polymerase chain reaction was implemented for the amplification

of the ribosomal internal transcribed spacer (ITS2) gene using primers (Cohuet et al., 2003; Koekemoer et al., 2002). ITS2 ribosomal DNA (rDNA) is a commonly used molecular marker for identification of closely related species including malaria vectors mosquitoes (Mishra et al., 2021). Positive controls of *An. gambiae* and *An. arabiensis* (from insectary) were analysed with the samples from the field to identify *An. gambiae s.l.* specimens. Similarly, I used positive controls of *An. funestus s.s.* and *An. rivulorum* as positive controls during the identification of *An. funestus* siblings. The PCR in a 10 µl (per sample) was prepared by mixing PCR mix of 2 µl of 5XHot Firepol Blended Master Mix (Ready to Load), primers (0.5 µM each), DNA template (2 µl) and nuclease-free water (5 µl). The thermal recycling conditions involved initial denaturation for 5 min at 95°C, after which 30 cycles of denaturation followed for 30 s at 94°C, annealing for 30 s at 50°C, extension for 30 s at 72°C and final extension for 5 min at 72°C. Kyratec Thermal Cycler (SC300T-R2, Australia) was used for the thermal reactions. Agarose gel-electrophoresis (2.0%) stained with 2 µl ethidium bromide against a 100 bp DNA ladder (Bioline, A Maridian Life Science @ Company, UK) and positive control was conducted to identify the species.

3.13 Statistical analysis

The overall response rate of released mosquitoes was defined as the number of mosquitoes leaving the release chamber in either direction of the olfactometer, hence non-responders remained in the release chamber. The approach of introducing a single gravid female into a cage in egg-count bioassays rather than groups of mosquitoes and only the responed mosquitoes included in the analysis. This has several advantages. It enables analysis of choice bioassays on a binary outcome, the count of eggs laid by an individual mosquito, and observation of skip oviposition (OKal, 2015). The analysis of numbers of responding females rather than the number of eggs laid with equal choice and different choices allow comparison of odds of success and avoid inflated type I error rates due to the skewed number of eggs laid by different individuals of females of the same batch (OKal, 2015). Choice experiments using olfactometers, BG-sentinel trap, WHO tube bioassays and egg count cage bioassays were analysed with generalized linear models with quasibinomial distributions fitted to cater for overdispersion. The proportions of gravid females responding to the ‘test’ (as opposed to the ‘control’) in two-choice experiments with two different choices were compared to the proportion of gravid mosquitoes responding to the ‘test’ in the experiments where ‘test’ and ‘control’ treatments were the same (water vs. water) (Okal et al., 2015). The experiment was

included as the fixed factor and the 'equal choice' experiment was used as a reference to estimate the odds ratios (OR) and their 95% confidence intervals (CI). The number of responders was included as a dependent variable. All reported mean proportions and their 95% CIs were estimated based on the model by transforming the log odds (logit) of the outcome to the odds scale and from the odds scale to the probability scale. The OR for the reference group is 1 (unity) and values greater than 1 implicates more attraction of the test treatment and values less than 1 shows less attraction compared to the control treatment.

Generalised estimating equations (GEE) with Poisson distribution fitted to a log function and exchangeable correlation matrix were used to test for associations between graminoid plants and the abundance of early instar *Anopheles* larvae for the field larval survey experiments. The same model was used to test for the proportion of adult mosquitoes attracted to the *C. rotundus* and river water for the *An. funestus* attraction bioassays using BG-Sentinel traps in Ahero. The cluster ID in which habitats were located and nights of mosquito collection (for larval survey and *An. funestus* oviposition attraction bioassay experiments, respectively) were included in the model as repeated measurement. A GEE model was also used to analyse associations between graminoid plant species and the presence of early instar *Anopheles* larvae. Here the presence of early instar *Anopheles* larvae as a dependent variable was included in the model with binomial distribution fitted to a logit function and exchangeable correlation matrix to analyse its association with graminoid plant species of the habitats (independent variable). The presence and abundance of early instar *Anopheles* larvae (rather than eggs which are difficult to identify from field samples) were used as dependent variables as a proxy for oviposition. This is based on recent work confirming that early instar density correlates with the abundance of females selecting a habitat for oviposition (Odero et al., 2019). The mean numbers of early instar larvae and the mean number of the response of gravid mosquitoes to each treatment for the *An. funestus* mosquito trapping and their 95% CI were analysed using the parameter estimates of the models. The statistical outputs were reported as incidence rate ratios (RR) for the abundance of the first instar larvae and the density of the adult *An. funestus* mosquitoes captured using BG-Sentinel traps, and odds ratios (OR) for the presence of first instar larvae with their 95% CI. R statistical software version 4.0.3 was used for the analyses (R Core Team, 2020).

3.14 Ethical considerations

An experimental permit to implement this study was obtained from the Kenya Medical Research Institute's Ethical Review Committee (Protocol no. 593; Appendix 2) and National Commission for Science, Technology and Innovation (License No: NACOSTI/P/20/6222; Appendix 3). The proposal for this study was approved by the School of Graduate Studies, Maseno University (Appendix 4). Written consent was obtained from volunteers for arm feeding (Appendix 5). The potential harms caused by mosquito bites such as local irritation, itching and discomforts were clearly explained to the volunteers before they start the arm feeding. This was alleviated by providing the volunteers with antihistamine lotion. The number of mosquitoes allowed to bite was also regulated to prevent excessive irritation. Information about the volunteers was not collected and unique identity codes were used to represent them and used to troubleshoot when there were increased mortality rates and reduced egg-laying rates. The names of the volunteers do not appear in any database. The volunteers were informed that they were free to withdraw from some or all blood-feeding activities at any time they wish. The sampling of indoor resting mosquitoes was made after seeking verbal consent from the household heads.

Reports of the study results were made and submitted to School of Graduate Studies of Maseno University, Capacity Building and Institutional Unit (CBID) of *icipe* and German Academic Exchange Service (DAAD). The study results were presented at local and international conferences, symposia and workshops. Additionally, articles were published in peer-reviewed journals.

CHAPTER FOUR

RESULTS

4.1 Aquatic habitats survey

4.1.1 Survey of malaria vectors larval habitat types and graminoid plants

A total of 110 aquatic habitats were identified during the survey. As expected, given the targeted areas within 300 m of the lake shore, the most prevalent aquatic habitat types were swamps (65.5%, n=72) defined as permanent or semi-permanent water-logged sections of land with tall graminoid vegetation and/or floating plants (Plate 4.1A). Other habitats (see Plate 4.1B, 4.1C, 4.1D and 4.1E) included ponds formerly used for breeding fish but abandoned at the survey time (11%, n=12), rainfed puddles (9%, n=10), drainages (9%, n=10) and artificial pits (5.5%, n=6).

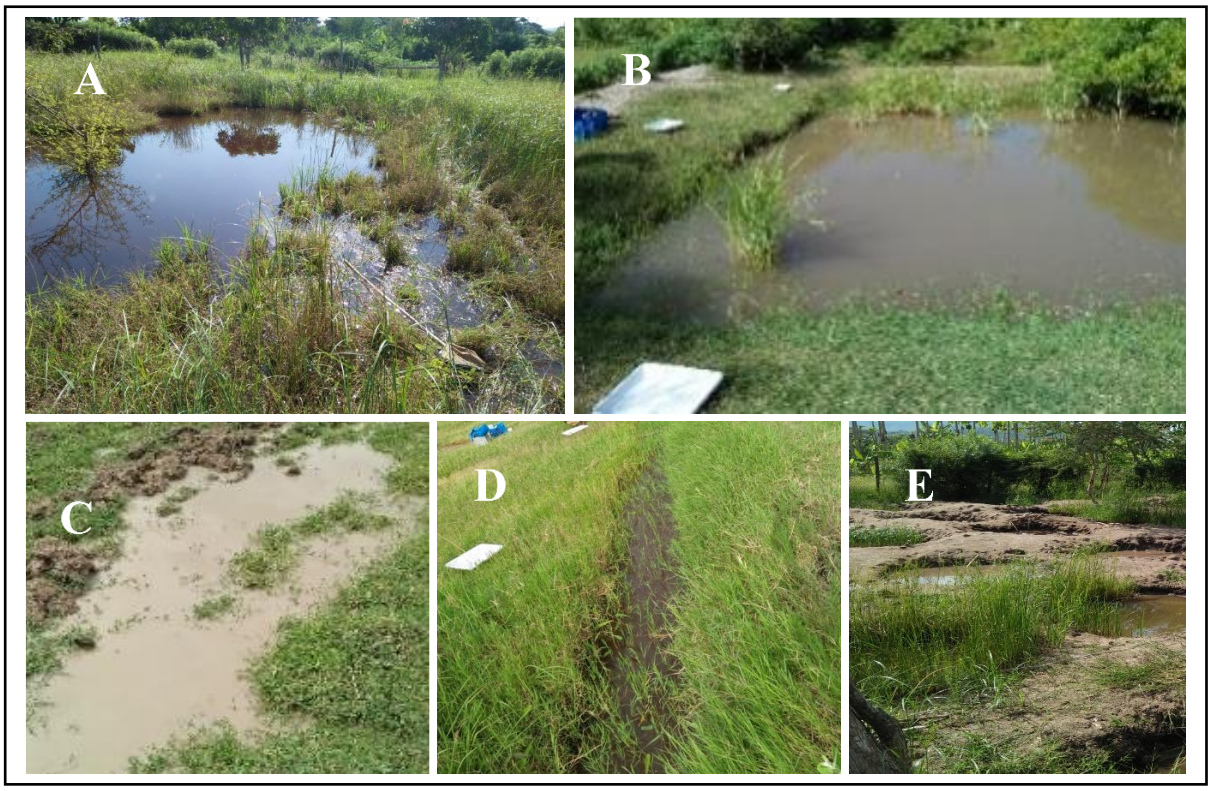

Plate 4. 1 Examples of habitat types. Swamp (**A**), Fishpond (**B**), Puddle (**C**), Drainage (**D**), and Artificial pit (**E**).

All the swamp habitats were bordered by graminoid plants along the water edges and had a high surface coverage. Similarly, 84% (32/38) of non-swamp habitats had graminoids along

their edges and 76% (29/38) had graminoids at their surfaces. Unexpectedly, swamp grasses were not the most frequently found graminoid plants in the survey. Representatives of the Cyperaceae family were found only in 39% of the aquatic habitats sampled. Among the Poaceae family, torpedo grass (*Panicum repens)* and Bermuda grass (*Cynodon dactylon*) were the dominant species (Plate 4.2).

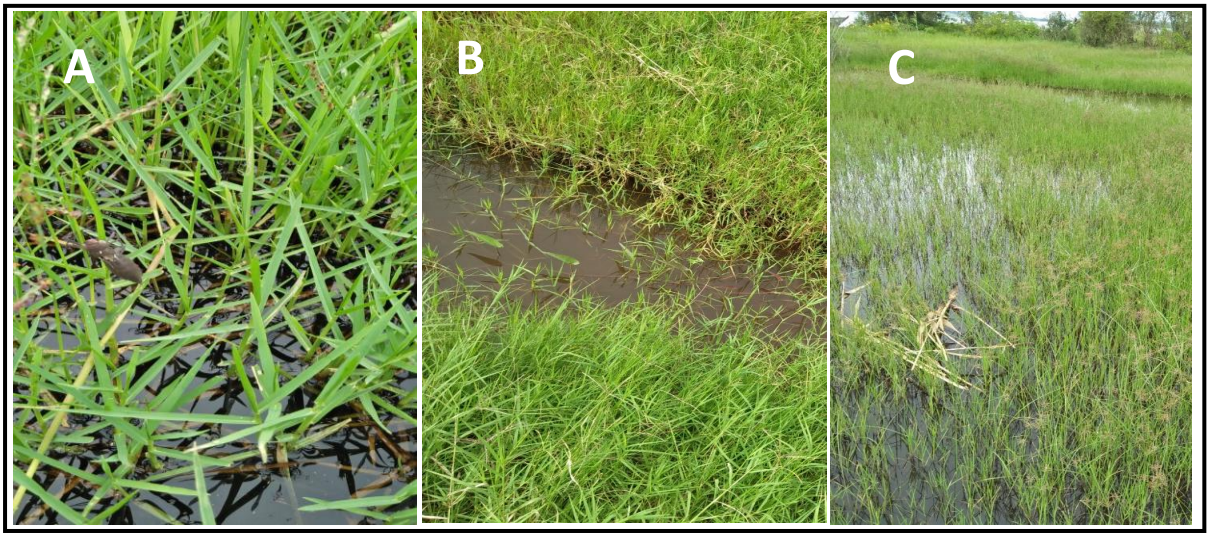

Figure 4. 2 The most dominant graminoid plants identified during the survey. *Panicum repens (Poaceae;* **A***), Cynodon dactylon (Poaceae;* **B***) and Cyperus rotundus (Cyperaceae;* **C***).*

Given that all non-swamp habitats were few in number, they were pooled for statistical analysis and the swamp habitats were used as the reference group (Figure 4.1). Early instar *Anopheles* larvae were found frequently during the survey in the habitat types: artificial pits (n=6, 100%), drainages (n=9, 90%), ponds (n=5, 42%), puddles (n=7, 70%) and swamps (n=61, 85%).

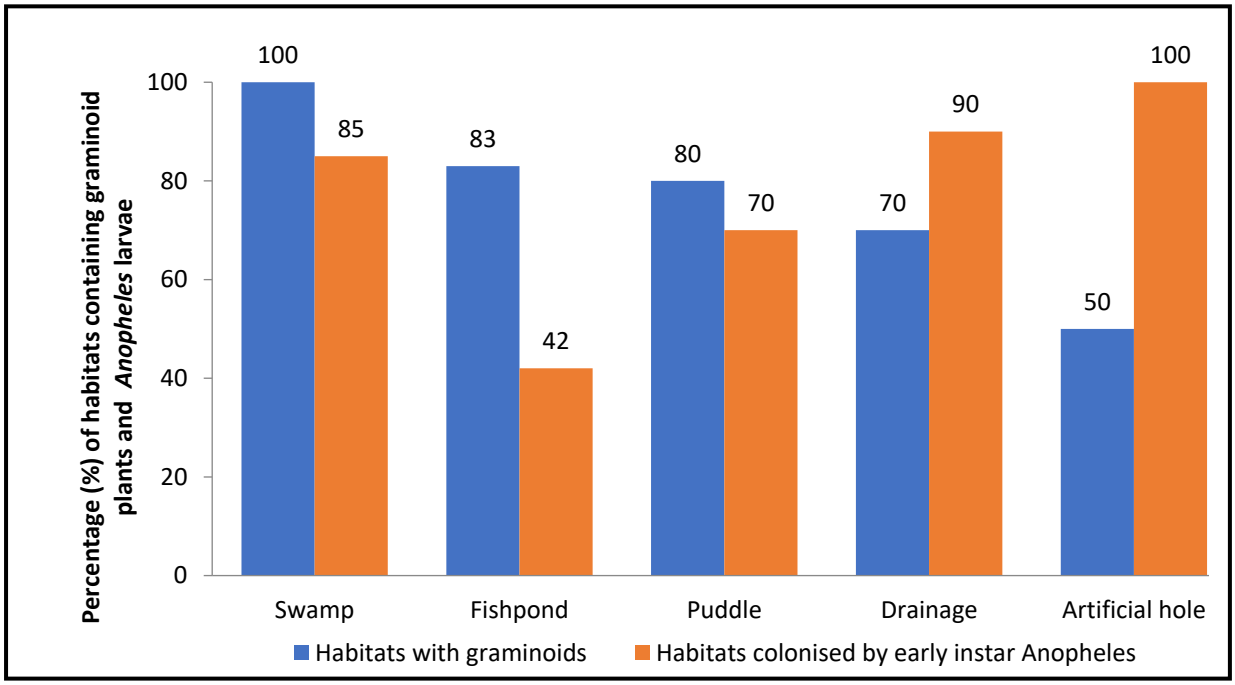

Figure 4. 1 Habitats containing graminoid plants and being colonised by early instar *Anopheles* larvae.

4.1.2 *Anopheles* mosquitoes species composition

A total of 14,145 early and late instar *Anopheles* larvae and 402 pupae were collected. Out of those, 4,650 emerged into adults and were morphologically identified (Table 4.1). *Anopheles gambaie s.l.* represented 96% of all *Anopheles* specimens collected. Molecular identification was done for a random sample of 10% of the *An. gambiae s.l.* (n=480) and revealed 100% *An. arabiensis* (Figure 4.2).

Table 4. 4 Species composition of *Anopheles* collected from habitats along the lake shore of Rusinga Island.

Anopheles spp	Number of mosquitoes	Percent composition
An. arabiensis *	4481	96.24
An. coustani	22	0.47
An. maculipalpis	2	0.04
An. pharoensis	67	1.44
An. rufipes	27	0.58
An. ziemanni	57	1.22

* Molecular identification of a random sample of 10% of the *An. gambiae s.l.* revealed 100% *An. arabiensis.*

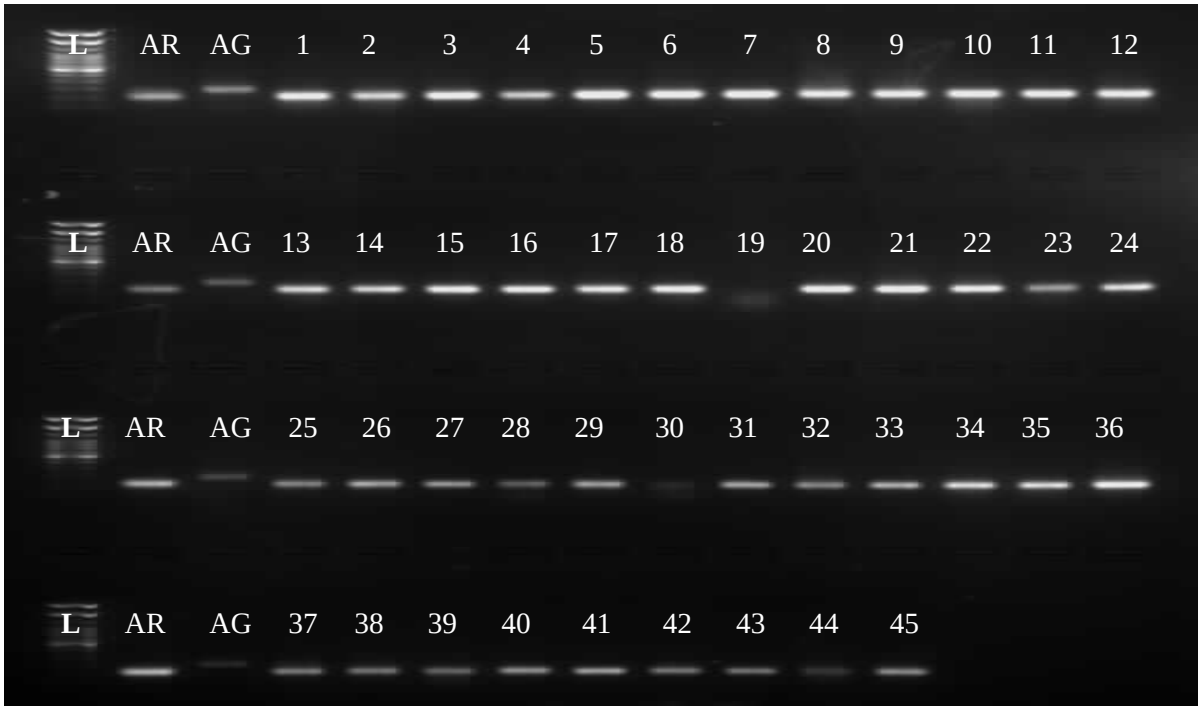

Figure 4. 2 Molecular identification of *Anopheles gambiae s.l.* collected on Rusinga Island. L=100bp ladder, **AR**= *Anopheles arabiensis*, **AG**=*Anopheles gambiae s.s.*, 1-18, 19-29, 31-45 are *Anopheles arabiensis*.

4.1.3 Association between graminoid plants and the presence and abundance of *Anopheles* larvae

Of the surveyed habitats, 42 (38%) were found covered by *P. repens* along their edges and 47 (43%) of the habitats at their surfaces. *Cynodon dactylon* was found covering the habitats both along the edges in 35 (32%) habitats and surfaces in 25 (23%) habitats. Overall, graminoid plants dominated in 96 habitats whilst forbs dominated only in five habitats during the survey. Nine habitats had no vegetations at their surface and five of them were colonized by early instar *Anopheles* larvae. Contrary to the hypothesis of this study, there was no significant association between the presence or abundance of early instar *Anopheles* larvae and the dominant graminoid plant present in a habitat (Table 4.2).

Table 4. 5 Association between dominant graminoid plants and the presence and abundance of *Anopheles* early instar larvae.

Graminoid plants	No. habitats	Mean (95% CI) of *Anopheles* early instar larvae	Presence of *Anopheles* early instar larvae OR (95% CI)	P-value	Abundance of *Anopheles* early instar larvae RR (95% CI)	P-value
Cyperus rotundus (Cyperaceae) *	14	57 (22.19-149)	1		1	
Cynodon dactylon (Poaceae)	25	99 (48-205)	1.1 (0.7-1.7)	0.762	1.7 (0.6-5.5)	0.35
Panicum repens (Poaceae)	47	84 (48-146)	1.2 (0.9-1.7)	0.305	1.5 (0.5-4.2)	0.99
Others (Poaceae)	10	58 (33-101)	1.4 (0.99-2)	0.057	1.01 (0.3-3)	0.48

*Selected as a reference based on initial hypothesis and earlier association of *Cyperus rotundus* with oviposition. OR= odds ratio, RR= rate ratio, CI= confidence interval.

4.2 Gravid *Anopheles gambiae s.s.* attracted to graminoid plants in two-port airflow olfactometer

After confirming that the olfactometer accuracy of generating valid and reproducible results with preliminary calibration experiments (Appendix 6), three sets of experiments were implemented. Equal choice experiment where the mosquitoes were provided with lake water in both chambers randomly allocated as 'test' and 'control', were conducted in parallel for all three sets of experiments. Expectedly, these reference tests resulted in an approximate 1:1 distribution of gravid females (Figure 4.5). Any preference for a specific test substrate in choice tests was expected to lead to a significant deviation from this balanced distribution.

Previous work (Lindh et al., 2015) implicated soil from the *Cyperus rotundus* collection site as attractive oviposition substrate for gravid *An. gambiae s.s.*. Consequently, I evaluated in a first step, whether wet soil from the location might be equally or more attractive in olfactometer bioassays than the live *Cyperus rotundus* plants in the same wet soil. However, the odds of a gravid female selecting the test chamber with the plants was nearly three-fold higher than in the reference experiment (OR 2.93; Figure 4.5). Removing the soil completely from the bioassay increased the odds further when compared to the reference (OR 4.95). Consequently, another four graminoid plants were tested and all of them released volatile chemicals attractive to gravid *An. gambiae s.s.* females (Figure 4.3) in the airflow olfactometer. The odds of finding a gravid female in the test chamber with the plants were 2.4-5 times higher than in the reference experiment, with the most profound effect induced by *Cyperus rotundus*. Even the drought-

resistant *Cenchrus setaceus* not naturally associated with mosquito breeding sites elicited a significant positive orientation towards the plants' odours (OR 2.41). The attractiveness of *Cyperus rotundus* was further investigated when presented in choice tests with the Poaceae species, *Panicum repens* and *Cynodon dactylon.* Chemical volatiles released from *Cyperus rotundus* were preferred over the other grasses, though the effect size was moderate.

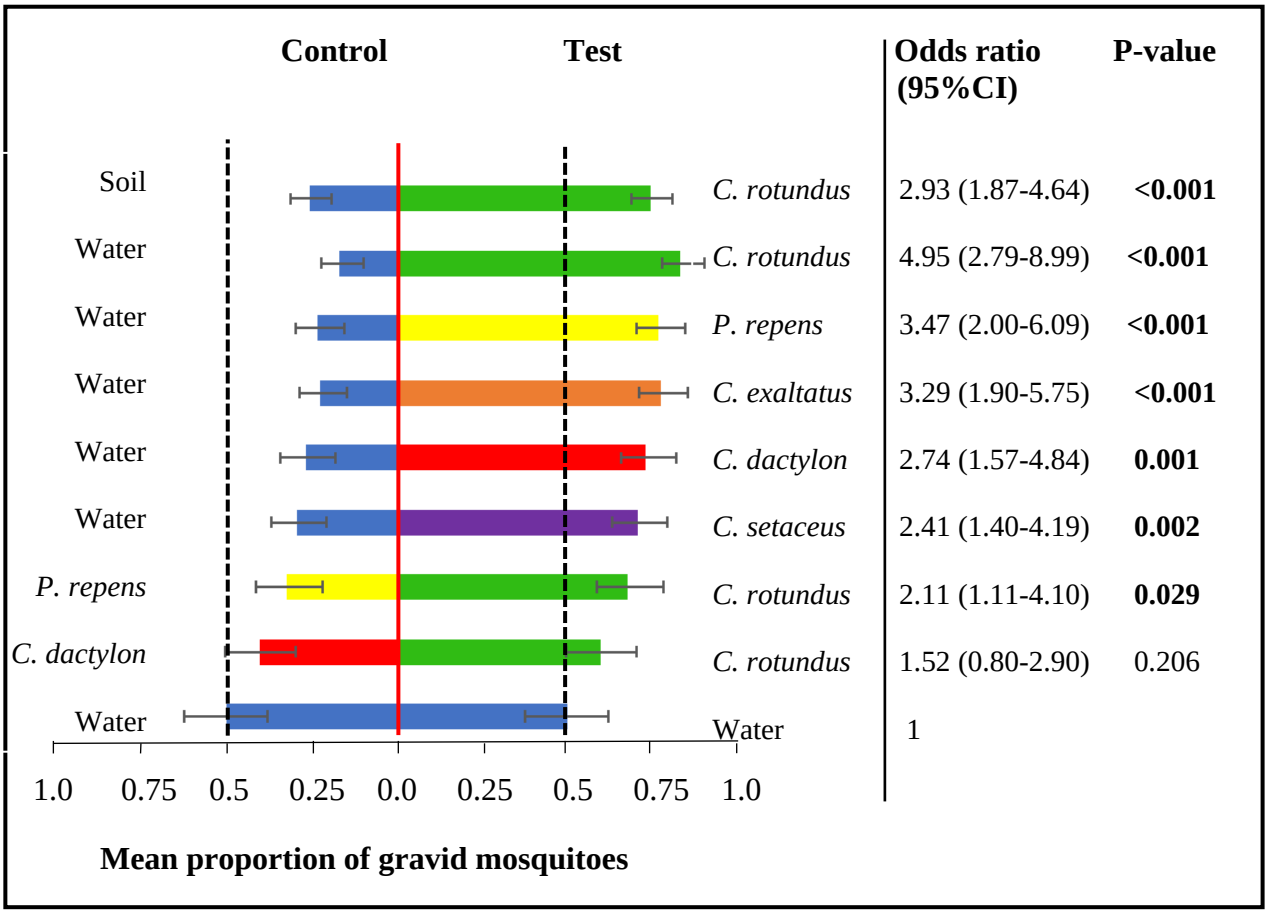

Figure 4. 3 Short-range attraction of gravid *Anopheles gambiae s.s.* to test substrates in choice experiments in two-port airflow olfactometers. The bars show the mean percentage with the 95% confidence intervals (CI). The outputs of the statistical analysis are presented as odds ratios (OR) and their 95% CI with the equal choice experiment as the reference. Each choice test was replicated over 16 different nights with 100 gravid *Anopheles gambiae s.s.* released per replicate. Each substrate type is designated by a specific colour.

4.3 Free-flying gravid *Anopheles gambiae s.s.* attracted to graminoid plants in large field-cage

Bioassays with free-flying gravid mosquitoes confirmed olfactometer results with higher proportions of the released gravid females trapped with BG-Sentinel traps containing live plants than with traps that contained water only (Figure 4.4). The odds of a female being captured in the test traps in the two-choice experiments were 1.5-2.5 times higher than in the reference experiment. Differences in the effect size of attraction between the plant species were not very pronounced under these more natural, long-range conditions, though *Cyperus rotundus* volatiles did slightly outcompete volatiles from *P. repens* in a similar way as in the olfactometer bioassays (Figure 4.6).

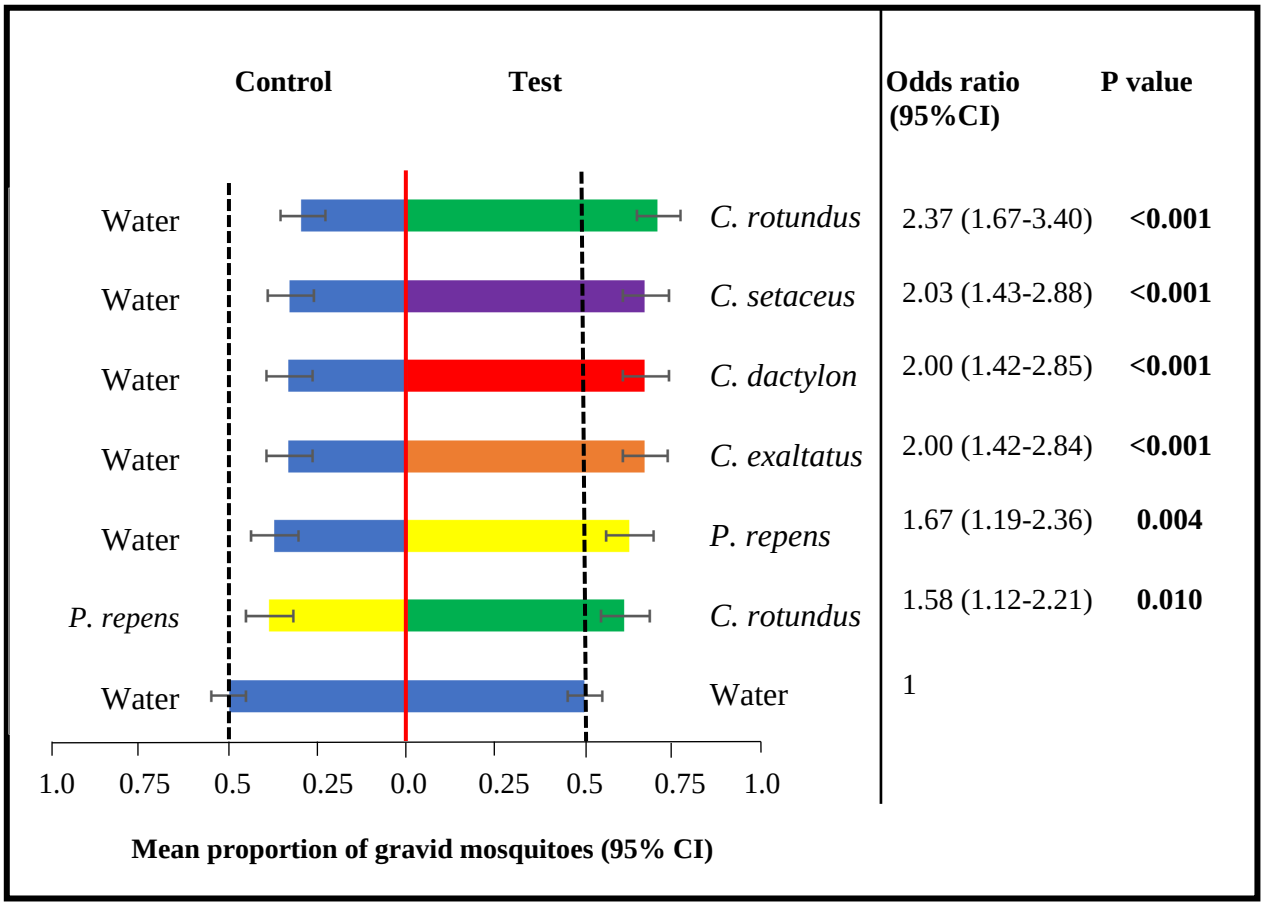

Figure 4. 4 Long-range attraction of gravid *Anopheles gambiae s.s.* to test substrates in choice experiments in large field cages. The bars show the mean percentage with the 95% confidence intervals (CI). The outputs of the statistical analysis are presented as odds ratios (OR) and their 95% CI with the equal choice experiment as the reference. Each choice test was

replicated over 16 different nights with 200 gravid *Anopheles gambiae s.s.* released per replicate. Each substrate type is designated by specific colour.

4.4 Volatile organic compounds identified from the graminoid test plants

Chemical analyses were done for 21 headspace samples: *Cyperus rotundus* (n=5), *Cynodon dactylon* (n=4), *Cyperus exaltatus* (n=4), *Panicum repens* (n=4) and *Cenchrus setaceus* (n=4). A total of 43 volatile organic compounds (VOCs) were detected with mass spectrometry (Table 4.3). The qualitative analysis shows that almost half of the detected compounds were sesquiterpenes.

Table 4. 6 Volatile profile of dynamic headspace sampling of aerial parts from *Cyperus rotundus* (CR), *Cyperus exaltatus* (CE), *Cynodon dactylon* (CD), *Panicum repens* (PR) and *Cenchrus setaceus* (CS).

Volatile compound		Area (%) composition ± SE					EAD Spec.	Physiol. stage	Ref
	RI	CR	CE	CD	PR	CS			
Primary Alcohol									
2-Ethyl-1-hexanol	1039	-	-	0.41 ± 0.322	-	-	-	-	
Aliphatic ketone									
Sulcatone	992	-	-	0.038a	-	-	Aa	G	(Wondwosen et a
Aliphatic Ester									
4-Hexen-1-ol acetate	1012	-	-	3.338 ± 1.867	-	-	-	-	
Cycloalkane									
1-isobutyl-1-cyclohexene	955	-	0.139a	-	-	-	-	-	
Cyclic ketone									
Cyclohexanone, 2,2,6-trimethyl	1043	-	-	0.102 ± 0.041	-	-	-	-	
Isophorone	1069	-	-	0.111 ± 0.088	-	-	-	-	
Aromatic									
1,4-Diethylbenzene	1056	-	-	-	-	1.433 ± 0.676	-	-	
Cymene	1062	-	-	-	-	0.351 ± 0.368	Aa, Ag	G	(Deletre et al., 20 Wondwosen et a 2018)
2,4-Dimethyl-acetophenone	1277	0.392 ± 0.324	-	-	-	0.514 ± 0.385	-	-	
β-Hydroxyethyl phenyl ether	1298	-	-	-	-	0.089a	-	-	
1H-indene, 1-ethylidene	1313	-	-	-	-	0.004a	-	-	
Alkyne									
4,6-Decadiyne	1063	-	-	-	-	0.475 ± 0.043	-	-	
Aromatic monoterpene									
Cumic alcohol	1271	0.418 ± 0.358	-	-	-	0.347 ± 0.308	-	-	
Monoterpene									
α-Pinene	942	-	-	0.035 ± 0.026	-	-	Aa	G	(Wondwosen et a 2018)

β-Pinene	980	0.632 ± 0.287	0.021a	0.042 ± 0.035	-	-	Aa, Ag	G, U	(Nyasembe et al. Deletre et al., 20 Wondwosen et a
Myrcene	994	0.452 ± 0.135	-	-	-	-	Ag	U	(Nyasembe et al. Meza et al., 2020
Limonene	1035	2.805 ± 1.127	1.043 ± 0.31	0.088 ± 0.069	0.037 ± 0.018	-	Aa, Ag	G,U	(Nyasembe et al. Deletre et al., 20 Wondwosen et a 2017)
Eucalyptol	1039	-	-	-	0.877 ± 0.27	-	-	-	
4-Thujanol	1078	-	-	0.076 ± 0.067	-	-	-	-	
1,1-Dimethyl-3-methylene-2-vinylcyclohexane	1121	1.554 ± 0.672	0.78 ± 0.591	0.188a	-	0.072a	-	-	
Camphor	1158	-	-	0.028 ± 0.03	-	-	-	-	
β-Cyclocitral	1234	-	-	0.118 ± 0.069	0.025 ± 0.016	-	-	-	
Sesquiterpene									
Unidentified M=[204]	1356	-	-	0.135 ± 0.06	-	-	-	-	
Ylangene	1362	-	-	-	0.115 ± 0.134	-	-	-	
Cyclosativene	1383	-	-	0.194 ± 0.081	-	-	-	-	
Copaene	1389	0.569 ± 0.372	-	0.044 ± 0.025	-	-	-	-	
γ-Elemene	1396	0.093 ± 0.028	-	-	-	-	Ag	U	(Nyasembe, 2016
β-Elemene	1404	3.64 ± 1.038	0.951a	0.069 ± 0.059	0.54 ± 0.19	-			
Cyperene	1418	0.584 ± 0.111	0.916 ± 0.514	-	-	-	-	-	
α-Gurujene	1419	-	-	0.134 ± 0.113	-	-	-	-	
Cedrene	1436	-	0.101 ± 0.073	-	-	-	Ag	U	(Nyasembe et al.
β-Caryophyllene	1438	3.517 ± 1.668	1.953 ± 0.641	0.141 ± 0.031	-	-	Aa, Ag	G, U	(Deletre et al., 20 Wondwosen et a Nyasembe et al., Meza et al., 2020
α-Bergamotene	1448	-	-	0.096 ± 0.082	-	-	-	-	
β-Ionone	1453	-	-	0.115 ± 0.009	-	-	-	-	
Humulene	1473	2.376 ± 0.96	0.429 ± 0.279	-	-	-	Ag	U	(Nyasembe et al. Meza et al., 2020
δ-Guaiene	1482	-	-	1.036 ± 1.696	-	-	-	-	
Germacrene D	1500	-	0.726 ± 0.471	0.126 ± 0.084	-	-	-	-	
α-Guaiene	1502	0.518 ± 0.2	0.132 ± 0.265	0.145 ± 0.163	0.072a	-	-	-	
α-Muurolene	1516	-	0.195 ± 0.109	0.099 ± 0.038	-	-	-	-	
δ-Cadinene	1535	-	0.796 ± 0.094	-	-	-	Ag	U	(Nyasembe et al.
Caryophyllene oxide	1609	0.281 ± 0.165	-	-	-	-	-	-	
Humulene epoxide II	1639	0.591 ± 0.887	-	-	-	-	-	-	
Hexahydrofarnesyl acetone	1853	-	-	1.463 ± 0.379	-	-	-	-	

RI - retention index calculated on a 15 m x 0.25 mm x 0.25 µm (5% phenyl)-methylpolysiloxane column. **SE** - Standard error. **a** – No standard error is calculated as the compound was only detected in one of the headspace samples. **Aa**-*Anopheles arabiensis*; **Ag**-*Anopheles gambiae s.s.*. **EAD** - electro-antennogram detection published for *Anopheles* species. **G**- EAD done for gravid females, **U**- EAD done for unfed females.

The second most common chemical class was monoterpenes, followed by a number of cyclic and straight compounds such as cyclic ketones, aliphatic esters and aromatic compounds. There was a slight overlap in the profiles of monoterpenes and sesquiterpenes which were identified from different plant species (Table 4.3). Compounds such as limonene, β-caryophyllene, β-elemene, 1,1-dimethyl-3-methylene-2-vinylcyclohexane and α-guaiene were present in the headspace of at least 3 out of 4 graminoid plants. Unlike the other graminoid species, *Cenchrus setaceus*, contained more aromatic compounds and had less overlap with the other species in its chemical profile. Overall, roughly 10% of the VOCs were detected in 4 of the 5 plants headspace, while around 65% of the VOCs were only detected from a single species. This shows the diversity of the headspace in the chemical environment of the malaria vector.

4.5 Oviposition responses of gravid *Anopheles gambiae s.s.* to synthetic chemicals

4.5.1 Attraction response in WHO tube bioassays

Preliminary experiments were conducted to evaluate if the WHO tubes generate valid and consistent results (Appendix 7). In the reference group, where distilled water and 200 µl of hexane were presented in the two sides of the WHO tubes, each side received nearly equal numbers of gravid females (OR 0.48, 95% CI 0.39-0.58; Table 4.4). In the two-different choices experimental set ups, β-elemene and β-pinene elicited significantly higher attraction of gravid mosquitoes. The odds of gravid *An. gambiae s.s.* selecting the test side of WHO tube baited with 0.1 ppm of β-elemene was higher by more than two-fold (OR 2.25, 95% CI 1.25-4.12) than the test sides in reference group (Table 4.4). The mosquitoes avoided higher doses (0.8 ppm and 1.6 ppm) of β-elemene. β-pinene was more attractive to the gravid mosquitoes at 0.8 ppm with an increase of mosquito preference by 1.82-fold (95% CI= 1.03-3.24, P=0.042) than the test in the reference group. Mosquitoes depicted a similar trend of attraction preference to 1.6 ppm limonene (OR=1.67, 95% CI=0.94-2.98, P=0.08) than in reference experiments, however, the difference was of borderline significance. Gravid mosquitoes avoided the side of a tube treated with α-humulene (OR=0.5, 95% CI=0.28-0.93, P=0.029) at 1.6 ppm in two different choices set ups than in reference experiments. ß-caryophyllene, caryophyllene oxide and α-cedrene induced no significantly different attraction in the gravid *An. gambiae s.s.* when compared to the reference group.

Table 4. 4 Mean proportion of *Anopheles gambiae s.s.* responding to control and synthetic chemicals in choice experiments.

Test substrate	Concentration	Mean proportion (95% CI)	OR 95% CI	P-value
Water		0.48 (0.39-0.58)	1	
β-Elemene	0.1 ppm	0.68 (0.57-0.77)	2.25 (1.25-4.12)	**0.008**
	0.2 ppm	0.53 (0.43-0.63)	1.23 (0.71-2.15)	0.465
	0.4 ppm	0.54 (0.43-0.64)	1.24 (0.70- 2.21)	0.450
	0.8 ppm	0.46 (0.36-0.57)	0.93 (0.53-1.63)	0.796
	1.6 ppm	0.40 (0.30-0.51)	0.72 (0.40-1.26)	0.249
β-Pinene	0.5 ppm	0.53 (0.43-0.62)	1.19 (0.69-2.05)	0.538
	0.1 ppm	0.51 (0.41-0.62)	1.14 (0.65-2.01)	0.655
	0.2 ppm	0.42 (0.33-0.52)	0.78 (0.45-1.34)	0.363
	0.4 ppm	0.53 (0.43-0.63)	1.20 (0.67-2.10)	0.524
	0.8 ppm	0.63 (0.52-0.72)	1.82 (1.03-3.24)	**0.042**
Limonene	0.1 ppm	0.52 (0.41-0.62)	1.14 (0.64-2.02)	0.653
	0.2 ppm	0.52 (0.42-0.63)	1.18 (0.66-2.10)	0.579
	0.4 ppm	0.55 (0.45-0.65)	1.33 (0.77-2.33)	0.311
	0.8 ppm	0.54 (0.43-0.64)	1.24 (0.71-2.17)	0.460
	1.6 ppm	0.61 (0.50-0.71)	1.67 (0.94-2.98)	0.080
α-Humulene	0.1 ppm	0.46 (0.35-0.56)	0.90 (0.51-1.59)	0.714
	0.2 ppm	0.45 (0.35-0.56)	0.89 (0.50-1.59)	0.703
	0.4 ppm	0.40 (0.30-0.51)	0.72 (0.41-1.29)	0.273
	0.8 ppm	0.42 (0.32-0.53)	0.79 (0.44-1.40)	0.425
	1.6 ppm	0.32 (0.23-0.43)	0.51 (0.28-0.93)	**0.029**
Caryophyllene oxide	0.1 ppm	0.48 (0.38-0.59)	1.01 (0.57-1.79)	0.973
	0.2 ppm	0.54 (0.44-0.64)	1.27 (0.73-2.25)	0.401
	0.4 ppm	0.51 (0.41-0.61)	1.10 (0.63-1.93)	0.731
	0.8 ppm	0.52 (0.42-0.63)	1.17 (0.66-2.08)	0.583
	1.6 ppm	0.46 (0.35-0.56)	0.90 (0.51-1.59)	0.715
β-Caryophyllene	0.1 ppm	0.50 (0.39-0.61)	1.07 (0.61-1.90)	0.809
	0.2 ppm	0.40 (0.31-0.51)	0.72 (0.41-1.27)	0.262
	0.4 ppm	0.51 (0.40-0.61)	1.11 (0.63-1.96)	0.730
	0.8 ppm	0.47 (0.37-0.58)	0.95 (0.54-1.68)	0.871

	1.6 ppm	0.50 (0.40-0.60)	1.07 (0.62-1.86)	0.816
α-Cedrene	0.1 ppm	0.54 (0.43- 0.64)	1.25 (0.70-2.23)	0.444
	0.2 ppm	0.41 (0.31-0.51)	0.74 (0.42-1.29)	0.287
	0.4 ppm	0.45 (0.35-0.55)	0.89 (0.51-1.55)	0.667
	0.8 ppm	0.43 (0.33-0.54)	0.83 (0.47-1.46)	0.508
	1.6 ppm	0.40 (0.31-0.51)	0.72 (0.41-1.27)	0.262

4.6.2 Egg-laying responses of gravid *Anopheles gambiae s.s.* to synthetic chemicals in cage bioassays

Preliminary cage bioassay experiments were conducted to determine the doses of the synthetic chemicals to be tested individually and as a component of blends (Table 4.5). Preliminary bioassays were conducted for β-elemene, β-pinene, blends of the two chemicals and blends comprising of other more chemicals varying their doses and compositions based on the WHO tube results. Those chemicals that showed no significant difference in attraction in WHO tube bioassays were only included in blends of the preliminary assays. Complete sets of experiments were carried out for the doses and blends showing promising results during the preliminary bioassays.

Table 4. 5 Summary of egg-count bioassays with gravid *Anopheles gambiae s.s.*.

Treatment 1 ('control')	**Treatment 2 ('test')**	**Total no of gravid *An. gambiae s.s.* responded (out of total tested)**	**Percent (%) laid in "test"**
Do gravid *Anopheles gambiae s.s.* show similar behavioural response to the synthetic chemicals as in the WHO tubes?			
Water	Water	46 (50)	49
Water	ß-Elemene 0.1 ppm	46 (50)	49
Water	ß-Elemene 0.2 ppm	44 (50)	48
Water	ß-Elemene 0.4 ppm	19 (20)	45
Water	ß-Elemene 1 ppm	17 (20)	50
Water	ß-Elemene 5 ppm	17 (20)	43
Water	ß-Pinene 0.8 ppm	47 (50)	50

Water	ß-Pinene 1 ppm	45 (50)	55
Water	ß-Pinene 3 ppm	19 (20)	48
Water	ß-Pinene 5 ppm	35 (40)	46
Water	ß-Pinene 20 ppm	14 (20)	38
Water	ß-Elemene 0.05 ppm + ß-Pinene 0.4 ppm	59 (70)	51
Water	ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm	46 (50)	55
Water	ß-Elemene 0.2 ppm + ß-Pinene 1 ppm	44 (50)	51
Water	ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm + Limonene 1.6 ppm + ß-Caryophyllene 0.8 ppm + ß-Caryophyllene oxide 0.2 ppm	37 (40)	37
Water	ß-Elemene 0.2 ppm + ß-Pinene 1 ppm + Limonene 2 ppm + ß-Caryophyllene 1 ppm + ß-Caryophyllene oxide 0.4 ppm	39 (40)	43
Water	ß-Elemene 0.1 ppm + ß-Pinene 0.8 ppm + Limonene 1.6 ppm + ß-Caryophyllene 0.8 ppm + ß-Caryophyllene oxide 0.2 ppm	18 (20)	32
Water	ß-Elemene 0.2 ppm + ß-Pinene 1 ppm + ß-Caryophyllene 1ppm + ß-Caryophyllene oxide 0.4 ppm	17 (20)	41
Water	ß-Elemene 0.05 ppm + ß-Pinene 0.4 ppm + ß-Caryophyllene 0.4 ppm	27 (30)	35

Nearly equal proportions of mosquitoes laid eggs in the “control” and “test” oviposition cups when both cups were treated with distilled water in the reference group. ß-elemene at 0.2 ppm was found to be significantly more attractive to gravid females than control inducing egg-laying response. The odds of females laying eggs in test cups treated with 0.2 ppm of ß-elemene in two choices bioassay experiments was 1.61 (95% CI 1.15-2.24) times higher than in test cups in the reference group (Figure 4.5). Similarly, the proportion of gravid mosquitoes laid in the test cup treated with ß-pinene at 1 ppm was 1.5-fold increased (95% CI 1.10-2.10) compared to the test cup treated with distilled water in the reference group. The proportions of gravid females laid eggs in the test cups treated with β-elemene at 0.2 ppm, β-pinene at 1 ppm, blend 1 and blend 2 were not significantly different from the proportions of mosquitoes laid in the test cups in the reference group.

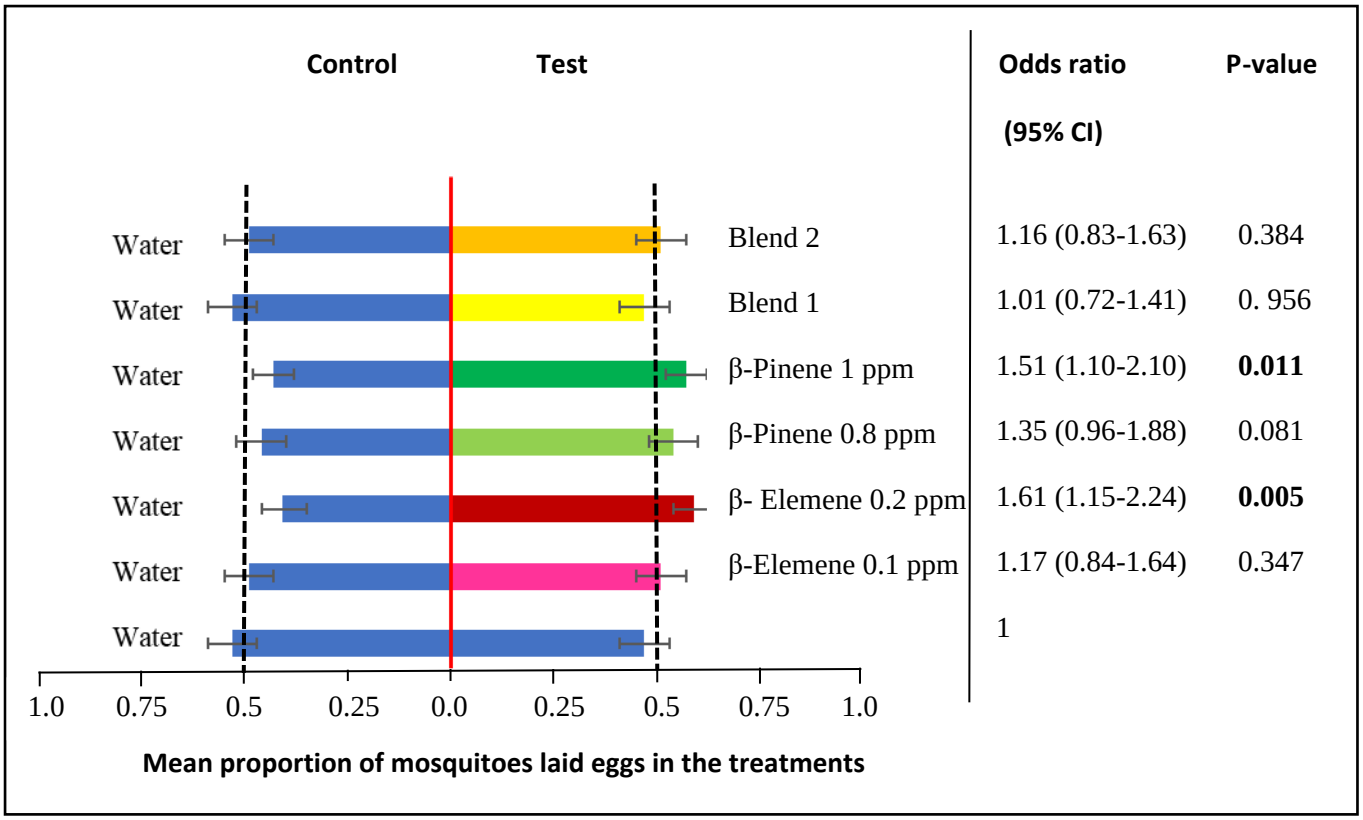

Figure 4. 5 Oviposition response of gravid *Anopheles gambiae s.s.* to distilled water and synthetic chemicals in choice egg-count experiments.

4.6 Oviposition attraction responses of *Anopheles funestus* to *Cyperus rotundus* and river water

4.6.1 Gravid *Anopheles funestus* showed no attraction to *Cyperus rotundus* in two-port airflow olfactometer

Laboratory and semi-field choice experiments (Sections 4.2 & 4.3), *Cyperus rotundus* was shown to be the most attractive graminoid plant tested for attraction of gravid *An. gambiae s.s.* and was chosen for this study. When river water was placed in both chambers of olfactometer, the proportions of mosquitoes in both chambers were balanced (nearly 50% in each chamber; Table 4.6). The odour orientation of gravid *An. funestus* towards the choice chambers was similar between river water ('control') and river water with *C. rotundus* ('test'; OR 0.91, 95% CI 0.47-1.74).

Table 4. 6 Proportion of gravid *Anopheles funestus* responding to river water and *Cyperus rotundus* in olfactometer.

Treatments	Mean proportion of mosquitoes in test	Odds Ratio (95% CI)	P-value
Water	0.50 (0.38-0.62)	1	
C. rotundus	0.48 (0.38-0.60)	0.91 (0.47-1.74)	0.77

4.6.2 Attraction of *Anopheles funetus* to *Cyperus rotundus* in houses

4.6.2.1 Cyperus rotundus attracted wild gravid Anopheles funestus in houses

When all physiological states were considered, traps treated with *C. rotundus* caught a greater proportion (RR 1.26, 95% CI 0.81-1.97) of *An. funestus* than the control trap containing water only although the difference did not reach a significant level (Table 4.7). The numbers of blood-fed and gravid *An. funestus* collected were 44 and 49, respectively. Most of females of *An. funestus* caught by control traps (90%) were unfed and the rest were blood-fed and gravid (5% each). Blood-unfed, blood-fed and gravid female *An. funestus* mosquitoes collected by test traps account for 85%, 7% and 8%, respectively. For analysis purpose, blood-fed and gravid mosquitoes were combined since their number was smaller and both are not host-seeking mosquitoes. The addition of *C. rotundus* to the traps increased the catches of blood-fed and gravid female *An. funestus* by 2-fold (95% CI 1.29-3.33) than the control traps. In house three and house six the proportions of blood-fed and gravid *An. funestus* mosquitoes were 1.75 (95% CI 1.87-6.04) and 3.56 (95% CI 1.69-7.51) times more likely to be collected compared to in house one, respectively.

Table 4. 7 Mean number (95% CI) of *Anopheles funestus* being trapped/trap/night by the control and test traps.

Factor		Mean no. per trap night (95% CI)	Rate ratio (95% CI)	P-value
***An. funestus* (male and female)**	Control	13.03 (9.10-18.65)	1	
	Test	15.72 (11.87-20.82)	1.10 (0.74-1.64)	0.633
Females *An. funestus*	Control	8.19 (5.34-12.6)	1	
	Test	11.94 (8.26-17.30)	1.26 (0.81-1.97)	0.307
Unfed *An. funestus*	control	7.39 (4.66-11.73)	1	
	Test	10.17 (6.64-15.57)	1.18 (0.71-1.95)	0.53

Fed and gravid *An.*	control	0.81 (0.51-1.27)	1	
funestus	Test	1.78 (1.31-2.41)	2.07 (1.29-3.33)	**0.003**

CI=confidence interval

4.6.2.2 Species composition and physiological states of mosquitoes captured

The species composition of the collected mosquitoes includes *An. funestus, An. arabiensis, An. coustani, An. pharoensis, Mansonia sp.* and *Culex sp.* A total of 1229 mosquitoes were trapped comprising 91.6% (n=1126) *Anopheles sp., Mansonia sp.* 1.5% (n=19) and 6.8% (n=84) *Culex sp.* using six modified BG-Sentinel mosquito traps during 12 collection nights. Of these 71% (n=876) were females and 29% (n=353) were males. *Anopheles funestus* was the predominant species caught accounting for 84% (n=1035) of the total catches with 70% (n=725) being females and 30% (n=310) being males. Of the 725 female *An. funestus,* 41% (295) were collected by control traps and 59% (430) were collected by test traps. The molecular identification was done for more than 550 female *An. funestus s.l.* samples and all were identified as *An. funestus s.s.*. Out of the collected female *An. funestus,* most of them (n=632) were not blood-fed (Table 4.). The proportion of blood-fed *An. arabiensis* was higher (24.5%) than the proportion of blood-fed *An. funestus* despite their lower number. 6% of the collected *An. arabiensis* was found to be gravid. One blood-fed *An. coustani* and one gravid *An. pharoensis* were also trapped.

Table 4. 8 Number of female mosquitoes in different physiological conditions trapped by control and test traps (n=36 trap nights for each treatment).

Mosquito species	Control trap			Test trap		
	Unfed	**Fed**	**Gravid**	**Unfed**	**Fed**	**Gravid**
*An. funestus s.s.**	266	14	15	366	30	34
*An. arabienis***	22	3	3	12	9	0
An. coustani	0	0	0	7	1	0
An. pharoensis	1	0	0	0	0	1
Mansonia sp.	8	3	0	4	1	0
Culex sp.	33	2	1	34	2	4

*Molecular analysis shows that all the captured *An. funestus s.l.* were *An. funestus s.s.*.
**Molecular analysis shows that all the collected *An. gambiae s.l.* were *An. arabiensis*.

CHAPTER FIVE

DISCUSSION

5.1 Survey of malaria vectors larval habitat types and graminoid plants

The work presented here was done with the aim of identifying graminoid plants for further behavioural and chemical ecology studies due to their association with habitats used by gravid malaria vectors for egg-laying. However, the presence of early instar *Anopheles* larvae in the majority of the surveyed habitats and the presence and high coverage of various graminoid plants did not allow us to analyse any statistically significant association. All the habitats surveyed provided excellent oviposition sites and favourable conditions for the development of immature stages based on the high and consistent number of early instar larvae as a proxy for oviposition and the associated high abundance of late instar larvae as an indicator for survival. The study, as implemented, did not allow infer specific plant-based factors with oviposition. Generally, the association between graminoid plants and *Anopheles* breeding sites as well as the presence and increased densities of *Anopheles* larvae in both temporary and permanent aquatic habitats have been shown before (Fillinger et al., 2004; Imbahale et al., 2011). It has been suggested that vegetation can protect mosquito immature stages from being washed off by river water (Dia et al., 2013) and from predation (Wondwosen et al., 2017, 2018). This study has several limitations that might be responsible for the negative results. The timing of the survey towards the end of the rainy season meant that all potential habitats were flooded and vegetation thrived. Habitats for oviposition were not a limiting factor and likely easy to identify without major cues for orientation. This might have been different if the survey had been implemented during the dry season. Furthermore, this survey was limited to locations close to the lake shores, biasing the study towards swampy habitats. Potentially a more rigorous evaluation of the plant coverage using standard methods such as a quadrant frame which might have provided more detailed information on plant numbers could have revealed more associations. However, given the high colonisation during the rainy season such method would be better applied during drier seasons. Lastly, due to high water levels during the peak rainy season, a number of habitats with swamp graminoids of the families Cyperaceae, Typhaceae, and Juncaceae were impossible to access, hence could not be sampled. This might also explain why only very few secondary malaria vector species and no *Anopheles funestus* were sampled, even though *An. funestus* is the major vector in houses in the study area (Minakawa et al., 2004; Mutuku et al., 2009; Paaijmans et al., 2007).

5.2 Oviposition attraction response of gravid *Anopheles gambiae s.s.* to graminoid plants

This study confirms and expands the evidence that odour cues released from graminoid plants play a role in the orientation of gravid *An. gambiae s.s.* females. The experiments in olfactometer, WHO tubes and modified BG-Sentinel traps allow to avoid the interference of visual cues and tactile cues with the test olfactory cues as the mosquitoes could not see and come in contact with the test substrates. mosquitoes the same batch and fresh substrates were used in each round of experiments to reduce the effect biological variability. Volatiles released from these plants add significant attraction to water vapour alone. Generally, all graminoid plant species tested, including the dry-land ornamental grass (Fish et al., 2015), *Cenchrus setaceus*, usually not associated with mosquito breeding sites, significantly attracted gravid females and behavioural differences in response to different test plants were not very pronounced especially under the more natural, longer-range trapping conditions.

Whilst the behavioural response of gravid *An. gambiae s.s.* mosquitoes appeared to be slightly stronger in reaction to the sedge, *Cyperus rotundus*, than to most other test plants, I was not able to exactly establish any unique differences in the chemical profiles that might explain this. This is likely, in part, due to the chemical sampling method. To the best of my knowledge, these bioassays are the first to use live plants rather than eluted headspace extracts for testing for attractiveness to gravid malaria vectors. The experiment was conducted to test the behavioural response of gravid females to plant volatiles under as natural conditions as possible. Plant volatiles react differentially with atmospheric oxidants, such as ozone, resulting in odour plumes that do not only include the plant-emitted volatile chemicals but also gradually include a blend of degradation products (Conchou et al., 2019), which might not be picked up during dynamic headspace sampling with filtered air. I had opted for headspace sampling since it is a non-destructive method for sampling the volatile profile emitted by plants which might consequently be detected by insects (Tholl et al., 2006). The pooled analyses of the headspace samples of this study suggest that there are variations between the chemical profiles of the different plant species. It is however unclear if these differences would be consistent over time and under different environmental conditions, and if they are responsible for the variations observed in attracting gravid females in the bioassays. The GC results have been highly variable between replicate plant samples of the same species with some samples not resulting in any detectable compounds. This is not unexpected, given that I have taken only a ‘snap-shot’ of volatiles released at a particular time point and without carefully standardizing plant age and

development. Some volatiles may be emitted in quantities below technical detectability, yet these might be functionally relevant for insect attraction (Conchou et al., 2019). Volatile organic chemicals emissions and concentrations are also affected by light, temperature, nutritional and soil-moisture conditions, and even by species composition of the neighbouring plant community (Assmann et al., 2000; Gouinguene & Turlings, 2002; Kfoury et al., 2017; Kigathi et al., 2009, 2019; Reichstein, 2003; Stewart-Jones & Poppy, 2006). Abiotic stresses, including stress induced by the air sampling itself when plant material is enclosed in plastic bags will also affect the volatile profile. Going forward, it will be desirable to sample under natural, yet varying environmental conditions and to compare results across different sampling strategies (Tholl et al., 2006) for a better understanding of the composition and concentration of compounds in the headspace of plants that might affect natural mosquito behaviour.

In this study, and across published work, the variation in the strengths of the behavioural response of gravid mosquitoes to varied graminoid plant species was very little, despite the fact that volatile profiles appear variable. The behavioural response of gravid *An. gambiae s.s.* induced by the wild graminoid plants in these bioassays was in the same ranges as those reported previously for *An. arabiensis* and *An. coluzzi* in response to low release rates of headspace extracts from rice plants (Wondwosen et al., 2016) and from the tropical African wetland grasses (Poaceae) *Echinocloa pyramidalis, E. stagnina* and *Typha latifolia* (Asmare et al., 2017). It was also in a similar range as observed for the attraction of unfed females to plant-based volatiles (Lahondère et al., 2020; Meza et al., 2020; Nyasembe et al., 2012). A limitation of this study was my inability to access equipment for electroantennography to determine exactly which volatile chemicals released from the test plants were detected by the gravid female's antenna. However, when comparing the volatile chemicals identified in this study with those published for rice plants and pollen from sugar cane and maize in the context of oviposition (Wondwosen et al., 2016, 2017, 2018), as well as with those published for a range of plants preferentially visited by malaria vectors for sugar feeding (Asmare et al., 2017; Meza et al., 2020; Nyasembe et al., 2018, 2012), it becomes apparent that there is significant overlap in the chemical compositions. Compounds reported here, such as limonene, α- and β-pinene, p-cymene, sulcatone, humulene, cedrene, β-myrcene, and β-caryophyllene, have previously been reported to elicit electrophysiological responses in gravid and unfed female *Anopheles* (Deletre et al., 2015; Meza et al., 2020; Nyasembe et al., 2018, 2012; Wondwosen et al., 2016, 2018, 2017) and many of them have been formulated into synthetic blends and shown to be attractive to unfed and gravid *Anopheles* under highly standardised experimental conditions

(Nyasembe et al., 2012; Nyasembe et al., 2014; Wondwosen et al., 2016, 2017). These compounds are among the most common VOCs emitted from plants (Knudsen et al., 2006) since they are synthesized through biosynthetic pathways common in most plants (Eisenreich et al., 1998; Nyasembe & Torto, 2014; Schwab et al., 2008).

In this study, three volatile chemicals, namely 1,1-dimethyl-3-methylene-2-vinylcyclohexane, α-guaiene and β-elemene, have not been tested previously, yet were detected frequently in four out of the five test plants. It might be useful to explore their potential to manipulate odour-orientation of *Anopheles* mosquitoes in follow-up studies, since they have been implicated as semiochemicals for other insect species (Asui et al., 2007; Darshanee et al., 2017; Mayo et al., 2016; Miao et al., 2020; Zhang et al., 2015). For example, 1,1-dimethyl-3-methylene-2-vinylcyclohexane was attractive to the beech leaf-mining weevil (Mayo et al., 2016), guaiene has been suggested to play a role in the attraction of the litchi stem-end borer (Meng et al., 2021) and β-elemene has been implied to contribute to attraction of the gravid tobacco moths (Miao et al., 2020) and the white-spotted longhorn beetle (Asui et al., 2007).

Myrcene**,** γ-elemene, humulene epoxide II and hexahydrofarnesyl acetone were specific to headspace samples of *Cyperus rotundus* in this analysis. This does, however, not necessarily imply that these compounds contributed to the attractiveness in these bioassays. Information on these compounds as info-chemicals for insects and specifically mosquitoes is scant and none of them have been tested with gravid malaria vectors. Both, unfed *Anopheles* and unfed *Aedes* mosquitoes showed electrophysiological activity to β-myrcene in previous studies (Lahondère et al., 2020; Meza et al., 2020). It was observed that myrcene elicits an avoidance behaviour in unfed *An. gambiae s.s.* when searching for sugar (Meza et al., 2020) or blood meals (Jaleta et al., 2016). γ-elemene was identified from plant headspace and found to be electrophysiologically active for unfed *An. gambiae* but behavioural implications were not studied (Nyasembe, 2016).

Gravid malaria vectors navigate a complex chemical environment in search for oviposition sites. It is plausible to assume that volatile chemical cues emanating from aquatic habitats and their surroundings are only used at relatively short-range, with visual cues and air movements guiding the gravid females' flight towards a water body (Beehler et al., 1993; Bentley & Day, 1989). Visual cues will include near-infrared radiation from slowly released heat from water bodies in the evening (Gibson, 1995), polarized light from water surfaces (Wellington, 1974) as well as ultraviolet light (Silberglied, 1979), all of which present strong long-range cues likely

used by gravid mosquitoes to evaluate the location and quality of potential oviposition sites (Day, 2016). In this context, it remains therefore unclear, if attractive, yet common, plant-based semiochemicals in odour-baited traps will be able to compete in an attract-and kill approach, with the complex interaction of cues provided by natural aquatic habitats. To date over 100 semiochemicals have been identified for mosquitoes of all physiological stages, yet synthetic odour-baited traps hardly play any role in contemporary surveillance and control of malaria vector mosquitoes (Wooding et al., 2020). Synthetic odour-baits mimicking human body odour have shown to perform poorly in attracting host-seeking *Anopheles* mosquitoes when presented in close vicinity to natural human blood hosts (Njoroge et al., 2021) and field evaluations of the oviposition attractant cedrol, showed that visual cues provided by an open water surface were essential in combination with the chemical cue to attract wild oviposition-site searching females (Lindh et al., 2015). In order to develop vector control interventions that manipulate the odour-orientation of malaria vectors in their natural environment, less emphasis might be placed in future on detecting more semiochemicals but more emphasis on how to formulate and present these chemicals in combination with other essential cues used by mosquitoes, to improve the efficacy of such interventions (Wooding et al., 2020).

5.3 Oviposition responses of gravid *Anopheles gambiae s.s.* to synthetic chemicals in dual choices bioassays

Attraction and egg-laying responses of gravid *An. gambiae s.s.* to synthetic chemicals found in the headspace of plants were determined with WHO tubes and cage bioassays. These results show that ß-elemene and ß-pinene when presented individually elicited a significantly higher attraction behaviour and induced oviposition responses in gravid *An. gambiae s.s.* mosquitoes. ß-elemene is a sesquiterpene (Zhai et al., 2019) whereas ß-pinene is a monoterpene (Knudsen et al., 2006).

This study is the first to report that ß-elemene is attractive to gravid malaria vectors eliciting oviposition response. Previously it was identified from *Cummiphora leptophloeos* leaf oil and detected by *Ae. aegypti* antennae (da Silva et al., 2015). The compound has also been reported for its strong larvicidal activity against larvae of *Anopheles subpictus, Aedes alibopictus* and *Culex tritaeniorhynchus* (Govindarajan & Benelli, 2016). Hence, it may be explored as a a strategy to control African malaria vector immature while attracting the gravid females. Similarly, ß-elemene was shown to be a major constituent of *Piper sp.* and *Commiphora erythraea* essential oils having larvicidal activities against larvae of *Culex restuans, Cx. pipiens*

and *Ae. aegypti* (Huong et al., 2019; Muturi et al., 2020). Additionally, the compound has been in use as traditional medicine specifically in China exhibiting high potential for the treatment of cancer (Zhai et al., 2019). Studies have shown that treatment of oesophageal squamous cell carcinoma, glioblastoma, myeloid leukaemia, lung cancer and malignant disease with ß-elemene in combination with other drugs has shown to be promising (Chang et al., 2017; Ma et al., 2016; Wang et al., 2012; Wu et al., 2020; Zhai et al., 2019; Zheng et al., 1969).

In the case of ß-pinene, it has been reported as an attractant for different mosquito species with different states individually and in blends. For instance, gravid *An. arabiensis* has demonstrated a strong attraction to a blend consisting of ß-pinene and other seven rice plant volatiles (Wondwosen et al., 2016). Similarly, a blend consisting of ß-pinene and other six compounds in their natural occurring doses were attractive to gravid *An. gambiae s.s.* and induced significantly higher oviposition (Milugo et al., 2021). However, when ß-pinene tested individually gravid females avoided laying eggs in water treated with the chemical (Milugo et al., 2021). In other studies, it strongly stimulated antennae of host-seek *Ae. aegypti* (Campbell et al., 2011) and significantly increased the number of eggs laid by gravid *Ae. Aegypti* (Waliwitiya et al., 2009). Compounds including ß-pinene, D-limonene, hexanal, ß-ocimene, (*E*)-linalool oxide and (*E*)-ß-farnesene attracted host-seeking *An. gambiae s.s.* both individually and as a blend (Nyasembe et al., 2012). Similarly, a blend comprising of ß-pinene, linalool oxide and ß-ocimene was two times more likely to attract host-seeking *An. gambiae s.s.* than the control linalool oxide previously reported as the highly attractive compound (Jacob et al., 2018).

Gravid females showed a weak attraction to limonene when tested individually in laboratory and it was not tested singly in egg-count bioassays. Previously, it was identified from headspace samples of rice plant as GC-EAD active and oviposition attractant as a blend with other compounds to gravid *An. arabiensis* (Wondwosen et al., 2016). It was also among the components of a blend of volatiles emanations of maize pollen attractive to gravid *An. arabiensis* (Wondwosen et al., 2017). Similarly, it has been shown that limonene was highly attractive to host-seeking *An. gambiae s.s.* when tested singly and in a blend with other compounds (Nyasembe et al., 2012). A conflicting result was reported by Jacob et al. that addition of limonene to the blend exhibited antagonistic effect on its attractiveness (Jacob et al., 2018). Other chemicals cedrene, β-caryophyllene and caryophyllene oxide did not significantly affect the behaviour of gravid *An. gambiae s.s.* under laboratory conditions. β-

caryophyllene and α-humulene were found to be oviposition deterrent for *Ae. aegypti* (da Silva et al., 2015). Similarly, β-caryophyllene oxide was found to be a strong repellent for *An. minimus* (Nararak et al., 2019).

Insects discriminate between host plants by detecting either volatiles specific to a given plant and closely related plant species or by recognition of specific blends and their ratios commonly present in several plant species (Bruce et al., 2005; McCormick et al., 2014). Host plant odour cues coding in several insect species largely depends on the VOCs emitted from many plant species than VOCs taxonomically specific to few plant species allowing them to adapt to the dynamic environment (Bruce & Pickett, 2011). This agrees with the present results since ß-elemene and ß-pinene were shared among the headspace samples of four and three of the tested plants, respectively. This suggests the role of these chemicals as salient odour cues in the attractiveness of the plants to gravid mosquitoes. They are among general plant VOCs produced by several plant families as reviewed by (Knudsen et al., 2006).

Selection of biologically important VOCs in insects' communications from the complex odour landscape is a critically relevant step to understand and use in further behavioural bioassay experiments (Magalhães et al., 2018). Electrophysiological bioassay with insect antennae can be deployed to select these biologically active compounds (Bruce et al., 2005; Cork & Park, 1996; Smallegange & Takken, 2010). Electrophysiological bioassay was not conducted for this study and perhaps this was a limitation of the study posing challenge on the choice of the components and ratios of blends for behavioural bioassays. The two individually attractive chemicals, ß-elemene and ß-pinene, when tested as blends at their optimal attractive doses, lower doses and higher doses were not attractive to the gravid mosquitoes. Insects often recognize blends consisting of three to ten plant volatiles which determine their behaviours (Bruce & Pickett, 2011) and these compounds perhaps include the trace ones (McCormick et al., 2014; Nyasembe et al., 2012). Considering this, blends of three to five components including ß-elemene, ß-pinene, limonene, ß-caryophyllene and caryophyllene oxide were examined using subtractive bioassays. However, the blends were failed to stimulate significantly higher egg-laying response in gravid *An. gambiae* than the distilled water. Conversely to the current study, a blend of rice emanations including three of these compounds ß-pinene, limonene, ß-caryophyllene, and other more compounds stimulated oviposition in *An. arabiensis* (Wondwosen et al., 2016). The results of this study may have been different if the right mixes of the compounds were identified and tested suggesting the ratio of the tested

compounds in the blend was not right and/or there were some missing chemicals responsible to make the natural attractive blends. Similar observation was reported in previous studies (Smallegange et al., 2010; Verhulst et al., 2011). They found that attractive synthetic blends of human odorants when they tested against the human hosts or natural human odorants dispensed using nylon matrix showed poor attraction. This shows the critical importance of the natural ratio and composition of the blend in determining the effectiveness in the oviposition attraction of chemicals (Bruce & Pickett, 2011; Wooding et al., 2020). Determining the bioactivity and attractiveness of chemicals singly and in a blend is very important for use in vector control and surveillance programmes (Nikbakhtzadeh et al., 2014). However, choosing the constituents and the optimum doses of the chemicals is extremely complex as a small variation in their quantity affects the overall attraction of the blend (Bruce et al., 2005; Bruce & Pickett, 2011; Nikbakhtzadeh et al., 2014). When plant VOCs are presented to insects singly they might be perceived as non-host cues (Bruce & Pickett, 2011). This is mainly because the meaning and behavioural influence of VOCs on insects delivered individually and as a blend are different (Bruce & Pickett, 2011). These have been demonstrated in earlier studies. Blends of eight GC-EAD active compounds released from rice plants, five compounds by maize pollen and eleven compounds released by sugarcane pollen when prepared considering their natural ratio were found to be attractive gravid *An. arabiensis* (Wondwosen et al., 2016, 2017, 2018). Subtraction of salient chemicals from blends adversely affect their attraction (Ignell & Hill, 2020) whereas subtraction of the redundant chemicals has no significant effect on their attractiveness (Bruce & Pickett, 2011). Therefore, the selection of bioactive compounds, their composition and their natural amount should be determined to develop an effective attractant.

5.4 Response of gravid *Anopheles funestus* to *Cyperus rotundus*

5.4.1 Response of gravid *Anopheles funestus* to volatiles of *Cyperus rotundus* in two-port olfactometer

This study presents for the first time an attempt made on testing of attraction of gravid wild *An. funestus*. Oviposition behaviour of *An. funestus* is poorly understood as its colonization in laboratory conditions is quite challenging (Ngowo et al., 2021). Studies of *An. funestus* in general has been neglected for several years due to this less adaptability of the vector in laboratory settings (Coetzee & Fontenille, 2004). This failure is mainly attributed to the limitation of knowledge about their biology because of the cryptic nature of larval habitats (Ngowo et al., 2021). Only two strains of *An. funestus* from Angola and Mozambique have

been successfully established at Vector Control Reference Laboratory, South Africa (Hargreaves et al., 2000; Hunt et al., 2005). In this study, similar attraction responses of gravid *An. funestus* to *C. rotundus* and river water were observed in olfactometer bioassays. Various reasons might be attributed to the absence of attraction of *An. funestus* to *C. rotundus*. This can be partly because of the impact of the controlled environment with small space limiting the free flight in the olfactometer that may have influenced their response. A fitness characterization study in a laboratory has shown that the field colony of *An. funestus* laid fewer eggs compared to the insectary colony (Ngowo et al., 2021). This can be evident by the higher proportion of gravid *An. funestus* collection by BG-Sentinel traps treated with *C. rotundus* than by the traps treated with only river water in houses (see section 5.3.2). This suggests that such studies with *An. funestus* mosquito should be done in the field settings. Moreover, this might be because the mosquitoes used in the bioassays were not ready to lay or they were not truly gravid. This might be because eggs development and maturation can occur without mating but egg laying in most mosquitoes species depend on insemination (Chambers & Klowden, 2001; Clements, 1999).

5.4.2 Response of gravid wild *Anopheles funestus* to *Cyperus rotundus* in houses

Gravid *An. funestus* trapping was done using modified BG-Sentinel mosquito traps treated with river water and *C. rotundus* in houses in the field. The modified BG-Sentinel *traps* performed well in catching a high number of all physiological stages malaria vectors indoors. When all physiological states were considered treatment of the traps with the plant increased the overall catches of female *An. funestus* mosquitoes than those being trapped by control traps. However, the difference was not significant. This suggests that *C. rotundus* might not have a significant influence on the preference of non-blood fed mosquitoes which account for the largest portion of the mosquito catches. Additionally, replication might not be sufficient to have the power at a significant level. Another study conducted in Ahero similarly found that most of *An. funestus* mosquitoes trapped while exiting houses using exit traps were unfed (Degefa et al., 2019). In the same study, it has been reported that more than 94% of *An. funestus* collected using CDC light traps from both indoors and outdoors were unfed. This might be an indication that the mosquitoes were trapped before they visit hosts or the use of bed nets protected the community from mosquito bites (Ndenga et al., 2016). Moreover, the capture of higher proportions of host-seeking females by the control and test traps might be due to the presence of water since mosquitoes need water for a drink. Despite their fewer numbers, treating the traps with *C. rotundus* had increased the catches of blood-fed and gravid *An. funestus* by more than two

times compared to control traps showing the influence of plant odour cues on mosquito oviposition preference.

Four species of *Anopheles* mosquitoes such as *An. funestus s.s.*, *An. arabiensis*, *An. coustani*, and *An. pharoensis* were captured by the traps indoors which are similar to the findings of a previous study (Degefa et al., 2017). The authors collected mosquitoes from inside of the houses using CDC light traps and spray sheet collections (SPC) in Ahero. In the study area *An. funestus* was the most abundant mosquito species collected. Ogola et al. sampled *An. funestus* both indoors and outdoors in Ahero and have found that more than 75% of the catches were from indoors collection (Ogola et al., 2017, 2018). The presence of higher density of *An. funestus* indoors in the study area highlights that it is still more endophilic. Ogola et al. found that the main blood meal sources for the majority of the blood-fed *An. funestus* females was humans (Ogola et al., 2017, 2018). Conversely to the present finding, a study by Degefa et al. found that the indoor and outdoor vector population in Ahero was dominated by *An. arabiensis* (Degefa et al., 2017). Another study conducted in Ahero during dry season shows nearly 55% of the indoor mosquitoes were *An. arabiensis* while *An. funestus* accounted for about 37% (Mburu et al., 2017). This is perhaps partly due to the seasonal difference of mosquito sampling as they conducted the collection during the short rainy season (September to October) unlike the present study was conducted in during rainy season (March). Additionally, the rice cropping cycle relates to the rainy season and contributes to the presence of breeding sites for *An. funestus*. During the short rainy season, the available breeding habitats for *An. funestus* may be fewer than that of *An. gambiae s.l.*. Water pools and puddles during the short rainy season serve as breeding sites of *An. gambiae s.l.* (Mwangangi et al., 2010).

The molecular identification of the collected samples shows that for all the amplified specimens of *An. funestus* group all were found to be *An. funestus s.s.*. Similarly, all the collected *An. gambiae s.l.* were identified to be *An. arabiensis.* This result is similar to the findings of Jacob et al. who conducted a study in the same area (Jacob et al., 2018). Another study also reported similar findings of all the *An. funestus s.l.* and nearly 99% of *An. gambiae s.l.* sampled indoors in the same study area were being *An. arabiensis* and *An. funestus*, respectively (Degefa et al., 2017).

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

In summary, the hypothesis that graminoid plants might be positively associated with the presence and abundance of early instar *Anopheles* larvae could not be confirmed. Immature stages of malaria vectors are predominant in aquatic habitats densely covered with short graminoid plants without showing preferences for a specific plant species during the wet season.

Plants of the Cyperaceae and Poaceae family release chemical cues that induce gravid females to orient to breeding sites with short and long-range effect. *C. setaceus*, which is usually not associated with aquatic habitats, attracted gravid mosquitoes in similar way.

The chemical profile of attractive graminoid plants revealed some ubiquitous and several unique volatiles which might be explored for use in odour-based vector control and surveillance strategies.

Few synthetic chemicals originated from attractive graminoid plants elicited attraction and egg laying responses in gravid *An. gambiae* when tested individually.

The volatiles of *C. rotundus* might be a potential candidate to be considered for further study for identification of putative oviposition blends that can be used in control and surveillance tools.

The utilization of these chemical cues for attract-and-kill trapping strategies must be explored under natural conditions to investigate their efficiency when in competition with complex interacting natural cues.

6.2 Conclusions

1. This study illustrates that early instar malaria vectors occur in high abundance in aquatic habitats densely vegetated with graminoid plants from both the Poaceae and Cyperaceae family. Field data did not suggest any oviposition preference for any of the graminoid plant species. Three grass species dominated in the aquatic habitats and were selected for further evaluation in the laboratory and under semi-field conditions.

2. This study confirmed that gravid *An. gambiae* use chemical cues released from graminoid plants of both the Cyperaceae and Poaceae families to orientate. No evidence to confirm the previous report that volatile chemicals released from Poaceae family are more attractive to gravid *Anopheles* mosquitoes than those released from Cyperaceae.
3. Chemical profiles largely show common plant volatiles released by most plants; including limonene, α- and β-pinene, p-cymene, sulcatone, humulene, cedrene, β-myrcene and β-caryophyllene which have previously been reported to elicit electrophysiological responses in gravid and unfed female *Anopheles*. The results suggest that plant volatiles provide a more general cue for gravid malaria vectors rather than vectors being highly adapted and evolved in context to specific plant species and environments.

4. β-elemene and β-pinene elicited significant short-range attraction and egg-laying responses in gravid *An. gambiae* when tested individually. The behavioural response was associated with specific concentrations.
5. Oviposition behaviour of *An. funestus* is poorly understood as its colonization in laboratory conditions is quite challenging (Ngowo et al., 2021). Experiments in the field with experimental huts and access to natural populations provide a good alternative to studying of this species. The field study with BG-Sentinel traps suggests that odour cues from *C. rotundus* attracted free flying wild gravid *An. funestus*. The modified BG-Sentinel traps performed well in catching a high number of vectors indoors (all physiological stages) and might be a good alternative collection tool to a light trap for *An. funestus* by only adding water.

6.3 Recommendations

1. All the tested graminoid plants attracted gravid malaria vectors in a short and long-range bioassays. This could inform future identification of gravid malaria vector attractants should consider all the predominant graminoid plants found in natural aquatic habitats.

2. Little is known about the odour-orientation of gravid *An. funestus* due to challenge in keeping the species in the insectary. Experiments directly in the field setting have shown promise and should be continued.

3. β-elemene and β-pinene attracted and elicidted egg-laying response in gravid *An. gambiae* when tested individually. The study recommends the chemicals as potential gravid attractive candidates for further study in semi-field with freely flying mosquitoes and in the field settings before use in vector surveillance and control.

6.4 Suggestions for further research

1. Habitat surveys should be repeated during dry season when swamp grasses stronger associated with water. Assessment of plant coverage a quadrant frame might have provided more detailed information on plant numbers and might reveal especially during dry seasons more associations.
2. Field collection of plant VOCs could provide important insight in validity of experimental headspace collections. These field collections of the VOC during different seasons might reveal changes in response to flooding.
3. Electro-antennogram investigations with gravid malaria vectors would be desirable to identifiy bioactive plant volatiles to develop odour-blends.
4. Overall, there is need to invest more research into developing odour-blend formulations that can compete with cues from natural habitats to improve surveillance and control of vectors.

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APPENDICES

Appendix 1: O**viposition substrates used in behavioural bioassays with gravid *Anopheles gambiae* in two-port airflow olfactometers and in large field cages**

Treatment 1 ('control')	**Treatment 2 ('test')**	**No of replications**	**Total no of gravid *An. gambiae* recollected (out of total released)**
Calibration experiments Two-port airflow olfactometer bioassays			
Do the olfactometer bioassays result in reproduceable outcomes? What is the response rate that can be expected from released gravid mosquitoes?			
Water	Water	16	831 (1600)*
Empty	Empty	13	595 (1300)
Empty	Water	14	707 (1400)
Water	Hay infusion	12	710 (1200)

Note:

Prior to testing intact plants, the olfactometers were calibrated by evaluating their accuracy of generating valid and reproducible results and to gauge the response rate that can be expected under standard test conditions. This was done by providing (1) two equal-choices in both chambers, (both containing water and both being empty) and (2) by providing two different choices with predictable outcome (water vs. empty; hay-infusion vs. water).

Appendix 2: Research permit from Kenya Medical Research Institute Ethical Review Unit

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel:(254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1 April 11, 2019

TO: **DR. ULRIKE FILLINGER, (PRINCIPAL INVESTIGATOR),**
ICIPE,
P.O BOX 30772-00100, NAIROBI

Dear Madam,

RE: **NON-KEMRI PROTOCOL NO. 593 *(REQUEST FOR ANNUAL RENEWAL)*: USE OF DIRECT HUMAN ARM FEEDING AND HUMAN LANDING CATCHES IN SEMI-FIELD SYSTEMS IN BENCHMARKING KEY LIFE HISTORY TRAITS OF CULTURED MALARIA MOSQUITOES.**

Thank you for the continuing review report for period **April 27, 2018** to **March 13, 2019.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **April 26, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **April 25, 2020**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval by **March 14, 2020.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

Appendix 3: Research permit from National Commission for Science, Technology and Innovation

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **829305**

Date of Issue: **07/September/2020**

RESEARCH LICENSE

This is to Certify that Mr.. Getachew Eticha Bokore of International Centre of Insect Physiology and Ecology, has been licensed to conduct research in Homabay, Kisumu on the topic: ROLE OF GRASSES AND ASSOCIATED FUNGAL SPECIES IN OVIPOSITION SITE SELECTION OF MALARIA VECTOR MOSQUITOES IN WESTERN KENYA for the period ending : 07/September/2021.

License No: **NACOSTI/P/20/6222**

829305

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

Appendix 4: The proposal approved by the School of Graduate Studies, Maseno University

MASENO UNIVERSITY

SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PhD/SC/00024/018

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 11th September, 2019

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR GETACHEW ETICHA BOKORE - PhD/SC/024/018

The above named is registered in the Doctor of Philosophy Programme in the School of Physical & Biological Sciences, Maseno University. This is to confirm that his research proposal titled "The Role of Grasses and Associated Fungal Species in Oviposition Site Selection of Malaria Vector Mosquitoes." has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

DEAN SCHOOL OF GRADUATE STUDIES
MASENO UNIVERSITY
15 OCT 2019
P. O. Box PRIVATE BAG, MASENO

Appendix 5: Informed consent document for direct human arm feeding

Investigator – Oversight:

Dr. Ulrike Fillinger, Senior Scientist, Human Health Theme, *icipe*

Study Location: *icipe*-TOC, Mbita

Purpose of the activity requiring human volunteer (s): To feed un-infected cages of malaria mosquitoes that have conserved natural behaviours for use in research of new vector control tools.

Introduction to the research

Novel Oviposition Attractant for Malaria Mosquito project is one of the projects under human health department here at *icipe*-TOC whose aim is to study the behaviour of gravid (pregnant) mosquitoes towards controlling them. One of the most important behaviours of malaria mosquitoes that are gravid is to seek for habitats where they can lay eggs. Mosquitoes need to take blood in order for their eggs to develop and prefer to bite humans over all other potential blood hosts. In order for us to study and understand how we can protect humans from mosquitoes we implement experiments in the laboratory and in large cages that simulate the natural conditions. Additionally, we study the ecology and behaviour of malaria vector mosquitoes to enable us to control them with different tools. For this to work, we need mosquitoes that respond to humans in the same way they would in the wild. For this we need to rear them in our insectary under near to natural conditions, including feeding them on the forearm of human volunteers. You have been invited to volunteer for this activity.

Criteria to be considered before you can volunteer

In order to be considered as a volunteer, we must first establish that you do not have a serious skin reaction to the bites of mosquitoes. To determine this, we will first expose you to 10 mosquito bites only and assess your reaction after one day. Additionally, you will be tested for malaria using a rapid diagnostic test as you will not be allowed to feed mosquitoes if you have malaria in your system. If you do not have a skin reaction and/or malaria, you may volunteer to feed mosquitoes.

Procedures for mosquito feeding

Mosquito feeding is usually done at 6.30pm. You will be required to wash your arms with soap and water and then dry them completely. You will then be required to wear gloves. After this, you will insert your forearm inside a cage containing a maximum of 300 female mosquitoes for about 15 minutes. You are advised to hold your arms very still to avoid shaking off the mosquitoes as they feed. After 15 minutes, you will shake off the mosquitoes from your arms

and carefully remove them from the cages. It is forbidden to feed mosquitoes that have fed on someone else at an earlier time. This prevents any chance that a parasite can be transmitted to you by mosquito bites.

Why have you been invited to participate?

You have been invited to participate because you have shown an interest in the research that we carry out on mosquitoes at *icipe*. We require the participation of adults who are fluent English speaker so that we can be sure you understand the work required.

What are your commitments?

We ask that you are available at the adult mosquito insectary at *icipe* between 18:30 and 19.00 hours on selected nights per week according to the experimental timeline that will be discussed with you prior to the feeding. During the evenings that you will be feeding mosquitoes, we ask that you do not smoke or drink alcohol should you be the consumer, as these activities may affect the way that you smell to a mosquito and can affect the feeding success and survival of the mosquitoes.

Potential harm, injuries, discomforts or inconvenience, risks

Mosquito bites can cause local irritation, itching and discomfort at the bite site on the skin. This will be alleviated, if necessary, by applying an antihistamine lotion to the skin provided by the project. To prevent excessive irritation, the numbers of mosquitoes allowed to bite will be regulated to a tolerable level. If on subsequent feeds the irritation increases, you will not be allowed to feed mosquitoes again.

Potential benefits

You will receive no direct benefit from these biting activities and the investigations they support. However, the results will advance our knowledge of the mosquitoes that transmit malaria and may lead to new methods to control or eliminate this disease.

Alternative procedures:

Although artificial membrane feeding is an alternative method for giving blood to mosquitoes in colony or experimental cages, it has unnatural and unwanted side-effects on mosquito survival and behaviour.

Confidentiality

The project leader will not collect any personal information from you. Your name will not appear in any databases. Your identity will be coded and the original code book kept under lock. The use of unique IDs will help us troubleshoot in case we observe increased mortality rates, reduced egg-laying rates or other unusual observations due possibly to the differential attractiveness of individual volunteer [s] and the quality of their blood.

Consent forms will be kept under the personal control of the core researchers. **Consent forms and paper data records will be** locked in file cabinets in offices at *icipe*. **Electronic Data Records will be** stored in password protected files on *icipe* maintained servers with regular back-up. Research data will be retained for 3 years after the completion of the project. Records for completed projects will be stored in secure locations at *icipe* with the same care used when the project was active. Paper records will be shredded and incinerated after the 3 years of storage; electronic records will be deleted.

Reimbursement

Arm feeding is a voluntary activity. To cater for your transport to and from the station we provide you with an allowance of Ksh3000 per month. If you do not volunteer for this activity, you will continue to be employed for your participation in other activities within the *icipe* TOC.

Participation

Your willingness to participate in blood-feeding aspects of this project is strictly voluntary and is not a condition of employment, staff promotion or study. If you choose to volunteer, you will be providing a source of blood required by female *Anopheles* mosquitoes, because humans are their normal and natural blood source. The proposed research requires that some humans be bitten. This use of human blood to maintain mosquito colonies and to provide experimental mosquitoes is common practice throughout the world. To participate in these mosquito-production activities, you must be a healthy and malaria-free adult, you must not have a psychological aversion to being bitten and must not be prone to strong skin reactions, or any other kind of allergic reactions, to insect bites.

Contact

If you experience any difficult event related to your participation in the study, you should report it immediately to the local Principal Investigator, Dr. Ulrike Fillinger (*icipe* TOC Mbita) and Paul Ouma. If you have any questions or concerns, either before, during, or after the activity, you should first contact Dr. Fillinger (Cell: +254791845259; email: ufillinger@icipe.org) Getachew Bokore (+254702953068; email: egetachew@icipe.org or Paul Ouma (Cell: +254724054224; email: podera@icipe.org

Informed consent document for direct human arm feeding

You are about to participate in *icipe's* malaria research programme. To test mosquito behaviour, we need to produce mosquitoes that show the same behaviours as mosquitoes in the wild. These mosquitoes will however be free of malaria. Mosquitoes prefer to feed on humans in order to produce eggs and give rise to more mosquitoes. We therefore need help in feeding female mosquitoes so that they can lay eggs and produce more mosquitoes for experiments.

All experiments will be done in screen houses and mosquitoes will not be released outside these screen houses.

Should you volunteer to participate, you will be asked to insert your arm into one or maximum two cages of mosquitoes and allow them to feed on you for 15 minutes.
We will not collect any personal data, we will only indicate in our records your unique number and date of feeding. All paper records will be stored in a locked filing cabinet.

You are free to withdraw from some or all blood-feeding activities at any time you wish, but please give sufficient advanced warning (ideally 48 hours), so that a replacement can be found. You may refuse to explain why you are withdrawing, without any consequences.

Should you agree to participate in one or more blood feeding activities please sign your name below and you will receive a copy of this signed Consent Form and the Informed Consent Document. We will need a confirmation that you have read and understood the nature of the study, your responsibilities as a study participant, and the inconveniences associated with voluntary participation in the study. Should any further questions concerning your participation arise or on your rights as a volunteer, you may contact the head of this study, namely Dr. Ulrike Fillinger (Cell: +254791845259; email: ufillinger@icipe.org), Getachew Bokore (cell +254702953068; email: egetachew@icipe.org or Paul Ouma (Cell: +254724054224; email: podera@icipe.org)

_____

All my questions regarding my participation have been adequately answered. I have been made fully aware that I may revoke this consent at any time without penalty or loss of benefits.

Legal rights

You are not waiving any of your legal rights by signing this informed consent document.

I_____(name) hereby give consent for my participation in the study.

Signature_____ ID No. _____Date_____

Name of witness _____ Signature _____

ID No. _____ Date _____

Appendix 6: Preliminary olfactometer calibration experiments with gravid *Anopheles gambiae s.s.*.

Experiment	'Control' substrate	'Test' substrate	Percent (%) response of all released (95% CI)	Percent (%) attracted to 'test' of all responders (95% CI)
1	Empty	Empty	46 (38-53)	52 (46-58)
2	Empty	Lake Water	51 (43-58)	80 (75-84)
3	Lake Water	Lake Water	52 (45-59)	49 (44-54)
4	Lake Water	Infusion	59 (52-67)	29 (24-35)

CI-confidence interval

Note:

The preliminary calibration experiments helped gauge the performance of the bioassay design and apparatus. During the majority of the preliminary experimental runs, around 50% of the released mosquitoes responded, whilst the others remained in the release chamber. This proportion could not be increased even when the experimental set up was modified. Hence, for all following experiments, it was defined that for a viable outcome the response rate must be 50% or above. When two equal choices of water were provided in the chambers, the released gravid mosquitoes distributed equally between the two chambers as expected (Table 4.5). When both chambers were empty, mosquitoes still responded, likely flying upwind in search of cues, and again distributed equally between the two chambers. The response rate, however, was overall slightly lower (46%) than when water was provided. When a choice between water in one chamber and no substrate in the other chamber was provided, > 80% of the responding females chose water. This confirmed that water vapour acts as an attractant for gravid mosquitoes. Moreover, it was confirmed that fermented three day-old hay infusion repels gravid *An. gambiae*. Out of all responding females, >70% oriented away from the infusion and towards the chamber with water.

Appendix 6: Preliminary olfactometer calibration experiments with gravid *Anopheles gambiae s.s.*.

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1	Empty	Empty	46 (38-53)	52 (46-58)
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Appendix 7: Preliminary WHO tubes calibration experiments with gravid *Anopheles gambiae s.s.*.

Treatment 1 ('control')	**Treatment 2 ('test')**	**Total no of gravid *An. gambiae* recollected (out of total tested)**	**Percent (%) attracted to 'test'**
Do the WHO tube bioassays result in reproduceable outcomes?			
Water	Water	52 (90)	52%
Water	Hay infusion	59 (129)	25%

Note:

The preliminary calibration experiments of the WHO tubes with the known gravid *An. gambiae* attractant (water) and repellent (three days old Bermuda grass infusions) substrates used to determine the reproducibility of the outcomes. The bioassays with equal choices (distilled water in both sides of the tubes) expectedly showed nearly equal number of gravid mosquitoes (52% in test; Appendix 7) in both sides of the tubes. 75% of mosquitoes preferred the tubes treated with distilled water over the repellent infusions confirming the reproducibility of the experimental set ups.