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The Identification of cereal volatile compounds that attract gravid malaria mosquito, *Anopheles arabiensis* Patton (Diptera: Culicidae)

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Dedication

This thesis is dedicated to my beloved parents Wondwosen Hailu and Bizuayehu Bogale who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve.

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List of abbreviations

ACT	Artemisinin-Based Combination Therapy
BG	Biogent
BH-660	Bako Hybrid-660
CDC	Cener for Disease control
E-MS	Capillary Electrophoresis Mass Spectrometry
CI	Chemical Ionization
DEET	*N*,*N*-Diethyl-*meta*-toluamide
DDT	Dichlorodiphenyltrichloroethane
EAG	Electroantennogram
EI	Electron Ionization
FAO	Food and Agriculture Organization
FID	Flame Ionisation Detector
GC-EAD	Combined gas chromatography and electroantennographic detection
GC-MS	Gas Chromatography-Mass Spectrometry
GOBP	General Odorant Binding Protein
IR	Ionotropic Receptor
IRS	Indoor Residual Spray
ITN	Insecticide Treated Net
IVM	Integrated Vector Management
LC-MS	Liquid Chromatography-Mass Spectrometry
LDP	Low Density Polyethylene
LLIN	Long-Lasting Insecticidal Net

MLB	Mosquito Landing Box
MM-X	Mosquito Magnet – X
OBP	Odorant Binding Protein
OR	Odorant Receptor
ORN	Olfactory Receptor Neuron
OBET	Odour-Baited Entry Trap
PBP	Pheromone Binding Protein
PMD	p-Menthane-3, 8-diol
RT	Retention Time
SPME	Solid Phase Microextraction
SFC/MS	Supercritical Fluid Chromatography/Mass Spectrometry
SIT	Sterile Insect Technique
TMA	Trimethylamine
VOC	Volatile Organic Compound
WHO	World Health Organization
ZM-521	Melkassa-2 (ZM)-521

Abstract

Malaria is a mosquito-vectored infectious disease, which causes serious human health problems in many endemic areas. The dominant malaria vectors in sub-Saharan countries are *Anopheles gambiae, Anopheles coluzzii, Anopheles funestus* and *Anopheles arabiensis*. Of these, *An. arabiensis* is the most widely distributed, versatile and opportunistic vector. The distribution of this vector is frequently correlated with a preference for larval habitats associated with natural and domesticated grasses (Poaceae). Over the past ten thousand years, humans have successfully cultivated grasses and altered the landscape, creating *An. arabiensis* favourable environments that contain excellent habitats for both larvae and adults. This may indicate that a pre-existing adaptation or preference by *An. arabiensis* for grass habitats may have co-evolved with human agriculture; to create highly suitable conditions for both the vector and malaria transmission.

The distribution and dispersal of *An. arabiensis* among various larval habitats is mainly driven by the choice of the gravid mosquito for an egg-laying site. To maximise the fitness of their offspring, female mosquitoes should carefully search for habitats with high nutriment, minimal competition and reduced risk of predation. Selection of oviposition site impacts on the survival and development stages of the immature mosquitoes, since larvae are minimally mobile and mature without parental care. The larval habitats also affect adult density, distribution and vectorial capacity. Several cues from the breeding site, such as visual, tactile and olfactory signals, assist gravid mosquitoes to select a suitable oviposition site. Among these, the olfactory cues play a major role in the breeding site selection process, similar to that which has been documented for in other fitness-related activities, such as blood host seeking and sugar feeding.

However, few studies have documented the odour-mediated oviposition behaviour of malaria vector mosquitoes.

The present studies investigated the effect of olfactory cues emanating from breeding sites associated with the domesticated grasses, rice, maize and sugarcane, on the oviposition preference of *An. arabiensis*. The volatile compounds associated with the plants and pollen of these grasses were collected by headspace volatile collection, from the above-ground plant tissues of two cultivars of rice (MR1 and MR2), and the pollen of two cultivars of both maize (Bako hybrid-660 and Melkassa 2-521) and sugarcane (Coll-48 and EAK 71-402). Behavioural responses of gravid *An. arabiensis* to the collected headspace volatile extracts were tested under laboratory conditions, using a two-port olfactometer and a two-choice oviposition bioassay. Headspace volatiles from both cultivars of rice plants as well as maize and sugarcane pollen attracted gravid *An. arabiensis* and stimulated oviposition. However, the response of the mosquitoes to the volatiles of each crop differed, indicating that females prefer the volatile profiles of specific cultivars. Through combined gas chromatography and electroantennographic detection (GC-EAD) analyses of the headspace volatiles extracts of MR3 rice, BH-660 pollen and sugarcane pollen revealed eight, five and seventeen bioactive compounds, respectively. These compounds were identified using combined gas chromatography and mass spectrometries (GC-MS), revealing that only α-pinene and nonanal were shared among each cereal. Synthetic blends of MR3 rice, BH-660 pollen and sugarcane pollen odour elicited significant attraction and oviposition stimulation of gravid *An. arabiensis*. Subtractive blends were inferior to that of the full synthetic blends. The synthetic blend of MR3 rice was also tested under semi-field

conditions showing gravid *An. arabiensis* are significantly attracted to the full blend when compared to a solvent control.

The results show that gravid *An. arabiensis* prefer oviposition sites associated with domesticated grasses and that this selection is driven by olfactory cues. Synthetic blends were demonstrated to elicit the complete behavioural repertoire of gravid *An. arabiensis* mosquitoes. The synthetic blends identified in the present studies are novel for malaria vectors and show great potential to be integrated with other components of Integrated Vector Management (IVM).

Key words: *An. arabiensis*, attraction, stimulation, cultivars, gravid, oviposition.

Chapter I

General Introduction

1.1. Global malaria transmission dynamics

Malaria is the main mosquito-borne human parasitic disease worldwide, primarily affecting people in tropical and sub-tropical regions. An estimated 3.2 billion people in 97 countries are at risk of malaria infection and malaria-associated diseases (World Malaria Report, 2015), of which 1.2 billion are at high risk of morbidity (World Malaria Report, 2014; 2015). In 2014, an estimated 198 million malaria cases and 584 000 deaths occurred globally, with similar numbers reported for 2015; 214 million malaria cases and 438 000 deaths (World Malaria Report, 2015). Most of these cases and deaths are estimated to have occurred in Africa (88% cases, 90% deaths), followed by South-East Asia (10% cases, 7% deaths) and the Eastern Mediterranean region (2% cases and deaths). In Africa, 80% of the casualties occurred in 15 countries, among which Democratic Republic of Congo and Nigeria accounted for more than 35% of the global total of estimated malaria deaths (World Malaria Report, 2015).

Malaria also has a tremendous effect on the socio-economic development of many countries. In sub-Saharan Africa, the disease is estimated to have cost nearly US$ 300 million annually since 2001, for case management only. In 2014, about 77% was spent on resources used for patient care service delivery and 23% on commodities for diagnosis and treatment (World Malaria Report, 2015). In the developing world, malaria continues to have serious negative impacts on productivity, and this has caused disastrous effects on agriculture through the loss of labor,

knowledge and assets. Moreover, agricultural activities can aggravate the risk of malaria and are implicated in causing some of the socio-economic burden by creating suitable breeding site for malaria vectors (Kebede *et al*., 2005; Mwangangi *et al*., 2008; Jaleta *et al*., 2013). In addition, the proximity of villages to fields increases the chance of biting by vector mosquitoes and thereby transmission of the diseases (Ijumba and Lindsay, 2001).

Globally, there has been a reduction of malaria cases by 18% and mortality by 48% since 2000 (World Malaria Report, 2015). In sub-Saharan African countries, mortality due to malaria has since declined 71% among children under five and 66% among all age groups. However, despite a global strategy for malaria control, billion of lives in the world are still at risk due to the increase in density and distribution of malaria vectors (World Malaria Report, 2015). A number of factors contribute to the increase and expansions of malaria vectors, of which, the development of insecticide resistance (Ranson *et al*., 2011; Trape *et al*., 2011), expansion of agriculture (Kebede *et al*., 2005) and climate change (Ramasamy and Surendran, 2012; Srinivasulu *et al*., 2013) are among the most important.

The intensive use of a chemical-based strategy resulted in the development of insecticide resistance and resurgence of vectors (Ranson *et al*., 2011; Zhou *et al*., 2011; World Malaria Report, 2015). Vector density and distribution have also increased in response to agricultural activities. This is due to the availability of larval nutrients and breeding grounds provided by agricultural fields (Lacey and Lacey, 1990; Kebede *et al*., 2005; Mwangangi *et al*., 2008; Jaleta *et al*., 2013). Moreover, climate change, which affects temperature, rainfall and humidity, has created ideal conditions for vector mosquitoes to breed and transmit malaria (Ramasamy and

Surendran, 2012; Beck-Johnson *et al*., 2013), which now expand into new areas where they exacerbate conditions in regions with endemic malaria (Ramasamy and Surendran, 2012; Wimberly *et al*., 2012; Beck-Johnson *et al*., 2013).

The use of multiple tools and strategies can impact the malaria transmission cycle, creating a more successful overall strategy (Beier *et al*., 2008). Choices about vector control are best considered with reference to a hierarchy of interventions. This hierarchy follows a historic development of vector control methods and begins with environmental management strategies, which if effective in the local setting, can yield the most sustained long-term benefits at the lowest cost (Barik *et al*., 2008). Other methods, that offer higher impact, more partial, limited or shorter-term solutions, can then cumulatively be added until the target or threshold set for reduction of disease transmission levels is met. One such method is biological control, which primarily is used against larval stages (Bale *et al*., 2008). Over the last two decades, adult vectors have been controlled using indoor residual sprays (IRSs) and long-lasting insecticide-treated nets (LLINs), two intervention methods that specifically target against indoor host seeking and/or resting mosquitoes (World Malaria Report, 2015). These methods alone have reduced malaria cases worldwide by up to 78% (World Malaria Report, 2015). Future vector interventions based on the use of LLINs or IRS are, however, in jeopardy, due to shortage of funds, the development of insecticide-resistance (Enayati *et al*., 2010), the increasing importance of outdoor-biting vector populations, as well as the heritable and plastic changes in vector behaviour in response to malaria vector control interventions (Russell *et al*., 2011; Killeen *et al*., 2014).

The use of traditional control strategies singly for intervention of vector control has paved the way for the vector to adapt and come up with new mechanisms of resistance. These have been documented in different species of mosquitoes, including *An. gambiae* (Protopopoff *et al*., 2013), *An. arabiensis* (Russell *et al*., 2011; Killeen *et al*., 2014), *An. funestus* (Riveron *et al*., 2015), *Cu. quinquefasciatus* (Yadouléton *et al*., 2015) and *Ae. aegypti* (Kasai *et al*., 2014). This provides the motivation for new malaria interventions that target other aspects of the mosquito biology, including oviposition site selection, searching for sugar sources, mating, outdoor feeding and resting. Since several of these behaviours are mediated by olfaction, one novel way to reduce the interaction between vectors and humans is to interrupt the odour-mediated behaviours of the mosquitoes (Michaelakis *et al.*, 2007; Nikbakhtzadeh *et al*., 2014; van Loon *et al*., 2015). So far, most research has focused on developing attractants from human and non-human hosts of the malaria mosquitoes to be used for the management of blood-seeking female mosquitoes (Okumu *et al*., 2010; Nikbakhtzadeh *et al*., 2014; van Loon *et al*., 2015; Webster *et al*., 2015). An area of research that seeks more attention is the oviposition behaviour of these *Anopheles* mosquitoes. By targeting mosquitoes that search for a site to lay their eggs it should be possible to target mosquitoes that have taken their blood meal either indoors or outdoors, increasing the number of females removed from the local population compared to the indoor-centric control methods currently in use. In this thesis I will present the behavioural and physiological responses of gravid *An. arabiansis* to olfactory cues emanating from different domisticated grasses including maize, rice and sugarcane. I will also show the effect of different cultivars or development stages of these grasses on attracting gravid malaria vectors. The odours identified in this thesis could be used for sustainable and fruitful management of *An. arabiansis*.

1.2. Objectives

1.2.1. General objective

Development of a novel and environmentally safe method for malaria vector control, by investigating the behavioural and electrophysiological response of gravid *Anopheles arabiensis* Patton to domesticated grass crop volatiles (maize, rice and sugarcane) (Poaceae).

1.2.2. Specific objectives

- To evaluate attraction and oviposition responses of gravid *Anopheles arabiensis* to BH-660 and ZM-521 cultivars of maize pollen volatiles under laboratory conditions
- To determine attraction and oviposition responses of gravid *Anopheles arabiensis* to volatiles of MR1 and MR3 rice cultivars at tillering, booting and flowering stages under laboratory conditions
- To investigate attraction and oviposition responses of gravid *Anopheles arabiensis* to volatile compounds of Coll-48 and EAK 71-402 sugarcane cultivars under laboratory conditions
- To identify volatile compounds from maize, rice and sugarcane cultivars through behavioural and electophysological responses of gravid *Anopheles arabiensis*
- To evaluate and compare the attraction of gravid *Anopheles arabiensis* mosquitoes to identified bioactive compounds from rice under semifield conditions

Chapter II

Literature Review

2.1. Malaria parasites: prevalence and transmission

Malaria is caused by *Plasmodium* parasites in the family Plasmodiidae, and was first identified in 1907 with the independent discoveries of *P. cynomolgi*, *P. inui*, and *P. pitheci* in monkeys (Garnham, 1966). The family comprises more than 100 species that are found in the blood of reptiles, birds and mammals (Gilles and Warrell, 1993). In humans, malaria is caused by four well-characterised *Plasmodium* species, *P. falciparum, P. vivax*, *P. malariae* and *P. ovale*. In addition, human cases of malaria due to *P. knowlesi* have also been recorded in certain forested areas of South-East Asia. *Plasmodium knowlesi* causes malaria among monkeys and is transmitted to humans when an *Anopheles* mosquito first bites an infected monkey and then a human (White, 2008). *Plasmodium falciparum,* together with *P. vivax,* are the predominant species, accounting for about 95% of all malaria infections (Vangapandu *et al*., 2007). *Plasmodium falciparum* is responsible for most deaths in the endemic malaria parts of the world, except for India and South America, where *P. vivax* is more common (Ashley *et al.,* 2006; World Malaria Report, 2015). *Plasmodium vivax* has a wider distribution than *P. falciparum*, since it can develop in its *Anopheles* hosts at lower temperatures and can survive in wider environmental conditions (Gilles and Warrell, 1993). Malaria caused by *P. vivax* occurs in South-East Asia (74%), the eastern Mediterranean region (11%) and Africa (10%). More than 80% of *P. vivax* malaria cases are estimated to occur in Ethiopia, India and Pakistan (World Malaria Report, 2015). The risk of infection with *P. vivax* in Africa is low because of the absence of the Duffy antigen in many human populations (Zhao *et al*., 2012). The Duffy blood group antigen serves,

not only as a blood group antigen, but also as a receptor for a family of pro-inflammatory cytokines, termed chemokines, and as a receptor for *P. vivax* malaria parasites (Mercereau-Puijalon *et al*., 2010). The distribution of *P. malaria* coincides with that of *P. falciparum* worldwide, and this species causes a long lasting and chronic infection. *P. ovale* is prevalent in West African countries, New Guinea, the eastern part of Indonesia and the Philippines, causing tertian malaria (Walker *et al.,* 2009).

The infection by the malaria parasites causes intense mortality in endemic and epidemic-prone areas of the world, partly due to the resistance of the parasites to many anti-malarial drugs, particularly chloroquine and sulphadoxine-pyrimethamine (Kokwaro, 2009). Resistance occurs when parasites are exposed to a lower than lethal dose of the drug, as a result of poor treatment practices, inadequate patient adherence to prescribed anti-malarial regimens and substandard forms of anti-malarial medicines. Resistance against many drugs have been reported in *P. falciparum*, *P. vivax* and *P. malariae* (World Malaria Report, 2014; 2015). Although artemisinin-based combination therapies (ACTs) have been shown to be effective against all malaria parasites, parasite resistance to artemisinin has already been identified in Southeast and is spreading (Gueye *et al*., 2014). New drugs need to be developed as the parasites have a high potential to develop resistance to these anti-malarial drugs.

2.2. Malaria vectors

Infected female mosquitoes of the genus Anopheles transmit malaria. There are approximately 537 recognised species of *Anopheles*, of which 41 are vectors of epidemiological importance (Harbach, 2013). The severity of malaria transmission by the *Anopheles* vector species is highly

dependent on the vector ovipostion site selection, density, feeding behaviour, resting site choice and distribution (Gilles and Warrell, 1993).

The principal malaria vectors in Sub-saharan Africa are members of the *An. gambiae* and *An. funestus* species complexes. The *An. gambiae* species complex contains *An. gambiae sensu stricto, An. coluzzii, An. arabiensis*, *An. bwambae, An. melas, An. quadriannulatus*, *An. amharicus* and *An. comorensis* (Coetzee *et al*., 2013). Of the *An. gambiae* complex mosquitoes, *An*. *gambiae* sensu stricto*, An*. *coluzzii and An. arabiensis* are the most efficient malaria vectors (Sinka *et al*., 2012; Harbach, 2013). The *An. funestus* species complex is also diverse, comprising nine sibling species, *An. parensis*, *An. aruni*, *An. confusus*, *An. funestus*, *An. vaneedeni*, *An. rivulorum*, *An. fuscivenosus*, *An. leesoni* and *An. brucei* (Gillies and Coetzee, 1987). Of these, *An. funestus s.s.* is the predominant and principal vector species, both in numbers and geographical distribution (Gillies and Coetzee, 1987; Sinka *et al*., 2012; Harbach, 2013).

Mosquitoes display a holometabolous life cycle that consists of four stages, egg, larva, pupa and adult. The four larval instars are aquatic and most species have been found to feed actively on algae, yeast, bacteria, organic matter, small aquatic organisms and detritus (Merritt *et al*., 1992). The reserve deposits of fat and glycogen carried over from the larval stage serve as sources of energy for flight, reproductive fitness, survival, longevity and bigger size of adult mosquitoes (Gary and Foster, 2001; Manda *et al*., 2007). After adult emergence, both male and female mosquitoes feed on sugar sources (*e.g.* nectar, honeydew) for their immediate energy requirements (Gary and Foster, 2004). Males continue to feed on sugar for the remainder of their

life cycle; however females of most mosquito species require a blood meal to lay a first batch of eggs. After obtaining a blood meal and egg development (2-3 days in the tropics), females search for a suitable breeding site and oviposit 50-200 eggs at a time depending on the mosquito species. The nutritional status and insemination of mosquitoes influences its ovipositional behaviour (Bentley and Day, 1989). Oviposition in mosquitoes occurs in two stages (Clements, 1999): an initial pre-oviposition behaviour followed by oviposition. The pre-oviposition behaviour has two phases: an initial behavioural response of gravid females that result in the arrival at potential oviposition sites. For mosquitoes to orient toward (attraction) the ovipostion site, they use olfactory and visual cues. Subsequently, gravid female evaluate the suitability of the site and ultimately accept to lay her eggs (stimulation). For many mosquito species, selection of oviposition sites occurs during twilight. The oviposition preference differs between species, and is the topic of ongoing research (Himeidan *et al*., 2013; Afify and Galizia, 2015).

Anopheles arabiensis is the main vector of malaria in Ethiopia, and one of the dominant malaria vectors associated with agricultural ecosystems (Kebede *et al*., 2005; Jaleta *et al*., 2013). The association with agricultural crops, particularly with rice, maize and sugarcane, in sub-Saharan African countries, including Ethiopia, Burundi, Cameroon, Kenya, Sudan and Tanzania, is well documented (El Gaddal *et al*., 1985; Audibert *et al*., 1990; Boudin *et al*., 1992; Ijumba *et al.,* 2002; Kebede *et al*., 2005; Mwangangi *et al*., 2008; Muturi *et al*., 2008; Jaleta *et al*., 2013). Cultivation of rice, maize and sugarcane provides breeding sites and nutrients directly from the plant (*e.g.* pollen) and indirectly from microorganisms for mosquito larvae (Lacey and Lacey, 1990; Ye-ebiyo *et al*., 2000; Kebede *et al*., 2005; Jaleta *et al*., 2013).

2.3. Factors affecting the distribution of malaria vectors

The main factors regulating malaria transmission are the density of hosts, vectors and parasites in a geographic area. Any change in the environment, including climate change and agricultural expansion can alter the density of hosts, vectors and parasites and contribute to the emergence and spread of malaria transmission (McMichael *et al*., 1996).

2.3.1. Change in climate conditions

Climate change, much of which is caused by alterations in the environment by anthropogenic activities, greatly affects the life of humanity itself and other organisms. The primary impacts of climate change are an alteration in atmospheric temperature, rainfall and humidity that affect the biology and ecology of disease vectors and consequently increase the risk of disease transmission (Srinivasulu *et al*., 2013).

Malaria vectors and parasites have an optimal range of temperature for survival and development. If the temperature is above or below the optimal, it will have a negative impact on the survival of the vectors and the parasites (Srinivasulu *et al*., 2013). However, a limited rise in temperature within the optimal range increases the rate of vector development and the density, as well as the human biting rate (Martin *et al*., 2008). In addition, malaria parasites will complete their extrinsic incubation within the female mosquito in a shorter time and thereby increase the proportion of infective vectors (Hay *et al*., 2000). Due to increasing temperature, as a result of climate change, the distribution of malaria mosquitoes has increased to areas where they have not been observed before. This includes the highlands of sub-tropical and temperate zone countries (Ramasamy and Surendran, 2012; Wimberly *et al*., 2012; Beck-Johnson *et al*., 2013).

The global climate change also affects rainfall, which has a direct effect on humidity. Humidity is the amount of water vapour in the air, which has a significant role in the survival of the vector mosquitoes. An optimal humidity significantly increases mosquito survival, however if the humidity is below or above the optimal it is known to decrease the lifespan of mosquitoes (Ramasamy and Surendran, 2012). Increased rainfall may increase the presence of disease vectors by expanding the size of existent larval habitats and by creating new breeding grounds. This explains why there is, most often, a peak in malaria transmission just after the rainy season in tropical countries (Ramasamy *et al*., 1992). However, excessive rainfall can wash away the immature stages of the vector temporarily and thereby lower the rate of malaria transmission (Ramasamy and Surendran, 2012).

2.3.2. Expansion of agricultural activities

The spread of vector borne diseases, particularly malaria, has co-evolved with humans, but malaria has only had a significant effect on humans in the past 10000 years, after the advent of agriculture, animal domestication and increased population densities (Livingstone, 1971). The slash and burn agriculture in West Africa 2000-4000 years ago resulted in the clearing of tropical forests and an increase in sunlit pools of water, the preferred breeding place of *An. gambiae* (Livingstone, 1971). Today, the vector density and distribution is increasing dramatically as a result of expansion of agriculture to feed the ever-growing population (Kebede *et al*., 2005; Mwangangi *et al*., 2008; Jaleta *et al*., 2013). Agricultural activities in most of the developing countries are small hold farms that are usually located near the household. Hence, suitable breeding sites, nearby hosts, are created for the vectors, which are suitable for enhanced survival

and transmission of the disease (Ye-ebiyo *et al*., 2000; Kebede *et al.,* 2005; Mwangangi *et al*., 2008; Jaleta *et al*., 2013). As a result, crop irrigation is blamed for increasing the malaria vector population and transmission by prolonging the breeding season and providing nutrients for immature vectors (Ye-ebiyo *et al*., 2000; Ijumba and Lindsay, 2001; Kebede *et al.,* 2005; Mwangangi *et al*., 2008).

Wild grasses (Poaceae) are commonly associated with natural larval habitats for malaria mosquitoes (Sinka *et al*., 2010). However, the most productive larval habitats are irrigated cultivations of domesticated grasses, including rice, maize and sugarcane, creating a spatial link between the vector and the people, and thereby increasing the risk of malaria transmission (Kebede *et al*., 2005; Mwangangi *et al*., 2008; Mwangangi *et al*., 2010; Jaleta *et al*., 2013). The grasses provide the larvae with shelter from abiotic stress and biotic threats, as well as nutrients, directly from shed pollen, and indirectly from the rhizosphere, supporting edible microorganisms (Lacey and Lacey, 1990; Ye-ebiyo *et al*., 2000; Mwangangi *et al*., 2006).

2.3.2.1. Rice cultivation

Rice (*Oryza*) is the second most productive agricultural commodity worldwide, both in metric tonnes and income produced (FAO, 2012). As such, rice is the leading staple cereal crop providing food and economic income for more than half of the world's population, with ca. 90 % produced and consumed in Asia, and the remainder distributed between Africa, South and Central America. Rice is predominantly produced using irrigation (ca. 75 %) to expand the crop cultivation into areas with irregular rainfall, as well as into arid and semi-arid regions (Keiser *et al*., 2002). While increasing rice crop productivity through the use of irrigation undoubtedly

enhances food security, its cultivation creates ideal environments for disease vector mosquitoes (Lacey and Lacey, 1990; Muturi *et al*., 2006; Mwangangi *et al*., 2006; Mwangangi *et al*., 2010), which poses a significant risk to human health.

Vector mosquito-rice associations have been long recognised. In the 17th century, rice cultivations in Spain, Portugal, Czech Republic and North America maintained a high vector population, with a reported high transmission of malaria (Sharma *et al*., 1994). Today, rice cultivations provide a favourable habitat for at least 140 mosquito species worldwide, with *Anopheles*, comprising 89 of the species, being the most dominant genus (Lacey and Lacey, 1990). It is estimated that about 25% of the *Anopheles* species of epidemiological importance thrive in rice fields, resulting in elevated human health risks in many parts of Africa, Asia and South America (Lacey and Lacey, 1990). For instance, there are recorded proliferations of vector *Anopheles* species associated with rice irrigation, including *An. arabiensis*, *An. gambiae s.s.* and *An. funestus* in Africa (Muturi *et al*., 2006; Diuk-Wasser *et al*., 2007; Mwangangi *et al*., 2008; Mwangangi *et al*., 2010), *An. annularis* in Sri Lanka (Ramasamy *et al*., 1992), *An. campestris* in Indonesia (Walsh *et al*., 1993) and *An. albitarsis s.l.* in southeastern Brazil (Forattini *et al*., 1994).

Compared with cultivations of other domesticated grasses, rice cultivations have the highest demonstrated larval density and malaria transmission (Ijumba and Lindsay, 2001; Muturi *et al*., 2006; Muturi *et al*., 2010). A possible reason for this is that each phenological stage of rice requires water, which creates breeding sites for mosquitoes and other organisms (Lacey and Lacey, 1990; Mwangangi *et al*., 2006; Mwangangi *et al*., 2010). In addition, the application of nitrogen fertilisers and insecticides may also increase the density of immature vectors. The

application of nitrogenous fertilisers enhances available larval food resources by increasing detritus and microbial communities (Mutero *et al*., 2004). Mutero *et al*. (2004) also reported that ammonium sulphate fertiliser reduces turbidity of water in rice fields thereby making them visually more attractive for ovipositing *An. arabiensis*. The application of insecticides can create an enemy free habitat for larvae by decreasing the population of predators and competitors (Service, 1977), particularly when the populations of *Anopheles* mosquitoes are, themselves, resistant to the insecticides (Ranson *et al*., 2011; Trape *et al*, 2011; Toé *et al*., 2014). This is due to the intense selective pressure caused by the exposure of females to these insecticides within houses (IRS and LLINs; Ranson *et al*., 2016) and the ability of the mosquito larvae to develop resistance with much faster rate than their enemies (Service, 1977).

2.3.2.2. Maize cultivation

Maize (*Zea*), also called corn, ranks third among cereal crops in the world following wheat and rice (FAO, 2012). Maize is believed to have originated from central Mexico and has become one of the most versatile crops grown under broad geographical and environmental conditions. The major maize production regions are located in the temperate regions of the United States, China, Brazil and Mexico. However, maize is also produced in significant quantities in India and Central America, as well as in Southern and Eastern Africa (Ranum *et al*., 2014). In Africa, maize was introduced after 1500 BC and 95 % of the production is consumed by humans, rather than being used as livestock feed or industrial raw material as it is in other parts of the world. In Ethiopia, maize is a major staple food, and is largely produced and consumed by small-scale farmers that comprise 80% of Ethiopia's population. However, maize cultivation is creating ideal

breeding sites for malaria vectors, negatively impacting on the human population (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a; Kebede *et al*., 2005; McCann, 2005).

Similar to rice, maize cultivation is associated with a high density of vector mosquitoes as well as a high malaria transmission (Kebede *et al*., 2005; Muturi *et al*., 2006; Mwangangi *et al*., 2008). A leading cause for the increase in the density of the vector, besides the irrigation-created breeding water, is nutrients that the mosquito larvae acquire from maize pollen (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a; Kebede *et al*., 2005; McCann, 2005). Maize pollen is produced in large quantities by numerous florets and each tassel produces 2–5 million pollen grains over approximately 8 days (Hoeft *et al*., 2000). Maize pollen grains are relatively larger (90-100 μm) than those of other grasses, are dispersed by wind and can be carried over a distance of up to 60 m (Raynor *et al*., 1972). The pollen contains sugars and relatively high levels of starch, proteins, lipids, phosphorus, potassium and other inorganic minerals (Roulston and Buchmann, 2000). The pollen may provide an important source of nutrients for larvae of mosquitoes, as shown for *An. arabiensis* (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a). *Anopheles arabiensis* larvae that fed on maize pollen, particularly the BH-660 cultivar, had an advantage of increased size, reproductive fitness, longevity and resistance to insecticides as an adult when compared to those that fed on microorganisms alone (Ye-ebiyo *et al*., 2000; Oliver and Brooke, 2013). Consequently, high levels of malaria transmission have been correlated with BH-660 maize cultivation areas (Kebede *et al*., 2005; McCann, 2005).

2.3.2.3 Sugarcane cultivation

Sugarcane belongs to the genus *Saccharum* and it is believed to have been established as a domestic garden crop possibly as early as 2500 BC (Daniels and Roach, 1987). It is the top produced agricultural commodity by metric tonnes globally and is most notably used for sugar and alcohol production worldwide, especially in the tropics (FAO, 2012). Sugarcane is widely cultivated through irrigation, and like the other domesticated grasses discussed above; large-scale sugarcane irrigation schemes strengthen the existing connection between rainfall and the risk of malaria outbreak (Jaleta *et al*., 2013). Moreover, sugarcane cultivations that lack the proper maintenance of the irrigation canals, vector monitoring and IVM can result in an increase of the vector population (Ijumba and Lindsay, 2001) resulting in a higher risk of malaria transmission in sugarcane-irrigated agro-ecosystems, as has been reported in Wollega, Ethiopia (Jaleta *et al*., 2013), Miwani, Kenya (Githeko *et al*., 1993) and Mvuleni, Tanzania (Ijumba *et al*., 2002).

2.4. Malaria vector control interventions

Malaria control today largely relies on the interruption of the disease transmission cycle by either targeting the mosquito larvae through the source reduction of breeding sites or by killing the adult mosquitoes using indoor-applied insecticides (Yohannes *et al*., 2005; World Malaria Report, 2014; 2015). This also appears true for many populations of *An. arabiensis*, for which the combination of insecticide treated nets and indoor residual spraying, have been found to be effective control measures (West *et al*., 2014; World Malaria Report, 2015). However, in some studied populations, the resistance of *An. arabiensis* to many insecticides and a change of its behaviour from endophilic and endophagic (i.e. indoor resting and feeding) to exophilic and exophagic (i.e. outdoor resting and feeding) have been reported (Yewhalaw *et al*., 2011; Kitau *et*

al., 2012; Okumu *et al*., 2013). Such a behavioural switch exemplifies the limited effectiveness of existing approaches alone, since residual malaria transmission can be maintained by vectors that feed and rest outdoors, as well as by vectors feeding on non-human mammals (Killeen *et al*., 2014).

2.4.1. Chemical insecticides

Chemical control utilizes natural or man-made compounds (insecticides) to reduce the mosquito population at larval and adult stages to tolerable levels. Larvicides can help to reduce the overall mosquito burden by limiting the number of adult mosquitoes produced, under conditions where the majority of larval habitats are identified and treated (Keiser *et al*., 2005). For control of mosquito larvae, different chemicals, such as petroleum oils, Paris green (copper acetoarsenite), monolayer surface films, DDT, organophosphate-based larvicidal formulations, synthetic pyrethroids and insect growth regulators are commonly employed (Kamareddine, 2012). These larvicides are highly persistent and have non-target effects on aquatic non-target organisms as well as on mammals, and a low effectiveness due to selective pressure for resistance (WHO, 2006). Instead, organophosphorus compounds, such as carbamates, pyrethroids and Temephos (Abate), are recommended as larvicides as these are less persistent, break down quickly in the environment and are relatively safe for non-target organisms. The application of larvicides, such as fenthion (Baytex™, an organophosphorus larvicide) or triflumuron (Starycide™, a benzoylphenylurea insect growth regulator) to breeding sites plays an important role in controlling *Cu. quinquefasciatus* (Mathew and Kalyanasundaram, 2004).

For adulticides, insecticides, including pyrethroids, organophosphates and carbamates, are used as indoor residual spray (IRS). In 2014, IRS protected 116 million people globally (World Malaria Report, 2015), with more than 75% of the population at risk in Cape Verde, Gambia, Sao Tome, Principe and Zimbabwe (World Malaria Report, 2014). Even if IRS was not fully implemented in large parts of sub-Saharan Africa, the evidence over several decades has confirmed the effectiveness of the control in reducing the level of infection and incidence of malaria in these areas. The combined use of IRS together with LLINs caused a remarkable decline in the malaria burden during the last decade in Africa (World Malaria Report, 2015). However, the use of IRS and LLINs is limited, as they only target mosquitoes that feed and rest indoors (Killeen *et al*., 2014). Moreover, an increasing number of studies show that mosquitoes have developed resistance to the substances used (Ranson *et al*., 2011; World Malaria Report, 2014). As a result, existing insecticides should be replaced with new ones, which have yet to be developed. Even replacement will not solve the problem, but only delay it, as most insects are able to overcome an insecticide within 10 to 15 years of its introduction on the market (Yu *et al*., 2015). Hence, to overcome the limitation caused by chemical control, alternative control strategies need to be developed.

2.4.2. Non-chemical interventions

2.4.2.1. Environmental modification and source reduction

Environmental modification is the physical transformation of mosquito habitats to reduce larval development and survival (WHO, 1982). The environmental modification activities include flooding or draining of natural or artificial wetlands, the regulation of water levels in reservoirs, intermittent irrigation to agricultural fields (particularly rice) and changing water salinity (WHO,

1982; Rafatjah, 1988). Moreover, growing of shade vegetation or trees near breeding habitats also helps to reduce the abundance of mosquito vectors (Rafatjah, 1988). This method has been successfully implemented in large-scale interventions in Panama, Italy, Malaysia, Indonesia, the Tennessee Valley in the US and the Zambian copper belt (Laumann *et al*., 2010). For effective control of mosquito larvae, locally derived ecological concepts are adapted and applied (Knight *et al*., 2003). Yohannes *et al.* (2005) reported that source reduction of breeding habitat of mosquitoes by the community, resulted in a 49% reduction in *An. arabiensis* in the Tigray region. However, many modifications, including road, dam or pipeline construction, deforestation and irrigation can generate larval breeding sites if such projects are improperly designed and maintained (Knight *et al*., 2003).

2.4.2.2. Biological control

Biological control is a sustainable management strategy that utilizes one or more types of natural enemies to achieve an effective mosquito control (Bale *et al*., 2008; Bukhari *et al*., 2013). These control methods include microbial agents, such as bacteria, fungi, protozoans and viruses. Among the microbial agents, the bacteria *Bacillus thuringiensis israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) (Charles and Nielsen-LeRoux, 2000; Kamareddine, 2012), fungal species belonging to the genera *Coelomomyces*, *Culicinomyces*, *Beauveria*, *Metarhizium*, *Lagenidium*, *Entomophthora* (Ondiaka *et al*., 2015), protozoans, including *Vavraia culicis* and *Nosemia algerae* (Yap, 1985), and Densonucleosis viruses (Carlson *et al*., 2006) have all been found to be effective control agents (Kamareddine, 2012). Moreover, organisms such as nematodes, cyclopoid copepods, predacious aquatic insects and larvivorous fish are used for mosquito control (Knight *et al*., 2003; Kamareddine, 2012). Of these, the predatory fishes, *Gambusa*

affinis and *Tilapia nilotica*, are among the widely used biological control methods employed against mosquito larvae. These fishes have been used in Ethiopia, North America, Palestine, Israel, Italy and India to control malaria vectors (Chandra *et al*., 2008). In Pondicherry town, *Gambusia affinis* caused a 98% reduction in larval density of *An. stephensi* (Menon *et al*., 1978). Although biological control agents offer a sustainable solution, the methods are limited as the effect of the natural enemies or toxoids is slow and can cause resistance (*e.g.* fungi, bacterial toxins) (Bale *et al*., 2008; Wirth, 2010; Cory and Franklin, 2012).

2.4.2.3. Personal protection

There are many measures that can be taken as a personal protection against mosquito bites. These measures include air-conditioning, screens, insecticide-treated mosquito nets (ITNs), covering exposed parts of the body during biting periods and using repellents (Schoepke *et al*., 1998). Of these measures, ITNs have been regarded as the most powerful malaria control tool of endemic regions from 2001 to 2015, with a decline of malaria 69% (World Malaria Report, 2015). In sub-Saharan Africa, about 82% of those with access to an ITN utlize it for protection. Bed nets are available as LLINs and conventional nets treated with pyrethroids (permethrin, deltamethrin and lamdacyhalothrin). The conventional treated nets need regular treatment, while LLINs retain its biological activity for at least 20 WHO standard washes under laboratory conditions and three years of recommended use under field conditions. In Africa, this tool is effective against *An. gambiae s.s.* and *An. funestus s.l.,* which prefer to bite at night when people are in bed. However, the control of mosquitoes by treated nets is limited, since they only target mosquitoes that are endophagic and/or endophilic and is only effective if humans are underneath at the time of the vectors' biting activity. Hence, ITNs are ineffective in controlling exophagic or

exophilic vectors, such as *An. arabiensis*, and early-feeding vectors, such as *An. rivulorum* (Ranson *et al*., 2011). Moreover, mosquitoes have been shown to develop resistance to impregnated insecticides (*e.g.* pyrethroids), (Ranson *et al*., 2011) and the poor ventilation inside the net makes it uncomfortable to be used in warmer areas.

Repellents are another method of personal protection that prevents mosquitoes from approaching or settling (Mng'ong'o *et al*., 2011). Repellents delay the emergence of insecticide resistance and reduce the toxic effects of chemicals to human and non-target organisms. The repellents create a vector-free area both in- and outdoors depending on efficacy and application modality. Repellents can be used outdoors, in the early evenings and in the mornings in places where IRS and LLINs cannot be used (Sangoro *et al*., 2014). These repellents are either natural or synthetic compounds. Natural repellents have been used for centuries as a personal protection measure against blood feeding arthropods. These repellents are still used traditionally, either applied to the skin or clothing, or released into the air through evaporation or burning. Some examples of natural plants used for repelling mosquitoes include: *Cymbopogan martini* and *Cy. citratus* (Ansari and Razdan, 1994; Moore *et al*., 2007), *Corymbia citrodora* (Dubé *et al*., 2011), *Eucalyptus camaldulensis* (Dugassa *et al*., 2009), *Lippia javanica* (Govere *et al*., 2000), *Ocimum suave* (Dubé *et al*., 2011)*, O. kilimandscharicum* (Kweka *et al*., 2008) and *Juniperus procera* (Karunamoorthi *et al*., 2015). Synthetic repellents are compounds that are produced synthetically to deter mosquitoes. Of these, the synthetic repellent, DEET (*N,N*-diethyl-3-methlybenzamide) is considered the gold standard, which all other repellents are compared against (Leal, 2014). However, p-menthane-3,8-diol (PMD) is a natural compound that have remarkable ability to repel mosquitoes when compared to DEET (Maia and Moore, 2011).

2.4.2.4. Genetic control

Genetic control is a control method, which uses the advancement in biotechnology against malaria vectors. There are two approaches for genetically controlling the population. One of these approaches is the elimination or replacement of the population. This approach includes the oldest and most widely used genetic control method, the sterile insect technique (SIT). The SIT is based on the propagation of sterility in successive generations. The main aim of SIT is to eliminate the population of the vector by minimizing the chance of mating and reproduction (Toure *et al*., 2004). The technique has been applied in El Salvador in the 1970s, and caused more than 97% population reduction of *An. albimanus* (Lofgren *et al*., 1974). Sterility in mosquitoes can also be caused by artificial infection of male mosquitoes with various strains of *Wolbachia* bacteria. These bacteria live within insect cells, as well as some nematodes, and may be transferred from one generation to the next through the eggs. The *Wolbachia* infection can also shorten the lifespan of the mosquitoes, thereby limiting the time available for pathogens to develop and reduce the possibility of an infective bite. *Wolbachia* infected males have successfully been used for sterile-male control of *Culex* and *Aedes* mosquitoes (Alphey *et al*., 2013; Burt, 2014; Atyame *et al*., 2015). Another genetic control approach being investigated is the replacement of wild mosquitoes with transgenic non-biting mosquitoes or mosquitoes that are refractory to the pathogens (Toure *et al*., 2004). For instance, transgenic *An. stephensi* that over-express Akt (a protein that regulates insulin signalling) were 60–99% less likely to be infected with malaria parasites, and had an approximately 20% shorter lifespan than unmodified mosquitoes (Corby-Harris *et al*., 2010). However, the implementation of the transgenic approach faces large technical, public, ethical, legal and social hurdles (McLean and Jacobs-Lorena, 2016).

Hence, it is important to develop new control strategies that can be used together with existing IVM. One way to meet this goal is to strengthen our understanding of the behavioural and chemical ecology of malaria mosquitoes. To date, extensive studies on the host-seeking behaviour of *Anopheles* mosquitoes have resulted in considerable progress towards the identification of odourants from skin emanations of humans and other primary blood meal hosts, which are currently being tested in large-scale field trials (Okumu *et al*., 2010; Nikbakhtzadeh *et al*., 2014; van Loon *et al*., 2015; Webster *et al*., 2015). However, there is limited knowledge about the cues used by gravid *An. arabiensis* to activate, orient, fly upwind, locate and accept an aquatic habitat to lay their eggs (*e.g.* Lindh *et al*., 2015). Hence, the present study deals with the identification of attractants from the mosquitoes' breeding sites within domesticated grasses that can be used, with other control strategies, for management of malaria vectors.

2.5. Semiochemicals and mosquito behaviour

Semiochemicals, also known as infochemicals, are defined as chemical substances or mixtures emitted by an organism that induces a behavioural or physiological response in another organism (Law and Regnier, 1971). For mosquitoes, semiochemicals influence the interaction within (pheromones) and between species (allelochemicals). To date, pheromone communication has only been shown to be involved in the location of oviposition sites for a few mosquito species (Laurence and Pickett, 1982; Mendki *et al*., 2000), and is an area that requires further research. In contrast, allelochemicals have received considerable attention over the years.

Allelochemicals are subdivided into synomones, allomones and kairomones (Gullan and Cranston, 1994). Synomones are chemical substances produced by an individual of one species that benefits both the producer and the recipient, *e.g.* pollination of orchids by mosquitoes when

feeding on nectar (Gorham, 1976). Allomones are chemical compounds that only benefit the emitter (*e.g.* mosquitoes repellents (Karunamoorthi *et al*., 2015)), whereas kairomones are advantageous for the receiver. Kairomones play a significant role in mosquito behaviour as they are used to locate sugar sources, host animals and oviposition sites (Takken and Knols, 1999). Here, emphasis is given to the odour-mediated oviposition behaviour of mosquitoes, as it is one of the important factors for successful survival and growth of the less mobile larvae (Afify and Galizi, 2015).

In mosquitoes, chemical cues from the resources including sugar source, mating have a great role in searching behaviour of mosquitoes (Takken and Knols, 1999). Searching behavior is an active movement of insects to locate the resourse for their development and survival. Mosquitoes searching activities are influenced by spatial and temporal distributions of resources. Due to hierarchical nature of spatial distributions, mosquitoes initially use cues from habitat followed by patch then individual resources. Furthermore, for effective location of resource, searching should be restricting to the most productive times and often these activites depends on endogenous timing or on behavioral mechanisms (Bell, 1990). Mosquitoes locate their resource from long, medium and short distance and for these, chemical cues undoubtly have a major function. An insect oriented up- or cross-wind of the resource and the movement is usually occurs close to the resourse (Short-medium range movement). Orientation and displacement associated with long-rang movement is usually downwind and occurs within the first few tens of meters of the resource.

2.5.1. Sugar feeding behaviour

Adult mosquitoes need to feed on sugar sources, which include floral and extra-floral nectaries, honeydew, tree sap, rotting fruit and leaves damaged by insects, to augment their glycogen and lipid energy reserves at any physiological state (Gary and Foster, 2004). Sugar meals play a significant role in the longevity, fecundity, flight capacity and resource-seeking behaviour of mosquitoes (Gary and Foster, 2001; Manda *et al*., 2007). Mosquitoes use volatile compounds emitted from the host plants, including terpene and non-terpene compounds for locating sugar sources (Knudsen *et al*., 1993; Nikbakhtzadeh *et al*., 2014). These volatile compounds have been identified as attractants in many mosquito species, for instance *An. arabiensis* (Healy and Jepson, 1988), *An. gambiae* (Nyasembe *et al*., 2012) and *Cu. pipiens pipiens* (Mauer and Rowley, 1999).

2.5.2. Mating behaviour

Mating behaviour of mosquitoes is influenced by their physiological state and age as well as environmental conditions. For accurate recognition of potential mates, mosquitoes may use flight tones (Diabate *et al*., 2011), visual cues from the swarm (Tripet *et al*., 2004) and chemical cues (Diabate and Tripet, 2015). There is some evidence suggesting that contact pheromones may be used for conspecific recognition of mates in *Ae. aegypti, Ae. albopictus* and *Culiseta inornata* mosquitoes (Diabate and Tripet, 2015). In addition, males of *Aedes* and *Mansonia* are attracted to host odours, and often mate in close proximity to a host animal (Hartberg, 1971). However, the use of mating pheromones and kairomones needs further work to confirm that these cues play a role in mate recognitions.

2.5.3. Host-seeking behaviour

Female mosquitoes engage in host-seeking behaviour to obtain blood for the maturation of their eggs and to replenish energy reserves (Scott and Takken, 2012). The main disease vectors are highly anthropophilic, i.e. prefer to feed on humans, whereas other mosquito species are more opportunistic or otherwise specialised and feed on other mammals, birds, reptiles, amphibians and even fish (Lyimo and Ferguson, 2009). To locate their hosts, mosquitoes use chemical cues, visual cues and body heat (McMeniman *et al*., 2014; van Breugel *et al*., 2015). Compounds emitted from hosts that have been found to be attractive to mosquitoes include carboxylic fatty acids, lactic acid, ammonia, 1-octen-3-ol and carbon dioxide (CO_2) (Kline, 2006; Qiu *et al.*, 2007; Okumu *et al*., 2010; Webster *et al*., 2015). Of these, CO_2 is a key kairomone, emanated or exhaled by all potential hosts, and which attracts mosquitoes alone or synergises with other host-related compounds, such as ammonia, lactic acid, 1-octen-3-ol, carboxylic acids, tetradecanoic acid, 3-methyl-1-butanol and butan-1-amine (Kline, 2006; Qiu *et al.*, 2007; Okumu *et al*., 2010; Webster *et al*., 2015).

2.5.4. Oviposition site selection behaviour

The physiological state of a mosquito changes after taking a blood meal, leading to a behavioural shift over time from host seeking to oviposition site selection. To identify and discriminate among potential oviposition sites, female mosquitoes use visual, olfactory, gustatory and chemo-tactile cues (Bentley and Day, 1989). Of these, mosquitoes appear to rely heavily on olfactory cues, including pheromones and kairomones, to identify a suitable oviposition site (Navarro-Silva *et al.*, 2009). Oviposition pheromones can influence gravid mosquitoes to lay eggs in the same area as their conspecifics. This has been suggested by observations of gravid *An. gambiae*

and *Ae. atropalpus* mosquitoes that are attracted to breeding sites with low conspecific larval density over no conspecifics (Munga *et al*., 2006; Ogbunugafor and Sumba, 2008). Oviposition pheromones emanating from conspecific immatures may also act as repellents as reported in gravid *An. atropalpus*, *Ae. aegypti* and *An. gambiae* (Zahiri and Rau, 1998; Michaelakis *et al*., 2005; Munga *et al*., 2006). This is probably due to the identification by the ovipositing female of existing conspecifics as competitors. Egg-laying females may also be attracted by pheromones deposited on or next to eggs laid by conspecific or congeneric females. The first mosquito oviposition attractant pheromone, *erythro*-6-acetoxy-5-hexadecanolide was isolated from the apical droplets on the egg of *Cu. quinquefasciatus* and was shown to attract gravid conspecifics as well as *Cu. tarsalis* (Laurence and Pickett, 1982; Otieno *et al.*, 1988; Pickett and Woodcock, 1996). Mendki *et al.* (2000) later identified an oviposition attractant pheromone (*n*-heneicosane) for *Ae. aegypti* by analysing the water in larval breeding sites. In addition, caproic acid is another oviposition attractant pheromone for *Ae. aegypti,* which was identified from conspecific eggs (Ganesan *et al*., 2006).

Kairomones, which provide valuable information for oviposition site seeking gravid mosquitoes, may originate from microorganisms, predators, competitors and the surrounding vegetation of the breeding sites (Afify and Galizia, 2015). These cues have been shown to affect gravid mosquitoes differentially (Afify and Galizia, 2015). Microorganisms may directly release volatiles that serve as semio-chemicals for gravid mosquitoes, *e.g.* chemical cues emitted from bacteria and filamentous algae have been shown to attract gravid *An. gambiae* and *An. pseudopunctipennis* (Huang *et al*., 2006). Microorganisms may also indirectly affect oviposition site seeking gravid mosquitoes in their role as decomposers of organic material, which

inadvertently lead to the production of mosquito detected metabolites (Bentley and Day, 1989). For example, cedrol, which has been identified in soil infusion, attracts gravid *An. gambiae* (Lindh *et al*., 2015). Similarly, phenol, 4-methylphenol (*p*-cresol), 4-ethylphenol, indole, and 3-methylindole (skatole), found in infusions from fermenting plants, such as grass, hay, white oak, live oak, acacia, bermuda grass, alfalfa hay and bamboo, are oviposition attractants and stimulants for several mosquito species (Afify and Galizia, 2015). Volatiles emitted from living vegetation in and around water bodies may also function as chemical cues for oviposition site seeking gravid mosquitoes (Torres-Estrada *et al*., 2005). The volatiles associated with predators and competitors may, on the other hand, deter mosquitoes from ovipositing in habitats where these are present (Kiflawi *et al.*, 2003; Blaustein *et al*., 2004). For instance, the backswimmer *Notonecta maculate* repels ovipositing females of *Culiseta longiareolata*, *Cu. laticinctus* and *An. gambiae.* These cues provide valuable information for the gravid females not to deposition their eggs (Arav and Blaustein, 2006; Munga *et al*., 2006; Silberbush *et al*., 2010).

2.6. The olfactory system of mosquitoes

The olfactory system is one of the most sophisticated sensory systems in mosquitoes. Odour cues in the environment are detected by olfactory organs on the head including the antennae, maxillary palps (Takken and Knols, 1999; Pitts and Zwiebel, 2006; Syed and Leal, 2007) and proboscis (Kwon *et al.,* 2006). The main olfactory organ, the antenna, is divided into three parts; the scapus, the pedicellus and the flagellum (McIver, 1982; Pitts and Zwiebel, 2006). The scapus, also called the base, is the first segment and is used for antennal movement. The second segment, the pedicellus, contains a bulbous structure called the Johnston's Organ, which houses sensory neurons sensitive to the vibrations of the antennae (Clements, 1999). The flagellum forms the

main part of the antennae, and is divided into 13 distinct segmental units called flagellomeres. The female antennae house chemosensory sensilla on all flagellomeres, whereas the olfactory sensilla on the male antennae are restricted to the two distal segments (McIver, 1982; Pitts and Zwiebel, 2006). Female of *An. gambiae, An. quadriannulatus* and *Ae. aegypti* possess approximately 900-1500 sensilla on each of their antenna (McIver, 1982; Pitts and Zwiebel, 2006). The number of sensilla in *An. arabiensis* has not yet been described. The antennae of adult mosquitoes bear five types of sensilla that house sensory neurons sensitive to mechanosensory (chaetica), thermosensory (ampullaceae), as well as olfactory cues, including floral, blood host and oviposition stimuli (see below) (Clements, 1999; Fig 2.1 b).

The second most important olfactory sensory organ in mosquitoes is the maxillary palps. The maxillary palp consists of five segments, in which the 5th segment is reduced to a knob in female anophelines. The maxillary palp is considerably less complex than the antenna, as it possesses a single olfactory sensillum type, the capitate peg (aka *sensillum basiconicum*) which is distributed on the second, third and fourth segments (McIver and Siemicki, 1975). This sensillum type has been observed in all species studied, *e.g. An. gambiae, Cu. quinquefasciatus* and *Ae. aegypti* (Lu *et al*., 2007; Syed and Leal, 2007; Bohbot *et al.,* 2013). Capitate pegs house three olfactory receptor neurons (ORNs). The ORN with the largest spike amplitude (by convention the A neuron) is highly sensitive to carbon dioxide (CO_2) (Lu *et al*., 2007; Bohbot *et al.,* 2013; Omondi *et al*., 2015), while the next largest spike amplitude is from the the B neuron and is highly sensitive to (*R*)-1-octen-3-ol (Lu *et al*., 2007; Bohbot *et al.,* 2013; Hill *et al*., 2015). Both of these volatiles have been shown to be involved in host recognition (Lu *et al*., 2007; Bohbot *et al.,* 2013; Omondi *et al*., 2015). The proboscis, an accessory olfactory organ, is covered by numerous

chemosensory sensilla at its bulbous tip, made up of two lobes, known as the labella. Besides serving a role in gustation, the labella in *An. gambiae* also carry sensilla that are sensitive to odorant stimuli, including butyl amine and several aliphatic carboxylic acids, which appear to play a role in host recognition (Kwon *et al*., 2006).

2.6.1. Sensilla on antenna of mosquitoes

Sensilla are hair-like, cuticular, structures present on the chemosensory organs, which house the ORNs and three auxiliary cells, the thecogen, tormogen and trichogen cells. Sensilla have been referred to as ″kleinorgane" (Henke, 1953) and "organules" (Lawrence, 1966) because of their small size, however, regardless of their small size, sensilla are complex in structure and function (Shields, 2010; Fig 2.1 a). Olfactory sensilla are classified into single-walled and double-walled sensilla depending on their wall structure (Steinbrecht, 1997). In single-walled sensilla, the pores are connected to the lymph by pore channels, which allow the transportation of amphiphilic odour molecules to the sensillum lymph and hence to olfactory receptors embedded in the dendritic membrane surface of olfactory neurons (ORNs) (Steinbrecht, 1997). In contrast, double-walled sensilla are composed of hollow cuticular finger-like structures, which are fused to each other and form spoke-channels at the fusion points. It is likely that odour molecules enter the sensillum lumen of double-walled sensilla via these channels (Steinbrecht, 1997). Of the three types of chemosensilla found on the mosquito antenna, the trichoid sensillum is single-walled, whereas grooved pegs and coeloconic sensilla are double-walled (only found in anopheline) (Ismail, 1964; Mclver, 1982).

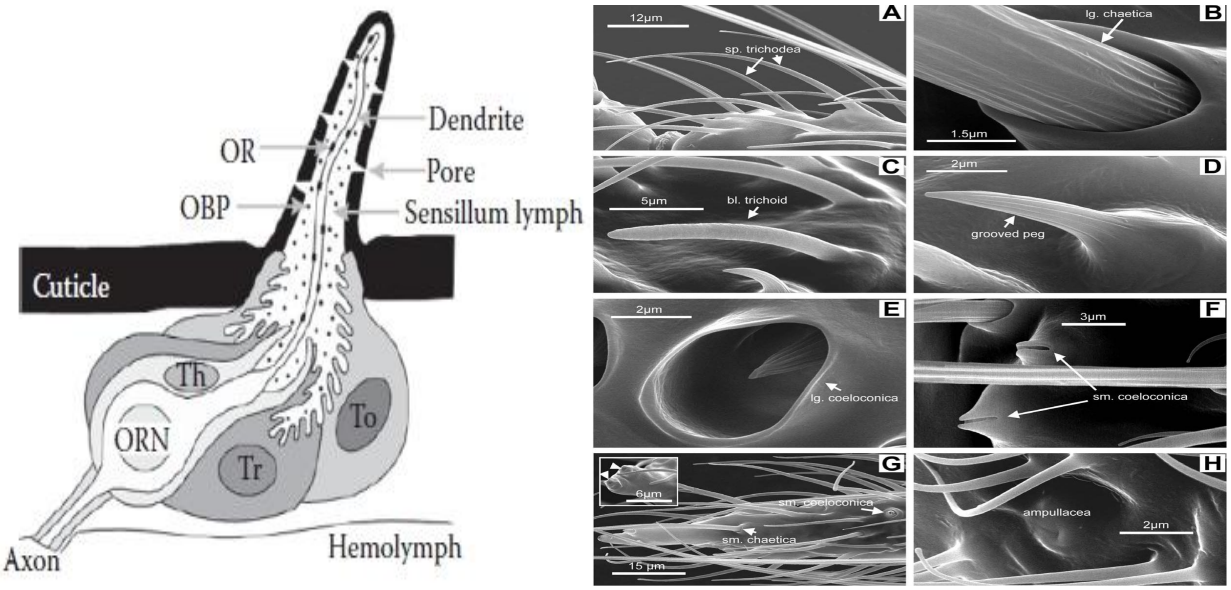

Figure 2.1 Sensilla of female *Anopheles gambiae* mosquitoes. a. Schematics of an olfactory sensillum, b. Sensilla types. (Pitts and Zwiebel, 2006).

In female mosquitoes, trichoid sensilla are the most abundant sensillum type, ranging from 200-1200 per antenna depending on species (Pitts and Zwiebel, 2006). The highest density of trichoid sensilla can be found on flagella 4 to 13 for *An. gambiae s.s., An. quadriannulatus, An. stephensi* and *An. maculipennis atroparvus* (Ismail, 1964; van den Broek and den Otter, 1999; Pitts and Zwiebel, 2006). Based on their external morphology, trichoid sensilla of *Anopheles* mosquitoes may be divided into three subtypes (McIver, 1982; Pitts and Zwiebel, 2006). The first subtype, sharp-tipped trichoid sensillum, is found on all 13 antennal segments, but significantly increase in numbers towards the more distal flagellomeres. The second subtype, short-sharp trichoid sensillum, is mostly distributed at distal flagellomeres and is less numerous than the sharp-tipped trichoid sensillum. The third subtype, blunt-tipped trichoid sensillum, is rather short and has a nearly consistent diameter from the base to the tip and is found in very low numbers on each of the segments (Qiu *et al*., 2006; Pitts and Zwiebel, 2006). Each trichoid sensillum contains one or

two ORNs (Ismail, 1964; Pitts and Zwiebel, 2006). In *An. gambiae*, *Ae. aegypti* and *Cu. quinquefasciatus* mosquitoes, these ORNs have been shown to respond to cues related to nectar resources, host animals and oviposition sites (Qiu *et al*., 2006; Ghaninia *et al*., 2008; Hill *et al*., 2009).

Grooved pegs are the second most abundant sensillum type, and are found in large numbers on the distal flagellomeres (Pitts and Zwiebel, 2006). Each female antenna has 10 to approximately 350 grooved pegs depending on the mosquito species. For instance, *An. gambiae s.s.* has 79 grooved pegs along the antenna, whereas *An. quadriannulatus* antenna has 114 grooved pegs (Pitts and Zwiebel, 2006). Grooved pegs are found in two distinct subtypes, long and short, described for *Aedes* and *Culex* species, as well as for *An. stephensi* (Mclver, 1982; Bowen, 1995). These sensilla contain two to four ORNs (McIver, 1982). Qiu *et al*. (2006) have showed the response of grooved peg in *An. gambiae* to lactic acid as well as other important host cues, including ammonia and butylamine.

2.6.2. Olfactory transduction in sensilla

Transduction is a process by which a chemical signal is converted to an electrical signal in the ORN. The process of transduction begins with the entrance of an odorant molecule, from the surrounding environment, into the aqueous sensillum lymph through the pores/spokes of a sensillum. In the sensillum lymph, odour molecules are recognized and bound to odorant binding proteins (OBPs). These proteins were first recognized in the silk moth, *Antheraea polyphemus* (Vogt and Riddiford, 1981), and since then large families of OBPs have been identified in many other insects, including mosquitoes (Pelosi *et al*., 2006). Odorant binding proteins are 120 to 150

amino acids long hydrophilic proteins, secreted by the auxiliary cells. Odorant binding proteins are classified into two subfamilies, pheromone binding proteins (PBPs) and general odorant binding proteins (GOBPs) I and II (Stengl *et al.,* 1999). The GOBPs have been shown to bind to olfactory cues present in *e.g.* food or oviposition substrates, whereas PBPs specifically bind to pheromones (Stengl *et al.,* 1999). Once bound to the OBPs, the protein-ligand complex is transported through the sensillum lymph to the olfactory receptors on the dendritic membrane of the ORN.

Olfactory receptors, which are found embedded in the dendritic membrane of the ORNs, are divided into odorant receptors (ORs) (Zwiebel and Takken, 2004), ionotropic receptors (IRs) (Croset *et al*., 2010) and Gustatory receptors (GRs) (Sanford *et al*., 2013). These olfactory receptors are sensitive and selective to different odorant molecules. Thereby, ORNs are able to encode odor quality, quantity and temporal change in odour concentration (Qiu *et al*., 2006). This information is then sent to the higher brain centre.

2.7. Methods in mosquito olfaction

Mosquito behaviour in response to olfactory cues may be analysed under laboratory, semi-field and field conditions using different methods, whereas physiological responses to olfactory cues commonly are analysed under laboratory condtions. Some of the methods used to identify behaviourally active volatile compounds, including collection of volatile compounds, behavioural and electrophysiological bioassays are described below.

2.7.1. Collection of volatile compounds

Volatile organic compounds (VOCs) have a relatively high vapour pressure, and include both human-made compounds and compounds produced by different organisms such as plants, animals and microorganisms. To analyse the behavioural and physiological role of the VOCs, these must first be collected. The source and nature of the VOCs determine the techniques used for volatile collections. Some of the techniques that are widely applicable to most types of semiochemicals are solvent extraction and headspace trapping (Agelopoulos and Pickett, 1998).

2.7.1.1. Solvent extraction

Solvent extraction has been used for the collection of volatile compounds in a number of studies (Ghosh *et al*., 2012; Otienoburu *et al*., 2012). However, due to the physical damage caused to the sample during the process of extraction, this technique needs to be used with caution. Depending on the state of matter of the sample to be extracted, different solvents are used that vary in volatility, polarity and molecular weight (Wells *et al*., 2003). For instance, for extraction of solid samples, non-polar solvents (benzene or dichloromethane), polar solvents (methanol) or mixtures of polar and non-polar solvents such as ethanol, benzene, acetone and hexane are used (Wells *et al*., 2003). By varying the type of the solvents, the full volatile organic profile of the sample can be obtained (Yalavarthi and Thiruvengadarajan, 2013).

2.7.1.2. Headspace volatile compound collections

Headspace volatile collection is a more preferred approach for collecting volatile compounds as this technique provides a more accurate volatile profile of a sample (Tholl *et al*., 2006). The technique has been developed in the 1980s to elucidate the odor compounds present in the air

surrounding various samples. The technique includes both static and dynamic headspace collections. In the process of headspace collection, the devices or materials used should be free of materials that retain any volatile compounds that could interfere with the volatile analysis (Tholl *et al*., 2006). The most common materials used for headspace volatile collections include glass, metal and special plastics, such as Teflon. In headspace volatile collection, volatiles from a sample are collected or trapped in polymer-based media, the most common of which are Super-Q, Tenax-TA, Carbotrap, Porapak-Q and activated charcoal adsorbents. These adsorbents have different polarities and affinities for VOCs. For instance, Super-Q and Tenax-TA have low affinity for low molecular weight or polar compounds, whereas Carbotrap has a wide affinity for organic compounds. Activated charcoal is less efficient in trapping aromatic aldehydes. Due to the different properties of the various adsorbents, more than one adsorbent may be employed to increase the trapping of VOCs. For example, Jhumur *et al*. (2007, 2008) used Tenax-TA and Carbotrap adsorbent for collection of volatiles from *Silene otites* (Caryophyllaceae). Volatiles that are trapped on the adsorbents are eluted with pure solvents or mixtures of low-boiling-point organic solvents into glass vials and then kept in a freezer for further use.

Static headspace volatile compound collection

In static headspace analysis, the air surrounding the sample emitting the volatiles remains constant (Tholl *et al*., 2006). This method is often used for sampling VOCs from low emitting plants and does not require any expensive equipment or instrument modification (Riter *et al*., 2003). The most commonly used static headspace analysis is solid phase microextraction (SPME). For SPME, the samples are first placed in a glass vial, flask or Teflon bag, and the inert SPME fibres, coated with either a liquid polymer or a solid adsorbent, is then introduced into the

container (Ulrich *et al*., 2000). During collection, the introduced fibre is exposed to the sample for a defined period of time. The amount of volatile compounds adsorbed on the fibre depends on its coating thickness and the type of sample (Ulrich *et al*., 2000). Solid phase microextraction is a fast and simple method for collecting volatiles and the fibres can be reused approximately 100 times (Wooten *et al*., 2002). Syed and Leal (2009), for example, used SPME to trap volatile compounds of live birds and humans of various ethnic backgrounds for GC-MS analyses. Accumulation of humidity, heat and deleterious chemicals on the fibre could interfere with the outcome of the volatiles profile (Tholl *et al*., 2006).

Dynamic headspace volatile collection

For dynamic headspace volatile collection, the compounds emitted from samples are trapped on an adsorbent, using a continuous flow of air in either closed or open containers (Tholl *et al*., 2006). The benefit of dynamic collections is that quantification is easier because volatiles are trapped at a known rate. Dynamic flow systems use active airflow (push), vacuum flow (pull), or combined air and vacuum flow (push-pull) systems to move headspace volatiles through a filter containing an adsorbent material volatile trap (Tholl *et al*., 2006). Alternatively, closed-loop stripping may be used when collecting volatiles with low volatile emissions. Closed-loop stripping (*e.g.* Healy and Jepson, 1988; Jhumur *et al*., 2008) is easy to set up in controlled climate containers, and allows for the simultaneous collections of several individual samples. For this reason, this system is usually chosen for screening purposes. However, due to accumulation of humidity and heat in the containers, which may interfere with the odour profile, it is essential to crosscheck obtained odour profiles with other systems (Tholl *et al*., 2006).

Figure 2.2 Pull headspace volatile collection of maize tassel.

In a pull system (Fig. 2.2), air regulated by a flow meter is pulled over a sample through an adsorbent that is connected to a vacuum pump. The system is simple to set-up, relatively inexpensive, portable and suitable for simultaneous collections from many containers, and for sampling in the field (Kessler and Baldwin, 2001). In 'push–pull' systems, filtered air is pushed into the headspace sampling container at a rate regulated by a flow meter, and then a portion of the air is pulled out of the container through an adsorbent connected to a vacuum pump at a defined rate controlled by a second flow meter (Tholl *et al*., 2006). The flow of air into the system is important in order to increase the collection of volatile compounds from the sample. Some push-pull systems prevent overpressure created in a container and are used for long period volatile collection. The 'push-pull' VOC collection technique offers more flexibility in regulating in- and outgoing airflow dependent on the emission rates of VOCs in the investigated sample. For instance, Nyasembe *et al*. (2012) and this study used the 'push-pull' system in their studies.

2.7.2. Behavioural bioassay techniques for mosquitoes

Behavioural bioassays are used to investigate the biological activity of mosquitoes in response to the collected headspace volatiles (Beeuwkes *et al*., 2008; Logan *et al*., 2008). The behavioural response of the mosquitoes can be analysed in still-air or moving-air, as well as in no choice or choice, bioassays. The most commonly used bioassays include cage, dual-port olfactometer, Y-tube and wind tunnel (Logan *et al*., 2008). The behavioural response of the mosquitoes can be recorded either through direct observation (Nyasembe *et al*., 2012; Chapters III, IV and V of this thesis) or via video tracking (Beeuwkes *et al*., 2008).

2.7.2.1. Still-air bioassays for mosquitoes

In still-air bioassays, the volatile compounds tested are loaded on dispensing materials and reach the test mosquitoes by simple diffusion. The most common still-air bioassays used are two-port olfactometers and cages (Puri *et al*., 2006; Kweka *et al*., 2011; Chapters III, IV and V of this thesis). In these bioassays, the behavioural response of the mosquito, moving toward or away from the odour source, is noted and recorded (Millar and Haynes, 1998).

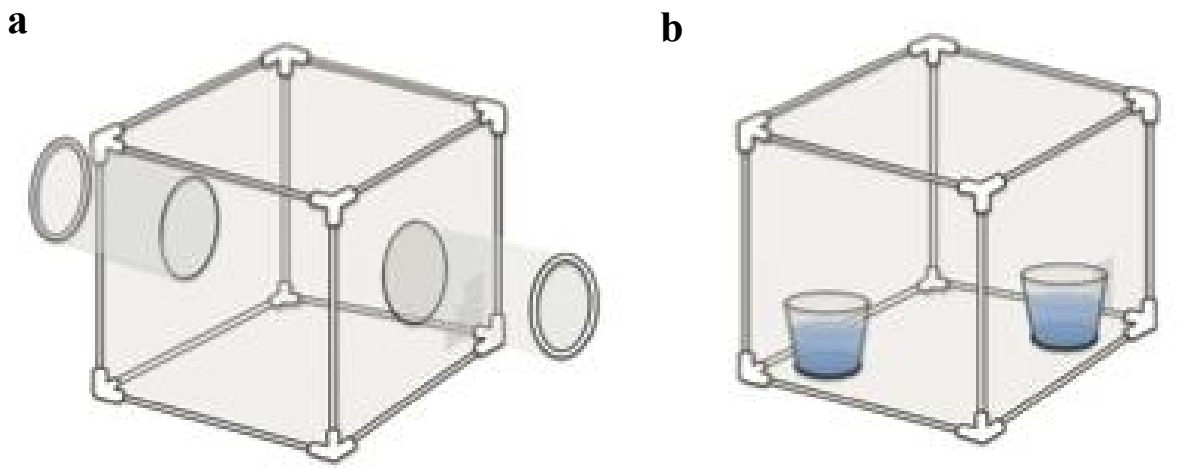

Figure 2.3 Schematic drawing of still-air behavioural bioassays. a. Two-port olfactometers, b. Cages bioassay

The two-port olfactometer is an assay with two arms, containing the odour lure or control, positioned either in opposite or parallel orientation to each other (Fig. 2.3 a). This bioassay provides a wealth of basic information on the mosquitoes' olfactory preference for short-range stimuli. This type of bioassay was used in this thesis and by Puri *et al*. (2006).

Cage bioassays are mostly used to determine the behavioural response of mosquitoes to short range odour stimuli (Isoe *et al*., 1995; Fig 2.3 b). This type of bioassay has, for example, been used to analyse the attraction to sugar and floral sources of *Aedes* and *Culex* mosquitoes (Brooks, 2014), the repellency of *N,N*-diethyl-meta-toluamide (DEET) to *An. gambiae*, *Cu. quinquefasciatus* and *Ae. aegypti* (Syed and Leal, 2008; Logan *et al*., 2010) and the attraction of *Ae. aegypti* to caproic acid (Ong and Jaal, 2015). Caproic acid is a pheromone which attracts ovipositing *Ae. agypti* mosquitoes. Cage bioassays are also the most commonly used bioassay to assess potential odorants for oviposition seeking mosquitoes. In this bioassay, the oviposition

stimulation of the mosquitoes is evaluated by counting the number of eggs laid on a test substrate compared to a reference substrate. For example, the oviposition stimulation of *Aedes* (Sharma *et al*., 2008), *Culex* (Millar *et al*., 1994), *An. gambiae s.s.* (Rinker *et al*., 2013; Lindh *et al*., 2015) and *An. arabiensis* in the present study were analysed using this bioassay.

2.7.2.2. Moving air bioassays for mosquitoes

In a moving air bioassay, the volatile chemical cues are carried downwind in an odour plume, and the behavioural responses of the mosquitoes are observed as they enter and leave the plume. For this purpose, a laminar airstream of uniform strength is used (Millar and Haynes, 1998). The most common moving-air bioassays are different types of olfactometers and wind tunnels. Olfactometers may be manufactured with one or multiple arms, each with a separate air source, while wind tunnels have only one source of air. Within the airstream, however, the wind tunnel may have multiple chemical emitters that can be charged with different test compounds.

Olfactometers, such as Y-tubes and dual-port olfactometers are used to assess the response of mosquitoes to a choice between an odour and a control or two different odours (Geier and Boeckh, 1999). The Y-tube olfactometer is commonly used to determine the upwind flight response of mosquitoes in relatively small arenas (Logan *et al*., 2008), whereas dual-port olfactometers are more suitable for mosquito species that perform better in larger arenas, such as *An. gambiae* (Smallegange *et al*., 2009).

Like olfactometers, the wind tunnel can be constructed with various designs to study mosquito behaviour (Cooperband and Cardé, 2006). Wind tunnel bioassays are used to exploit the upwind

flight response of mosquitoes (Millar and Haynes, 1998). Unlike olfactometers, wind tunnels are used to assess the rate of movement and choice of direction during flight. In the wind tunnel, the wind velocity and other parameters can be modified. In various studies, wind tunnels have been employed to evaluate the attraction of mosquitoes toward the odour source (Klun *et al*., 2013).

2.7.2.3. Semi-field and field behavioural bioassay

Compared to laboratory assays, semi-field and field assays provide researchers with the means to evaluate potential behaviorally active compounds under natural conditions (Qiu *et al*., 2007; Lindh *et al*., 2015; Mweresa *et al*., 2015). Semi-field bioassays, which became an essential component of mosquito research in the late 1990s, are generally large outdoor screened-in houses that are used to evaluate the orientation of several mosquitoes to odorants over short- to medium-range distance (0-20 m) (Okumu *et al*., 2010; Lindh *et al*., 2015; Fig 2.4). However, in field bioassays the behavioural response of mosquitoes to test odorants is evaluated over longer distances (>20 m) (Okumu *et al*., 2010). In field bioassays, in contrast with semi-field bioassays, the number of mosquitoes, the type of species and the environmental conditions cannot be fixed or controlled.

For semi-field and field bioassays, various traps and delivery materials are employed to investigate the efficacy of test odorants (Dugassa *et al*., 2012; Mweresa *et al*., 2015; Okal *et al*., 2015). The traps are designed depending on the species, behaviour and physiological state of the mosquitoes tested. To trap host- and oviposition-seeking mosquitoes, various traps have been employed, *e.g.*, odour-baited entry traps (OBETs) (Kweka *et al*., 2009), mosquito magnet model X (MM-X) traps (Okumu *et al*., 2010), CDC miniature light traps (Murphy *et al*., 2001), BG

sentinel traps (Lindh *et al*., 2015; Okal *et al*., 2015), electric nets (e-nets) (Dugassa *et al*., 2014), resting boxes (Kweka *et al*., 2009), OviART (Dugassa *et al*., 2013), Frommer updraft gravid traps and Box gravid traps (Reiter-Cummings gravid trap) (Braks and Cardé, 2007). Of these, the BG sentinel trap is the most effective one for collection of gravid mosquitoes, as it has a size, which is large enough to incorporate multiple attractants (Lindh *et al*., 2015; Wright *et al*., 2015).

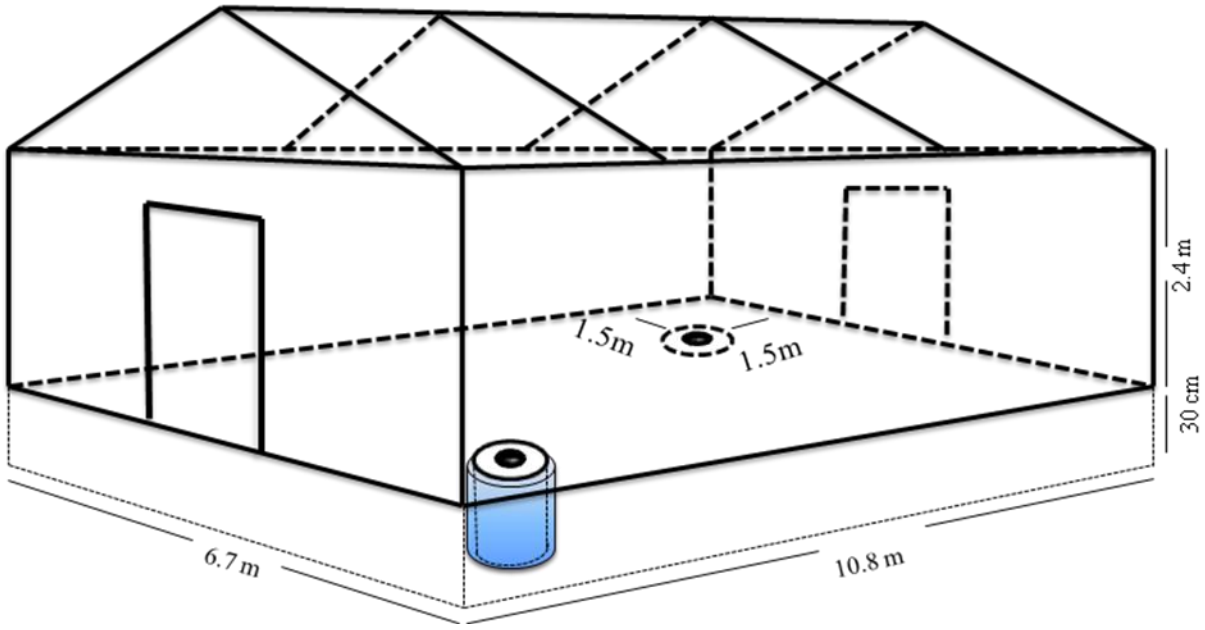

Figure 2.4 Schematic drawing of the outdoor screened house of semi field bioassay with BG sentinel trap dug in sand at a depth of 30 cm.

The delivery materials that are used to dispense the test odorants in semi-field and field bioassays should ensure stability of the impregnated active ingredients, sustained release of optimal odour concentrations while maintaining the ratio of all components to one another, and be easy to prepare for large-scale application (Okumu *et al*., 2010). The dispensing materials differ in their physico-chemical characteristics, such as porosity and chemical binding affinity, and as a result regulate the release rates of odour volatiles differently (Dekker *et al*., 2002). The

dispensing methods for test compounds include wicks (Owino *et al*., 2015), glass vials (Qiu *et al*., 2007), sealed polythene sachets (Torr *et al*., 2008) and nylon strips (Okumu *et al*., 2010; Mweresa *et al*., 2015). Compared to low density polyethylene (LDPE) sachets and open glass vials, nylon strips were found to provide a better release matrix for delivering synthetic attractants for host-seeking *An. gambiae* (Okumu *et al*., 2010). However, Mweresa *et al*. (2014) found that the textile materials, such as polyester, cotton and a cellulose polyacrylate blend perform equally well, or even better, than nylon as they are easy to use, locally available and relatively cheap when considered for large-scale application. Thus, there is a potential to develop locally available textile material as dispenser material in the future.

2.7.3. Electrophysiological bioassay

Electrophysiological bioassays are used to detect and measure the antennal response of insects to chemical cues. However, the electrophysiological bioassays do not provide information on how the cues affect the behaviour of the insect, i.e., the compounds may be either attractant or repellent or may not have a behavioural effect on its own. For olfaction, the most commonly used assays involve recordings from the whole antenna, electroantennogram (EAG), and combined gas chromatography and electroantennographic detection (GC-EAD) analysis. The latter method is useful when identifying active compounds from a complex odour blend.

2.7.3.1. Combined gas chromatography and electroantennodetection

Schneider developed Electroantennography (EAG) in 1957. This bioassay is used for investigating the antennal response of an insect to synthetic compounds or compounds in

complex odour blends eluting from a gas chromatograph (Wadhams, 1992). Electroantennography is assumed to measure the total response of the ORNs on the insect antenna when it is exposed to volatile stimuli, and is a method that does not require complicated instrumentation. With the development of the GC-EAD analysis by Arn *et al*. (1975), researchers are now able to screen for biologically active compounds in complex odour blends rather than testing the individual compounds separately. Combined GC-EAD detection has been used widely for analysing the antennal responses of several species of mosquitoes to a wide range of semiochemicals emanating from host animals, plants, microorganisms and oviposition or breeding sites (Jhumur *et al*., 2007; Syed and Leal, 2009).

For EAG analysis, the antenna is prepared by excising the head from an adult mosquito. Then, the distal end (1-2 flagellomeres) of the antenna is cut off and connected to a recording glass electrode filled with a buffered saline solution (Beadle-Ephrussi). The base of the antenna is fixed to a grounded reference electrode, filled with the same solution. Silver–silver chloride junctions are used to maintain the electrical contact between the electrodes and the input of a preamplifier. The recording electrode is connected to a high impedance DC amplifier interphase box. Preparations are held in a continuous, humidified, and charcoal-filtered air stream, the outlet of which is positioned close (ca. 0.5 cm) to the preparation.

For GC-EAD analysis, the behaviourally active headspace extract is injected onto a GC. The GC is equipped with a flame ionisation detector (FID) and a fused silica capillary column. A carrier gas, helium, nitrogen, argon or hydrogen (Robert *et al*., 2004), carries the injected extracts through the column. The extract sample is separated in the capillary column based on the

volatility of the compounds, the polarity of the compounds, the column temperature, the polarity of the column, the flow rate of the carrier gas and the length of the column. The GC column effluent is split so that one half of the injected sample goes to the FID and the other half goes via a transfer line to the antennal preparation outside the GC (Arn *et al*., 1975). The analogue signals of the GC and the EAD are both fed into an AC/DC data acquisition controller so that they can be aligned during subsequent analyses to identify those compounds that induce an electrical response in the antenna.

2.7.3.2. Identification of volatile compounds

Combined gas chromatography and mass spectrometry is an analytical method that separates, quantifies and identifies different substances within a test sample (Cork *et al*., 1990). Similar to the GC-EAD method, this analysis method also uses a GC for separating test compounds in complex odour blends (Robert *et al*., 2004). However, instead of a FID, a mass spectrometer acts as the detector. Mass spectrometry is a method that is used to identify the amount and type of chemicals present in a test sample by measuring the mass-to-charge ratio and abundance of gas-phase ions (Sparkman and David, 2000). In mass spectrometry, the molecules from the GC column are changed into ionised fragments. Fragmentation of the molecules is caused by ionization, either by electron ionization (EI) or chemical ionization (CI) (Fales *et al.,* 1972). Electron ionization is the most common form of ionization, and is caused by bombardment of molecules with free electrons. Chemical ionization is another ionization technique, which is produced through the collision of the molecules with ions of a reagent gas (methane, ammonia or isobutane) (Fales *et al.,* 1972). Once the molecules are broken into ionized fragments, the MS detects these fragments using their mass-to-charge ratio. The identification of compounds is

relatively easy compared with other analytical platforms, *e.g.* liquid chromatography mass spectrometry (LC-MS), capillary electrophoresis mass spectrometry (CE-MS) and supercritical fluid chromatography mass spectrometry (SFC/MS), as each compound has a unique or near unique mass spectrum that can be compared with mass spectral databases (Putri and Fukusaki, 2014). Combined gas chromatography and mass spectrometry analysis has been useful for identification of chemical compounds to many studies related to mosquito olfaction (Syed and Leals, 2009; Nyasembe *et al*., 2012; Lindh *et al*., 2015).

2.7.4. Application of semiochemicals

Olfactory cues play a great role in the life activities of mosquitoes (Takken and Knols, 1999). As a result, this area has received considerable attention of many researchers and has led to the identification of many semiochemicals (Takken and Knols, 1999; Takken and Verhulst, 2013; Afify and Galizia, 2015). These semiochemicals have been identified from host plants or animals or oviposition sites and are often used for monitoring and management of vector mosquitoes (Kline, 2006; Okumu *et al*., 2010; Matowo *et al*., 2013; Menger *et al*., 2014; Mweresa *et al*., 2015; van Loon *et al*., 2015; Afify and Galizia, 2015). For instance, for the intervention against sugar seeking mosquitoes, volatile compounds have been exploited from various host plants (Healy and Jepson, 1988; Mauer and Rowley, 1999; Nyasembe *et al*., 2012; Nikbakhtzadeh *et al*., 2014). In the case of host seeking mosquitoes, volatiles emanating from human and non-human hosts are used for monitoring and controlling vector mosquitoes (Kline, 2006; Okumu *et al*., 2010; Matowo *et al*., 2013; Menger *et al*., 2014; Mweresa *et al*., 2015; van Loon *et al*., 2015). Among the host volatile compounds used to control mosquitoes, CO_2 is one of the most common

kairomones, either alone or in synergy with other synthetic compounds (Kline, 2006). For example, CO_2 has been used in combination with 1-octen-3-ol (a common attractant released in the breath of vertebrates hosts) in mass trapping of *Anopheles* mosquitoes in USA (Kline, 2006). In addition, CO_2 has been used with other host-derived volatiles such as lactic acid, ammonia, carboxylic acids and tetradecanoic acid for significant reduction of Anophelinae and Culicinae mosquitoes (Qiu *et al.*, 2007; Okumu *et al.*, 2010; Matowo *et al*., 2013; Menger *et al*., 2014; Mweresa *et al*., 2015; van Loon *et al*., 2015).

Pheromones and kairomones isolated from breeding sites or the surrounding vegetation have also been used for monitoring and controlling oviposition seeking gravid mosquitoes (Afify and Galizi, 2015). For instance, n-heneicosane and 6-acetoxy-5-hexadecanolide pheromones were used for luring gravid *Ae. aegypti* and *Cu. quinquefasciatus* mosquitoes to ovitraps for surveillance and monitoring (Michaelakis *et al.*, 2007; Seenivasagan *et al.*, 2009). The kairomone AtrAedes lure, derived from chemicals isolated from grass infusion, and a blend containing trimethylamine (TMA) and nonanal were used for large scale monitoring and management of gravid *Ae. aegypti* and *Culex* in Brazil, respectively (Leal *et al*., 2008). Efforts are in progress to identify biologically active compounds from plants and soil infusions that are potentially attractant for gravid *An. gambiae* complex (this study; Lindh *et al*., 2015) that could be used for monitoring and controlling gravid *Anopheles* mosquitoes.

2.7.5. Integrated vector management

Integrated vector management using more than one control strategy has the possibility to provide an increase in the potential to eliminate the threat of vectors and minimise the development of vector resistance to control methods (WHO, 2012). For instance, a 95% reduction in malaria

incidence over a four-year period was observed as a result of combining water-source reduction activities and biological control in larval habitats (Sharma *et al*., 1986). Similar reports in a coastal flood plain of Haiti were obtained from 1969-1970 by using both water drainage techniques and larvicides (Schliessmann *et al*., 1973). In Sri Lanka, the human morbidity and mortality as a result of malaria were reduced based on community participation, ITNs and larval source management (Yasuoka *et al*., 2006). Moreover, a significant reduction in the vector mosquito population was observed in Tanzania by integration of ITN and IRS with the management of the larval habitat (Beier *et al*., 2008).

Sustainable management of vector mosquitoes has the potential to be enhanced by incorporating semiochemicals as a component of IVM. Successful stories of IVM that involved semiochemicals for malaria suppression have been witnessed in several countries of the world. For example, in Brazil, 3-methylindole (skatole) or grass infusion was integrated with *Bti* to control gravid *Cu. quinquefasciatus* (Barbosa *et al*., 2010). This same species has been controlled by integrating the pheromone 6-acetoxy 5-hacadecanolide with an insect growth regulator in Kenya (Otieno *et al*., 1988). *Culex pipiens s.s.* is also controlled by the integration of the pheromone 6-acetoxy-5-hexadecanolide with the larvicide Temephos (Michaelakis *et al*., 2007). Integrated vector management is not limited to management of Culicine mosquitoes; it is also widely used for management of Anopheline mosquitoes. For instance, the control device Mosquito Landing Box (MLB), baited with natural human foot odours combined with CO_2 and treated with a mosquitocidal agent (pyriproxifen), has been successful against outdoor-biting mosquitoes such as *An. arabiensis* when it is complemented with LLINs (Matowo *et al*., 2013). Additionally, mosquito repellents (i.e., Buzz-Off® petroleum jelly and essential oil blend) have

also been used with integration of LLINs or IRS for significant reduction of malaria vectors in Ethiopia (Yohannes *et al*., 2012; Deressa *et al*., 2014).

Chapter III

The role of rice volatiles in oviposition site selection by *Anopheles arabiensis*

3.1. Introduction

Oviposition site selection by malaria mosquitoes is a behaviour that needs to be exploited in future control strategies. Although existing indoor control methods, including indoor residual spray (IRS) and long lasting insecticidal nets (LLINs), have been effective in reducing indoor malaria transmission (O'Meara *et al*., 2010), we are now seeing a reduction in that efficacy (Ranson *et al*., 2016), with the emergence of insecticide resistance (Ranson *et al*., 2011; Trape *et al*, 2011; Toé *et al*., 2014), the resurgence of vectors (Zhou *et al*., 2011; Sinka *et al*., 2016), and a shift in indoor and outdoor vector behaviour (Killeen *et al*., 2013; Riehle *et al*., 2011;Sougoufara *et al*., 2014; Sinka *et al*., 2016) leading to a proportional increase in outdoor malaria transmission (Russell *et al*., 2011; Kitau *et al*., 2012; Mwangangi *et al*., 2013; Ojuka *et al*., 2015). Besides larval control, which has largely been disbanded in favour of IRS and LLINs (Fillinger and Lindsay, 2011) in contemporary sub-Saharan Africa, there are no effective control strategies that target the populations of outdoor-biting malaria vectors. This includes one of the dominant vectors, *Anopheles arabiensis* (Killeen *et al*., 2012). Gravid malaria mosquitoes, irrespective of indoor or outdoor feeding patterns, must seek for suitable aquatic habitats to lay her eggs. Identifying and manipulating cues from such habitats could provide important insights toward novel mosquito control tools.

Selection of oviposition sites for mosquito vectors of malaria is dependent on a number of physical and chemical factors. Gravid malaria mosquitoes are attracted to water bodies by water vapour (Okal *et al*., 2013), as well as olfactory cues from conspecific larvae (Rejmánková *et al*., 2005; Ogbunugafor and Sumba, 2008) microorganisms (Sumba *et al*., 2004) and soil (Lindh *et al*., 2015) associated with the sites. While habitat cues associated with the sites potentially attract

mosquitoes over a longer distance, these have not been fully explored. Emergent and other vegetation may provide such potential cues as grasses and other short vegetation often emerge from and surround natural larval habitats of malaria mosquitoes (Minakawa *et al*., 2006; Ndenga *et al*., 2011).

Governmental and non-governmental initiatives are systematically increasing the regions under irrigated cultivation throughout sub-Saharan Africa (FAO, 2007). Although irrigated cultivations of cereal crops, including rice, maize and sugarcane, provide food security, they also provide ideal larval habitats for malaria mosquitoes (Ye-Ebiyo *et al*., 2000; Mwangangi *et al*., 2006). This generally creates a spatial link between the vector and the people, thereby increasing the risk of malaria transmission. The cereal crops provide the larvae with shelter from abiotic and biotic threats, as well as nutrients directly from shed pollen and indirectly from accumulated detritus and associated microorganisms (Merritt *et al*., 1992; Ye-Ebiyo *et al*., 2000). Irrigated rice agro-ecosystems, in particular, create permanent mosquito breeding habitats (Mwangangi *et al*., 2010), thereby increasing malaria transmission risk compared with other cultivated cereals (Ijumba and Lindsay, 2001; Mboera *et al*., 2015; Diakité *et al*., 2016). Moreover, the agronomic activities in rice, including the addition of fertilizers and insecticides (Darriet *et al*., 2012; Reid *et al*., 2016), also affect larval density by increasing available nutrients (Mutero *et al*., 2004) and creating predator-free habitats (Service, 1977), respectively. Thus, female mosquitoes that select breeding sites within irrigated rice cultivation have the potential to increase their own fitness by providing their offspring with a selective advantages. We show that gravid *An. arabiensis*, one of the predominant mosquito species in rice cultivation (Muturi *et al*., 2006; Jarju *et al*., 2009) are attracted to the odour of rice, irrespective of the phenological stage of the plant, and identify a

synthetic blend of rice-volatiles that acts as a long-range attractant and elicits oviposition. The manipulation of the odour-mediated oviposition site selection behaviour of gravid adults has distinct potential as a tool in the future control of malaria vectors. Here, we take a significant step toward the development of a long-sought-after control tool for gravid malaria mosquitoes, by identifying the first synthetic lure from an odour blend.

3.2. Materials and Methods

3.2.1. Experimental mosquitoes

Anopheles arabiensis, Mwea and Dongola strains, were used for behavioural (International Centre of Insect Physiology and Ecology, Kenya; ICIPE) and electrophysiological (SLU, Sweden) analyses, respectively. The mosquitoes were maintained at 27±2°C, 75±5% relative humidity under a 12 h light/12 h dark photoperiod. For laboratory experiments, larvae were reared in distilled water and fed Tetramin® fish food (Tetra, Melle, Germany). Alternatively, for semi-field experiments, larvae were reared in water from Lake Victoria and fed on ground Go-Cat® food (Nestlé Purina Petcare company, Kenya). Adults were maintained in cages (30 cm × 30 cm × 30 cm; custom made or Bugdorm, MegaView Science, Taiwan), kept under laboratory (27±2°C, 75±5% relative humidity) or ambient outdoor conditions (ICIPE, Mbita, Kenya), and provided with 10% honey or sucrose solution *ad libitum.* Five days post-emergence, females were offered sheep blood from an artificial feeder (SLU, Hemotek, Discovery Workshops, Accrington, UK), rat blood (ICIPE, Nairobi) or the arm of a volunteer (ICIPE, Mbita) once every day for two days, 15-30 min *per* day. For all experiments, gravid females, 3 days post-blood

feeding, were selected by visually inspecting the enlarged pale white abdomen and used for bioassays.

3.2.2. Headspace volatile collections

Headspace volatiles of MR1 and MR3 rice cultivars (150 replicates per stage) were collected in Saudi Star Agriculture and Irrigation Project in Gambella, Ethiopia. These available cultivars have desirable agronomic characters, including high yield, aeration seeding, moderate resistance to lodging, and are able to grow under rain-fed and irrigated conditions. The above-ground parts of intact rice plants, in the field, at tillering, booting and flowering stages were enclosed in polyamide bags (Toppits, Cofresco, Germany). In addition, the headspace volatiles of water from natural breeding sites (200 replicates) were collected from 1 l poured into a Teflon bag. These sites were selected from pools at the edge of Lake Ziway, Ethiopia that contained *Anopheles gambiae sensu lato* larvae. A charcoal-filtered continuous airstream (1.0 l min^{-1}) was drawn by a Personal Air Sampler (PAS-500, Spectrex, Redwood City, CA, USA) over the rice plant, or by a diaphragm vacuum pump (KNF Neuberger, Freiburg, Germany) over the breeding water, onto an aeration column for 2 h. Aeration columns were made of Teflon tubing (6 cm × 3 mm i.d.), holding 35 mg Super Q (80/100 mesh; Alltech, Deerfield, IL, USA) between polypropylene wool plugs and Teflon stoppers. The columns were rinsed with 1 ml re-distilled *n*-hexane (LabScan, Malmö, Sweden) before use. Adsorbed volatiles were eluted with 300 µl re-distilled *n*-hexane. Headspace volatile extracts from each cultivar and phenological stage were pooled separately and then stored in glass vials at -80 °C until used for behavioural and electrophysiological analyses.

3.2.3. Two-port olfactometer

A two-port olfactometer was used to test the mosquito attraction preference for the headspace volatiles collected from the different phenological stages of the MR1 and MR3 cultivars, and from natural breeding water. All assays were conducted between 18:00 and 21:00 local time under red light conditions (icipe, Nairobi, Kenya), the peak oviposition activity as determined in pilot experiments. For each replicate, 10 gravid females were allowed to acclimatize for 5 min in a custom-made cage (22 cm × 30 cm × 12 cm; L:D:H) constructed of clear vinyl for easy viewing. Thereafter, two dental-wick odour dispensers (4 cm × 1 cm; L:D; DAB Dental AB, Upplands Väsby, Sweden) were simultaneously introduced into the cylindrical vinyl arms (13 cm × 9 cm; L:D) positioned at opposite ends of the cage. The ends of the cylindrical arms were covered by mesh. Mosquito attraction preference to the following treatments was analysed: a) headspace volatiles of breeding water *vs.* hexane control, b) headspace volatiles of each phenological stage of MR1 or MR3 *vs.* hexane, c) headspace volatiles of each phenological stage of MR1 or MR3 *vs.* headspace volatiles of breeding water, and d) headspace volatiles of the pooled phenological stages of MR1 *vs.* headspace volatiles of the pooled phenological stages of MR3. The behavioural response to the two rice cultivars was analysed to increasing release rates of the headspace volatile extract from all phenological stages of MR1 and MR3. Pilot studies were conducted to determine the period that no more mosquitoes accumulated in each arm which was five minutes. After five minutes, the behavioural responses of the mosquitoes were scored by counting the number of mosquitoes in each port. Ten replicates *per* treatment and *per* dose were performed. Between each trial the bioassay was cleaned with 70% ethanol and the position of the treatments changed to avoid bias.

3.2.4. Oviposition bioassay

The oviposition preference of gravid mosquitoes was analysed in a two-choice assay. Custom-made metal wire framed cages (30 cm × 30 cm × 30 cm) covered with white nylon mosquito netting were used. Two 100 ml polypropylene cups (Qingdao Ori-Color Industry and Commerce Co., Ltd., China), placed in opposite corners of the cages, and filled to the brim with distilled water or field collected breeding water, served as the oviposition substrate, as indicated. The position of the cups was exchanged between experiments. Treatment cups were conditioned by dosing the oviposition substrate with the headspace volatile extracts of the three phenological stages of MR1 and MR3 as described above; hexane was used as a control. An additional comparison between the headspace volatiles of the pooled phenological stages of MR1 *vs.* headspace volatiles of the pooled phenological stages of MR3 dosed into breeding water was performed for the oviposition assays. No significant difference in oviposition preference was found between the breeding and distilled water when compared directly against each other. Ten gravid mosquitoes were transferred from the maintenance per cage at dusk (18:00), and the numbers of eggs in the two cups were counted on the following day (09:00). All experiments were replicated ten times.

3.2.5. Electrophysiological analysis

Antennal responses of gravid female *An. arabiensis* to the pooled headspace extract of the three phenological stages of the MR3 rice cultivar were analysed using combined gas chromatography and electroantennographic detection (GC-EAD). An Agilent Technologies 6890 GC (Santa Clara, CA, USA) was equipped with a HP-5 column (30 m × 0.25 mm i.d., fused silica, 0.25 μm film thickness, Agilent Technologies), and hydrogen was used as the mobile phase at an average

linear flow rate of 45 cm s^{-1}. Each sample (2 µl) was injected in splitless mode (30 s, injector temperature 225°C). The GC oven temperature was programmed from 35°C (3 min hold) at 10°C min^{-1} to 290°C (10 min hold). At the GC effluent, 4 psi of nitrogen was added and split 1:1 in a Gerstel 3D/2 low dead volume four way-cross (Gerstel, Mülheim, Germany) between the flame ionization detector and the EAD. The GC effluent capillary for the EAD passed through a Gerstel ODP-2 transfer line, which tracked the GC oven temperature, into a glass tube (10 cm × 8 mm), where it was mixed with charcoal-filtered, humidified air (1.5 l min^{-1}). The antenna was placed 0.5 cm from the outlet of this tube. The antennal preparation was made by using the excised head, cutting the distal segment and inserting the distal end of the antenna into a recording glass electrode filled with Beadle–Ephrussi Ringer. The recording electrode was connected to a pre-amplifier probe (10 ×) and then to a high impedance DC amplifier interface box (IDAC-2; Syntech, Kirchgarten, Germany). The reference electrode, filled with Beadle–Ephrussi Ringer, was inserted into the head capsule, and grounded. To obtain consistent antennal response to the sample compound fifteen recordings were preformed.

3.2.6. Chemical analysis

The pooled MR3 headspace extract was analysed on a combined gas chromatograph and mass spectrometer (GC-MS; 6890 GC and 5975 MS; Agilent Technologies), operated in the electron impact ionization mode at 70 eV. The GC was equipped with fused silica capillary columns (60 m × 0.25 mm, 0.25 µm film thickness), DB-wax (J&W Scientific, Folsom, CA, USA) or HP-5MS (Agilent Technologies). Helium was used as the mobile phase at an average linear flow rate of 35 cm s^{-1}. Two micro-litres of the sample were injected. The temperature programmes were the same as for the GC-EAD analysis. Compounds were identified according to retention times

(Kovat's indices) and mass spectra, in comparison with custom made and NIST05 libraries (Agilent), and confirmed by co-injection of authentic standards: (±)-*α*-pinene (CAS no. 7785-70-8; Aldrich, 98%), (-)-*β*-pinene (CAS no. 18172-67-3; Sigma, 99%), 3-carene (CAS no. 13466-78-9; Aldrich, 90%), (±)-limonene (CAS no. 5989-27-5; Sigma, 97%), nonanal (CAS no. 124-19-6; Aldrich, 95%), decanal (CAS no. 112-31-2; Aldrich, 92%), *β*-caryophyllene (CAS no. 87-44-5; Sigma, 98.5%) and sulcatone (CAS no. 110-93-0; Fluka, 96%). For quantification, 100 ng of heptyl acetate (99.8% chemical purity; Aldrich) was added as an internal standard to a 20 µl aliquot out of the total 400 µl headspace extract.

3.2.7. Bioassay with synthetic blend

The assays were carried out in the same two-port olfactometer and oviposition bioassay that were used for the natural extract experiments (n=10). The synthetic blend mimicked the composition and ratio of compounds in the pooled natural extracts of the MR3 rice cultivar (Fig. 1j). Synthetic blends were prepared at different doses in half orders of magnitude between 1 to 1000 ng in pentane. Then, dose-response assays were conducted with the full blend against its solvent control. Subtractive bioassays followed in order to determine the relative activity of the identified components, after establishing the effective release rate (Fig. 1k, l).

3.2.8. Semi-field trials with the synthetic blend

Semi–field experiments were conducted in an outdoor screened-in enclosure (10.8 m long × 6.7 m wide × 2.4 m high) at ICIPE, Thomas Odhiambo Campus, Mbita, Kenya. The ground was covered with sand to a depth of 30 cm for placement of modified BG Sentinel traps (Biogents AG, Regensburg, Germany) (Okal *et al*., 2015). The traps, containing 4 l of distilled water, were

recessed in the corners of the screened house at a distance of 1.5 m from the two adjacent walls. The experimental design used was a randomized complete block design and was performed under ambient environmental conditions. Preliminary trials were conducted to determine the attractiveness of the full synthetic blend dissolved in heptane (Merck, DE), with release rates between 3 to 100 ng min^{-1} with four replications to identify the optimum release rate for the test system. The blend was released by diffusion from a wick dispenser made out of a 2 ml glass vial with a pin hole in the centre of the cap through which a cotton wick encased in Teflon protruded into the air (Birgersson *et al*., 2012). The wick dispenser allows for the release of all compounds in constant proportions throughout the experiment. Heptane was used as a solvent to allow for the release of compounds throughout the course of the experiment. Two hundred gravid females were released in the evening (18:00) and the numbers of mosquitoes recaptured in each catch bag were counted at 08:00. Thereafter, trials were carried out using the cost-effective release rate of 3 ng min^{-1} for 12 nights with the same number of gravid mosquitoes. Furthermore, controls vs. control trials were performed in which the attraction to both traps baited with heptane were evaluated, with the same number of mosquitoes and replications as above. Dissection of the females post-assay confirmed that 100% of the mosquitoes caught in the experiments were gravid.

3.2.9. Statistical analysis

The attraction (AP) and oviposition (OP) preference for the laboratory assays were determined using indices generated by (T-C)/(T+C); whereas a proportion (Pr) was calculated for the semi-field bioassays: Pr=T(C)/(T+C). T is the number of mosquitoes or eggs associated with the test odours and C the number of mosquitoes or eggs associated with the control odours. The

behavioural responses of gravid *An. arabiensis* in the two-port olfactometer and oviposition bioassay were analysed using a nominal logistic fit model, in which choice was the dependent variable, weighted by the number of 1) mosquitoes in the attraction assays and 2) eggs laid in the oviposition assays, with dose as the independent fixed effect and replicate as a random effect (JMP® Pro 12.0.1. SAS Institute Inc., Cary, NC, USA). Here, we report the χ^2 and *p*-value from the Likelihood Ratio Test. The data from semi-field experiments were analysed by using R software (version 3.00) with generalized linear model (GLM) using a quasi-binomial distribution in R statistical software version 2.13 (Team, 2013).

3.3. Results

3.3.1. Gravid malaria mosquitoes are attracted to rice odour

To assess whether *An. arabiensis* respond behaviourally to rice odours, we collected the headspace of two rice cultivars, MR1 and MR3, at three different phenological stages: tillering, booting and flowering. Gravid mosquitoes were significantly attracted, in a dose-dependent manner, to the headspace of both MR1 and MR3 cultivars in a two-port olfactometer (Fig. 3.1a), regardless of phenological state, when compared to the hexane controls (Booting, HEX *vs*. MR1: χ^2=6.104, P=0.0135; Booting, HEX *vs*. MR3:χ^2=13.55, P=0.0002; Tillering, HEX *vs*. MR1: χ^2=14.02, P=0.0002; Tillering, HEX *vs*. MR3:χ^2=13.13, P=0.0003; Flowering, HEX *vs*. MR1: χ^2=18.26, P<0.0001; Flowering, HEX *vs*. MR3: χ^2=6.083, P=0.0136; Fig. 3.2a-f) and the headspace of breeding water (Booting, BW *vs*. MR1: χ^2=9.628, P=0.0019; Tillering, BW *vs*. MR1: χ^2=8.207, P=0.0042; Flowering, BW *vs*. MR1: χ^2=18.26, P<0.0001; Booting, BW *vs*. MR3: χ^2=13.18, P=0.0003; Tillering, BW *vs*. MR3: χ^2=21.30, P<0.0001; Flowering, BW *vs*.

MR3: χ^2=6.083, P=0.0136; Fig. 3.3 a-f), suggesting that rice volatiles can attract over the short range. Similarly, gravid females preferred to lay their eggs in water treated with the headspace of all phenological stages of the MR1 and MR3 cultivars over controls (Booting, HEX *vs*. MR1: χ^2=14.58, P<0.0001; Booting, HEX *vs*. MR3: χ^2 =10.02, P=0.0015; Tillering, HEX *vs*. MR1: χ^2=9.607, P=0.0019; Tillering, HEX *vs*. MR3: χ^2=9.959, P=0.0016; Flowering, HEX *vs*. MR1: χ^2=13.34, P=0.0003; Flowering, HEX *vs*. MR3: χ^2=20.57, P<0.0001; Fig 3.4a-f; Booting, BW *vs*. MR1: χ^2=10.18, P=0.0014; Booting, BW *vs*. MR3:χ^2=9.631, P=0.0019; Tillering, BW *vs*. MR1: χ^2=9.837, P=0.0017,; Tillering, BW *vs*. MR3**,** χ^2=9.200, P=0.0024; Flowering, BW *vs*. MR1: χ^2=6.262, P=0.0123; Flowering, BW *vs*. MR3: χ^2=10.03, P<0.0015; Fig. 3.5a-f) in a two-choice oviposition assay (Fig. 3.1b), suggesting that the rice volatiles act as oviposition stimulants.

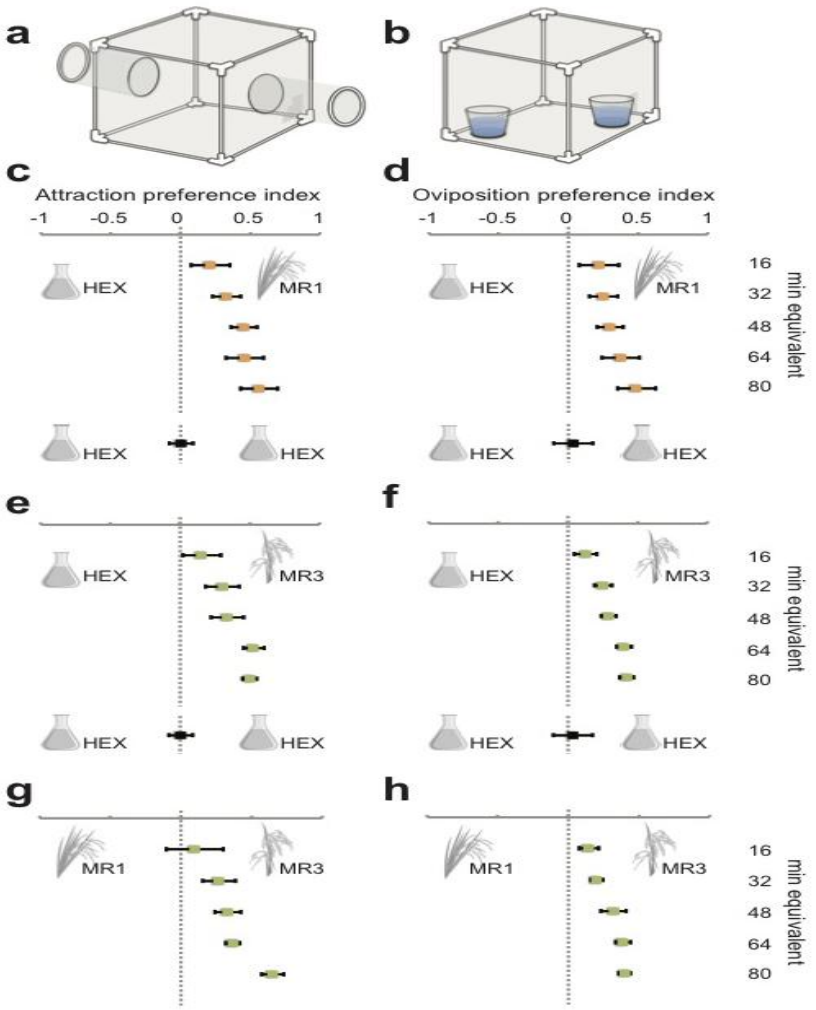

Figure 3.1 Gravid *Anopheles arabiensis* respond to rice odour. Diagrams of the two-port olfactometer (a) and oviposition (b) assays. Attraction (c, e) and oviposition (d, f) preference of mosquitoes to headspace volatiles of the MR1 and MR3 rice cultivars compared to hexane control, respectively. The headspace of the MR3 rice cultivar significantly attracted (g) and stimulated oviposition (h) of mosquitoes over that of the MR1 cultivar. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

As no significant differences were observed in the behavioural responses to the headspace of the phenological stages of either of the cultivars (MR1, Dose, χ^2=32.74, P<0.0001, Stage, χ^2=0.2267, P=0.8928; MR3, Dose, χ^2=29.53, P<0.0001, Stage, χ^2=0.3283, P=0.8486; Fig. 3.2; MR1, Dose, χ^2=30.63, P<0.0001, Stages, χ^2=0.3287, P=0.8485; MR3, Dose, χ^2=42.90, P<0.0001, Stages, χ^2=0.2532, P=0.8811 Fig. 3.3; MR1, Dose, χ^2=33.90, P<0.0001, Stage, χ^2=0.5059, P=0.7765; MR3, Dose, χ^2=42.89, P<0.0001, Stage, χ^2=5.426, P=0.0663; Fig. 3.5), the headspace extracts of each cultivar were pooled for use in subsequent behavioural assays. While the pooled headspace of both cultivars were attractive and stimulated oviposition in a dose-dependent manner when compared to hexane (HEX *vs*. MR1: χ^2=11.87, P=0.0006; HEX *vs*. MR3: χ^2=8.378, P<0.0038; HEX *vs*. MR1: χ^2=16.21, P<0.0001; HEX *vs*. MR3: χ^2=14.05, P=0.0002; Fig. 3.1c-f) and breeding water controls (BW *vs*. MR1:χ^2=11.51, P=0.0007; BW *vs*. MR3: χ^2=11.88, P=0.0006; Fig. 6a,b; BW *vs*. MR1: χ^2=11.34, P=0.0008; BW *vs*. MR3: χ^2=7.538, P=0.0060; Figs. 3.7a,b), the pooled MR3 headspace was preferred over that of MR1 in both assays (MR1 *vs*. MR3: χ^2=7.080, P=0.0078; MR1 *vs*. MR3: χ^2=5.822, P<0.0158, P<0.019; Fig. 3.1g, h and, MR1 *vs*. MR3: χ^2=4.740, P=0.0295; Fig. 3.7c). The observed behavioural preference indicates that gravid females can discriminate between odour profiles emitted by different rice cultivars.

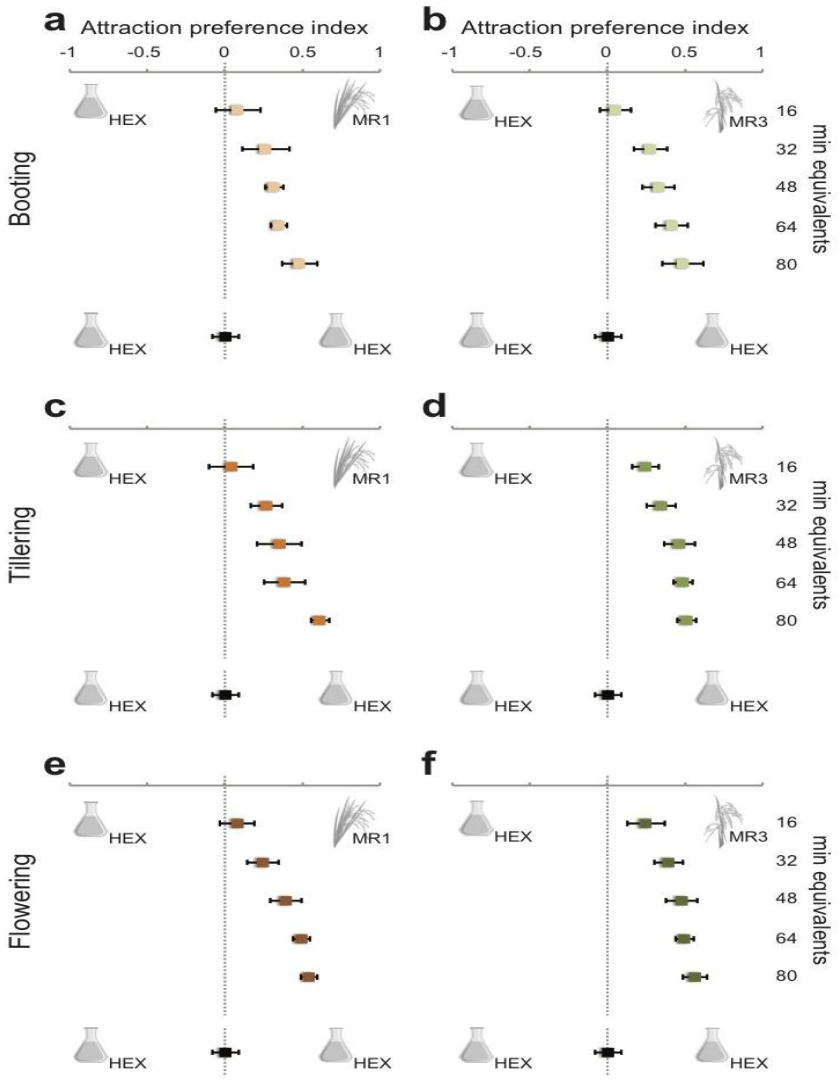

Figure 3.2 Attraction preference of *Anopheles arabiensis* to the booting, tillering and flowering stages of the MR1 (a, c, e) and MR3 (b, d, f) cultivars against hexane controls (HEX). Headspace volatiles of the three phenological stages of MR1 and MR3 rice cultivars elicited dose-dependent attraction preference. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

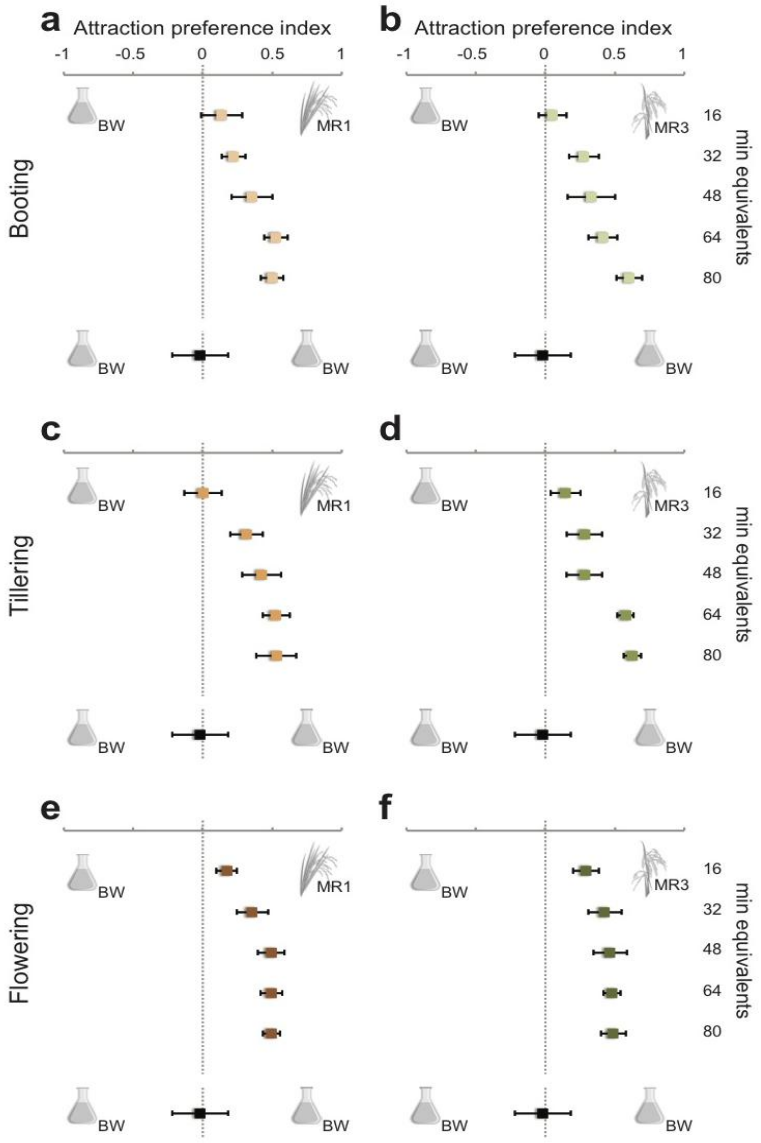

Figure 3.3 Attraction preference of *Anopheles arabiensis* to the booting, tillering and flowering stages of the MR1 (a,c,e) and MR3 (b,d,f) cultivars against the headspace of breeding water (BW). Headspace volatiles of the three phenological stages of MR1 and MR3 rice cultivars elicited dose-dependent attraction preference. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

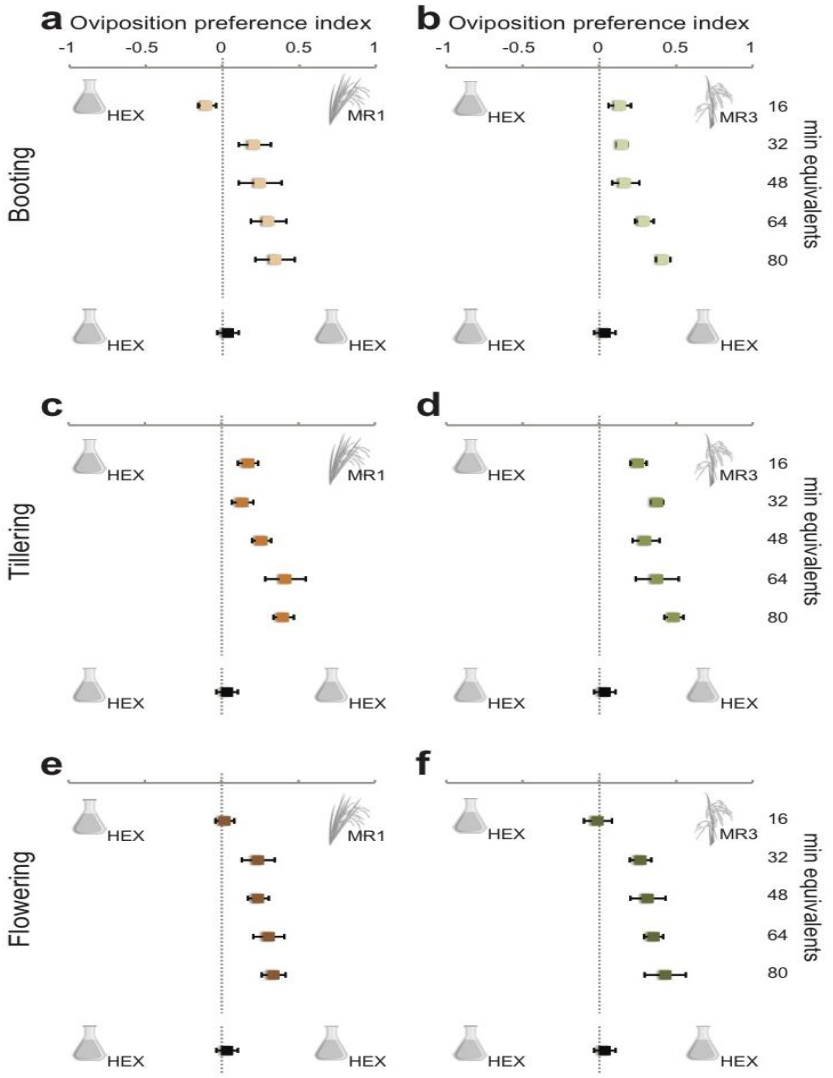

Figure 3.4 Oviposition preference of *Anopheles arabiensis* to the booting (a, b), tillering (c, d) and flowering (e, f) stages of the MR1 (left) and MR3 (right) cultivars against hexane controls (HEX). Headspace volatiles of the three phenological stages of MR1 and MR3 rice cultivars added to distilled water elicited dose-dependent oviposition. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

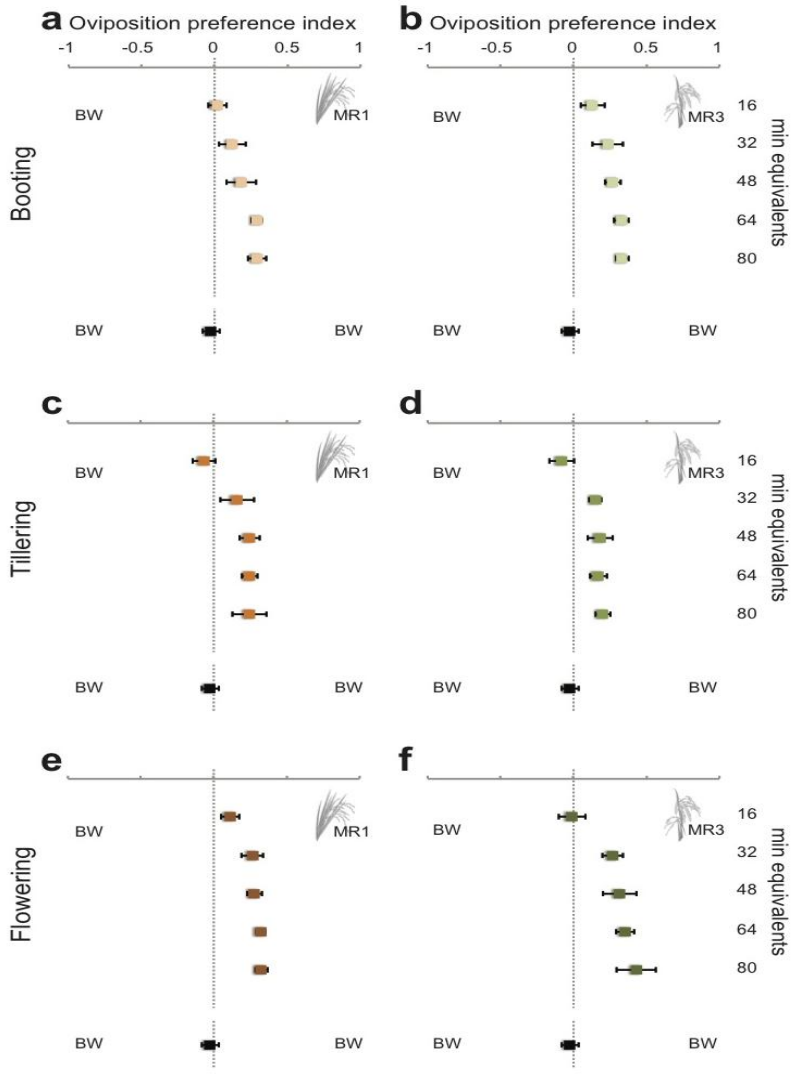

Figure 3.5 Oviposition preference of *Anopheles arabiensis* to the booting (a, b), tillering (c, d) and flowering (e, f) stages of the MR1 (left) and MR3 (right) cultivars against hexane controls in breeding water (BW). Headspace volatiles of the three phenological stages of MR1 and MR3 rice cultivars added to breeding water elicited dose-dependent oviposition preference. Different letters denote significant differences between doses. Ten replicates of 10 mosquitoes each were used in each behavioural experiment. Error bars represent standard errors of the mean.

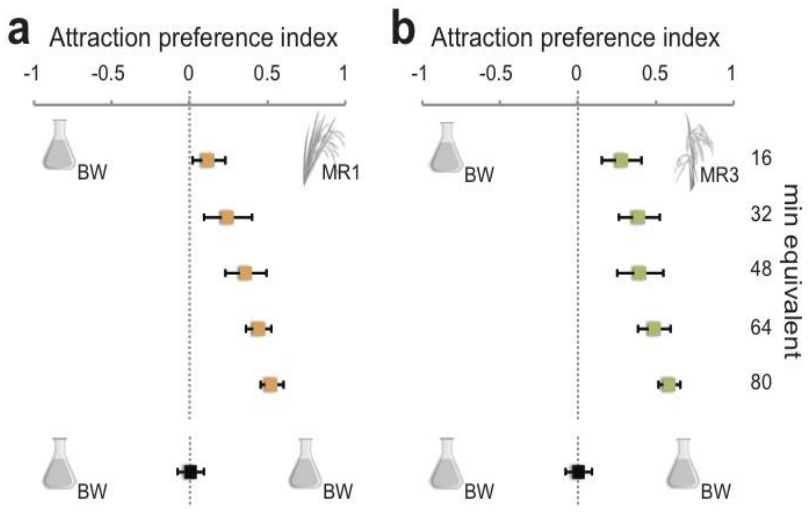

Figure 3.6 Attraction preference of *Anopheles arabiensis* to the pooled headspace of MR1 (a) and MR3 (b) cultivars compared to the headspace of breeding water (BW). Headspace volatiles of the MR1 and MR3 rice cultivars elicited a dose-dependent attraction preference to the headspace of the breeding water control. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

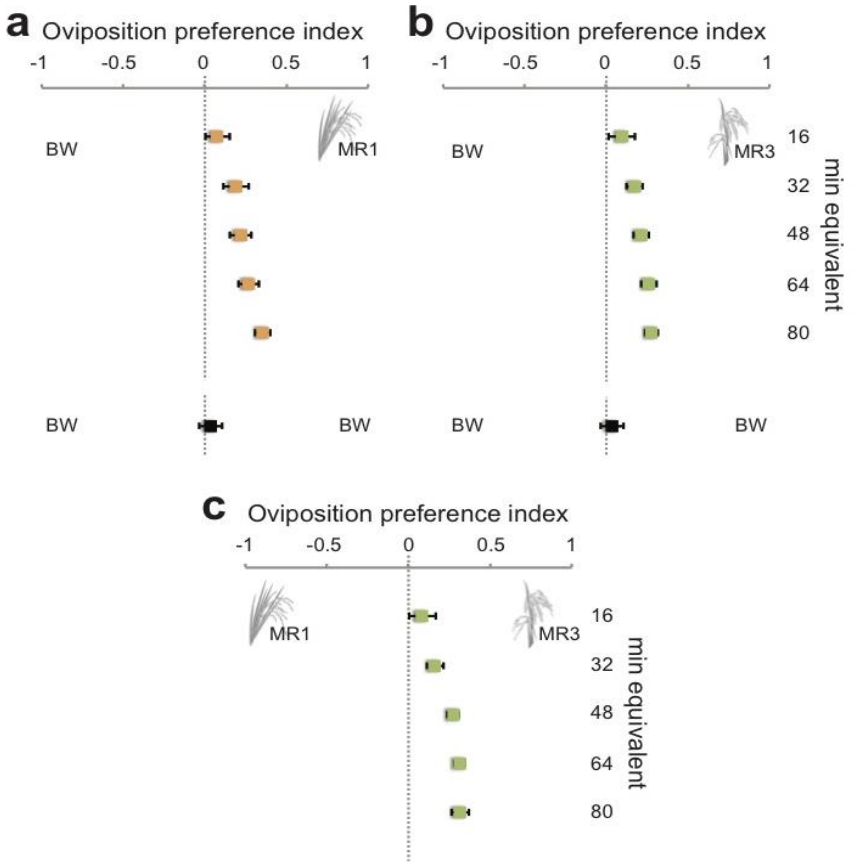

Figure 3.7 Oviposition preference of *Anopheles arabiensis* to the pooled headspace of MR1 (a) and MR3 (b) cultivars compared to hexane in breeding water (BW) and between the headspace of the cultivars in BW (c). Headspace volatiles of the MR1 and MR3 rice cultivars elicited a dose-dependent oviposition preference to the headspace of the breeding water control. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

3.3.2. A complex odour blend drives the oviposition behaviour

Of the eight compounds in the preferred MR3 headspace eliciting antennal responses in gravid female *An. arabiensis* (Fig. 3.1 c, d), limonene, sulcatone, decanal, nonanal *ß*-pinene, α-pinene, *ß*-caryophyllene and 3-carene were identified from MR3 rice cultivar. The overall volatile

release rate was 24 ng min^{-1}, with limonene, nonanal and *ß*-caryophyllene as the most abundant compounds (Fig. 3.8d).

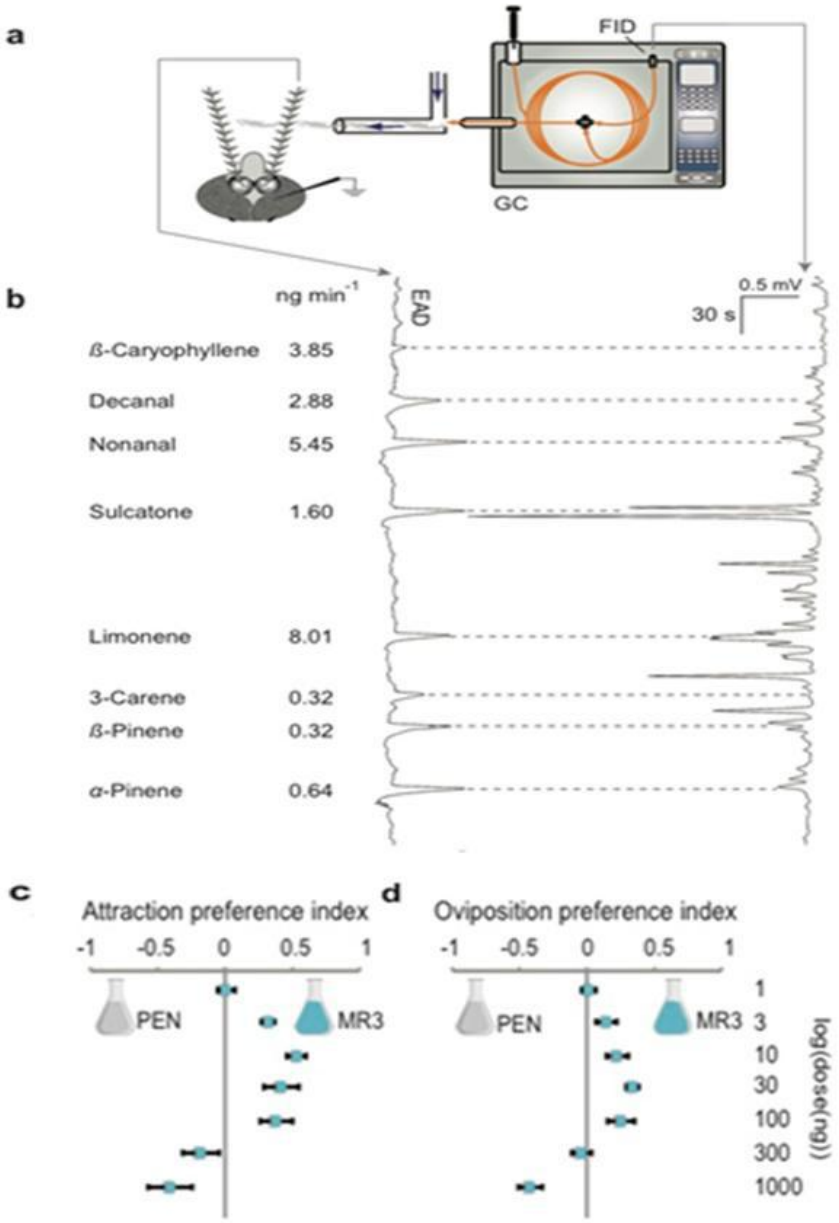

Figure 3.8 A synthetic blend composed of the bioactive compounds identified, in their natural ratio (b), elicited attraction (c) and stimulated oviposition (d) in gravid mosquitoes in a dose-

dependent manner. a, Schematic of the combined gas chromatograph (GC) and electroantennographic detection (EAD) analysis. FID, flame ionization detector. d, EAD traces depict voltage changes (mV) in response to the bioactive compounds in the headspace of the MR3 rice cultivar, eluting from the GC and identified by the FID. The identity and release rate of the bioactive compounds are shown at the left.

A synthetic blend of all eight GC-EAD-active compounds identified in the MR3 headspace, in their natural ratio, elicited short-range attraction and stimulated oviposition in gravid *An. arabiensis* in a dose-dependent manner (PEN *vs*. MR3: χ^2=18.34, P<0.0001; PEN *vs*. MR3: χ^2=32.93, P<0.0001; Fig. 3.8 c, d). Subtraction of individual components from the full blend released at the lowest effective dose tested, 10 ng, did not significantly reduce the attraction of gravid females compared to the full blend, with the exception of nonanal (χ^2=14.68, P=0.1002; Fig. 3.9). However, nonanal by itself was unable to elicit attraction equivalent to that of the full blend (Odds ratio=2.306, P=0.0225; Fig. 3.9). In contrast, the oviposition response of gravid females was significantly reduced for all the subtractive blends, except *β*-pinene (χ^2=22.09, P<0.0001; Fig. 3.10), however, not to the level of the full blend (χ^2=4.82, P=0.0282; Fig. 3.10).

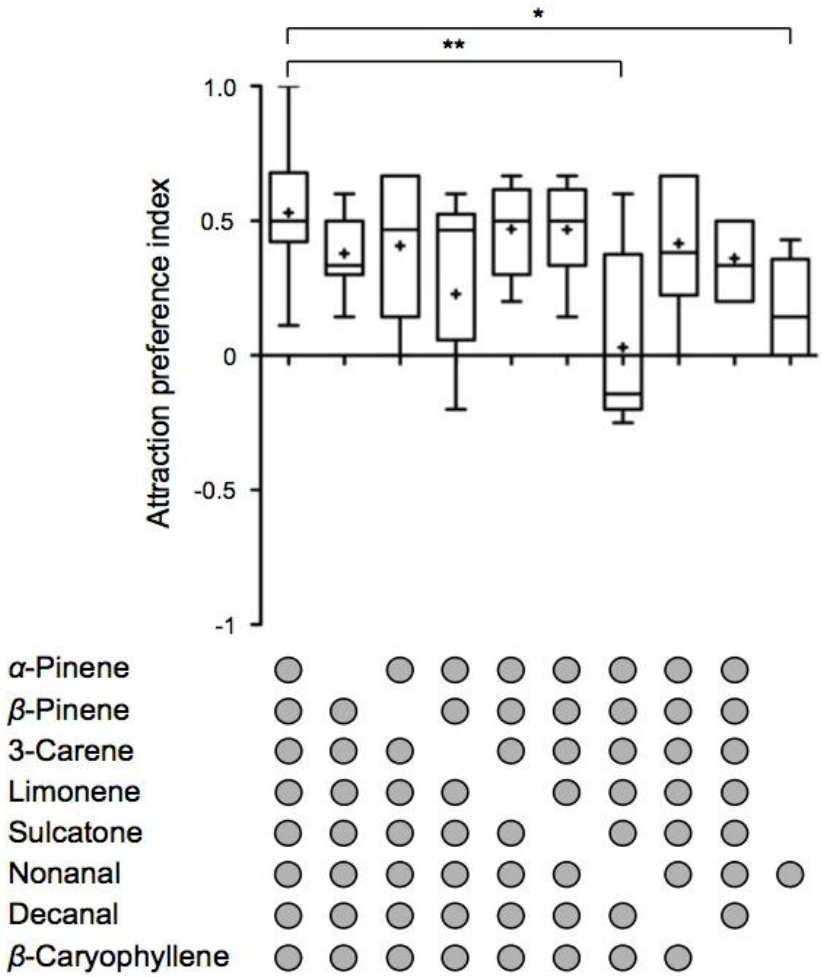

Figure 3.9 Attraction preference of *Anopheles arabiensis* to the full and subtractive synthetic MR3 blends against hexane controls. Overall, the subtractive blends were found to elicit a lower attraction preference compared with the full blend. Statistical significance (*** P<0.001; ** P<0.01; * P<0.05). Error bars represent standard errors of the mean**.**

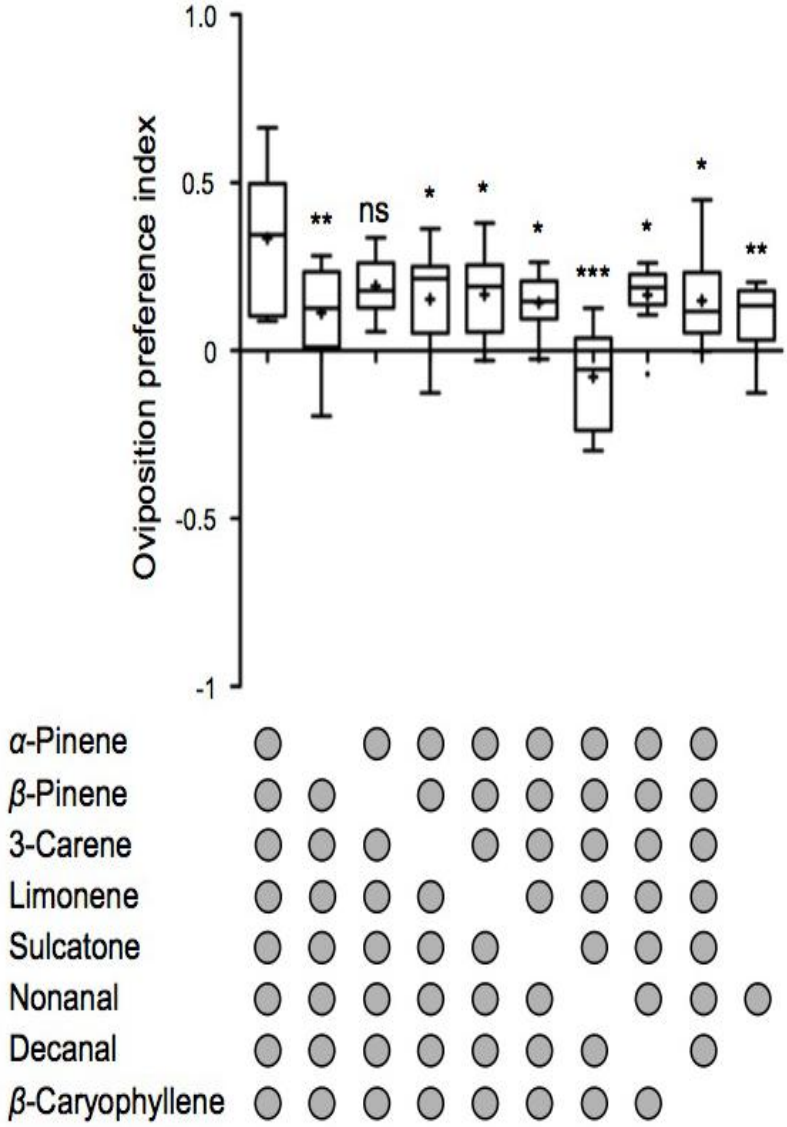

Figure 3.10 Oviposition preference of *Anopheles arabiensis* to the full and subtractive synthetic MR3 blends against hexane controls in distilled water. Overall, the subtractive blends elicited a reduced oviposition preference compared with the full blend. Statistical significance (*** P<0.001; ** P<0.01; * P<0.05). Error bars represent standard errors of the mean.

3.3.3. Long range attraction to the blend

An evaluation of the synthetic MR3 odour blend in large outdoor screened-in enclosures (Fig. 3.12a) revealed that oviposition site-seeking *An. arabiensis* were attracted over the long-range and caught in a dose-dependent manner in recessed BG sentinel traps (GLM, $F_{(7,24)}$=11.912, P<0.0001; Fig. 3.11). Based on this pilot experiment, the lowest effective release rate tested (3 ng min^{-1}) was used in further trials. Gravid *An. arabiensis* were significantly attracted to the full synthetic blend (3 ng min^{-1}; Pr = 0.74, 95% CI 0.68-0.79; Fig. 3.12b) when compared against the solvent control (heptane), whereas no significant difference was found between the solvent controls (Pr = 0.51, 95% CI 0.44-0.58; Fig. 3.12c).

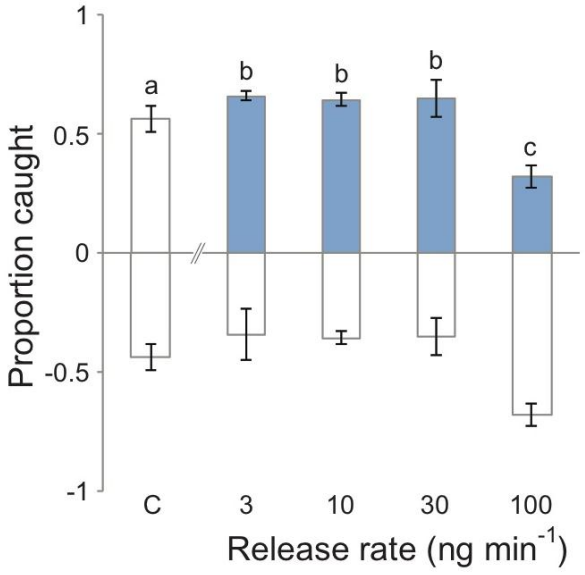

Figure 3.11 Pilot experiment to determine the effective release rate of the synthetic MR3 odour blend. Different letters denote significant differences between doses.

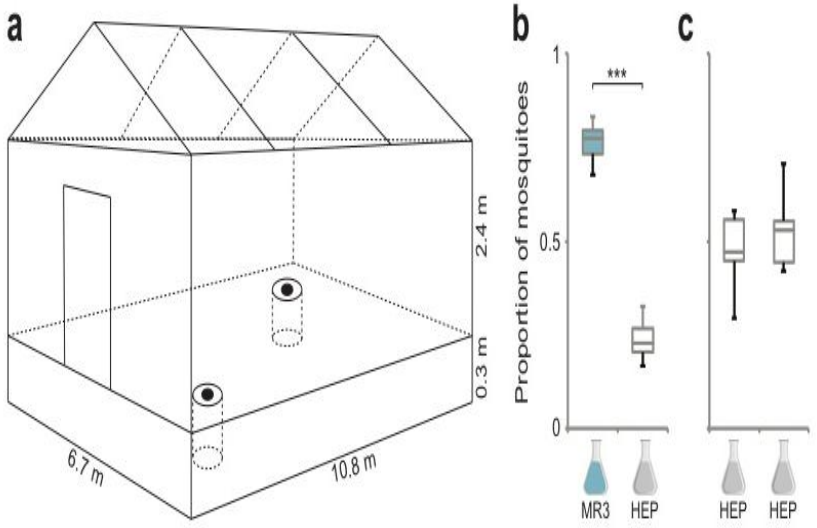

Figure 3.12 Long range attraction of gravid *Anopheles arabiensis* to the synthetic rice blend. a, Diagram of the semi-field screened-in enclosure and the recessed BG-Sentinel traps used to attract and capture free-flying mosquitoes, b, synthetic blend compared to a hexane control, c, between heptane controls.

3.4. Discussion

Gravid *An. arabiensis* are attracted and stimulated to oviposit by rice plant volatiles, strengthening the spatial association between the malaria vector and the agricultural landscape. Female mosquitoes were attracted to volatiles emitted by all stages of the rice plant, which concurs with field observations showing that the larvae are present in rice cultivation throughout the growing season(s) (Mutero *et al*., 2004; Mwangangi *et al*., 2010). Rice volatiles thus can play a significant role in the maintenance of vector populations, along with other aspects of rice cultivation, including water availability, insecticide- and fertiliser-use, and sunlit water surface (Service, 1977; Mutero *et al*., 2004; Muturi *et al*., 2006; Mwangangi *et al*., 2006; Jarju *et al*.,

2009; Mwangangi *et al*., 2010). The selective advantage for this association is yet to be determined. The enhanced availability of food in rice fields due to microbial growth in response to nitrogen surplus and water clarity following fertiliser application (Mutero *et al*., 2004), along with the reduction of competition and predators, by insecticide treatment (Service, 1977), are possible drivers for this adaptive selection.

Mosquitoes use volatiles originating from plants as attractants and stimulants for oviposition (Afify and Galizia, 2015). While gravid anopheline mosquitoes display preferences for volatiles associated with living plants (Torres-Estrada *et al*., 2005) and microbes associated with their larval habitats (Sumba *et al*., 2004; Lindh *et al*., 2015), culicines prefer those associated with the microbial fermentation of plant tissues (Mutero *et al*., 2004; Herrera-Varela *et al*., 2014). Here, we have identified a long-range plant odour-based attractant for gravid malaria mosquitoes, providing the integrated vector management community with new perspectives for developing a tool that targets gravid anophelines regardless of feeding and resting preferences. The current malaria vector control strategies in widespread use, the IRS and LLINs, which focus on indoor feeding and resting mosquitoes, may benefit greatly from the addition of a complementary odour-based tool targeted against gravid females.

3.5. Conclusions

Oviposition site selection is a key factor for the vector population dynamics and epidemiology of malaria. The propagation of the malaria vector, *Anopheles arabiensis*, in sub-Saharan Africa has significantly increased as a result of intensified agricultural activity, particularly in rice cultivation. Female mosquitoes that are able to select breeding sites within irrigated rice

cultivation provide their offspring with selective advantages. We show that gravid *An. arabiensis* are differentially attracted and stimulated to oviposit in response to collected headspace odours from two rice cultivars. Furthermore, we identify an eight-component synthetic rice odour blend that elicits long-range attraction of free-flying mosquitoes under semi-field conditions, as well as stimulates oviposition. The identification of cues from oviposition habitats provide important substrates for the development of novel control measures that target female malaria mosquitoes, irrespective of indoor or outdoor feeding and resting patterns.

Chapter IV

Electrophysiological and behavioural responses of gravid *Anopheles arabiensis* to maize pollen volatiles

4.1. Introduction

Oviposition site selection behaviour provides a much-needed target for vector control (Takken and Knols, 1999), as selection of oviposition sites is an essential part of the mosquito life history, and a critical factor in their survival and population dynamics (Bentley and Day, 1989). Gravid females should select enemy-free and productive habitats for their offspring, as mosquito aquatic stages are restricted in mobility within the maternally selected habitats (Bentley and Day, 1989; Afify and Galizia, 2015). Breeding habitats with reduced predator (Munga *et al*., 2006; Silberbush *et al*., 2010) and competitor pressures (Gimnig *et al*., 2002; Munga *et al*., 2006), as well as sufficient food availability, are vital for determining mosquito fitness (Yoshioka et al., 2012), and directly affects vectorial capacity and competence (Ye-ebiyo *et al*., 2000; Okech *et al*., 2007; Araujo *et al*., 2012; Oliver *et al*., 2013). Larval mosquito diets have hitherto been shown to contain microorganisms, including algae and bacteria, along with pollen and particulate organic detritus (Merritt *et al*., 1992; Ye-ebiyo *et al*., 2000; Gimnig *et al*., 2002; Kivuyo *et al*., 2014). Maize pollen, in particular, serves as an important source of larval nourishment for *Anopheles* mosquitoes (Kivuyo *et al*., 2014), which are adapted to breed in transient turbid water that can often be found associated with agricultural activities (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a). Feeding on maize pollen enhances larval development, increasing the likelihood of large

adults with increased longevity, fitness and resistance to insecticides (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a; Oliver *et al*., 2013; Kivuyo *et al*., 2014), and results in the intensification of malaria transmission (Kebede *et al*., 2005). Female mosquitoes that are able to detect breeding sites where maize pollen is abundant may thus provide their offspring with selective advantages, including survival and development (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a; Kivuyo *et al*., 2014). Identifying and manipulating sensory cues that mediate a female mosquito's ability to choose superior oviposition sites could provide important insights essential to developing novel mosquito control tools.

For many mosquito species, oviposition site selection is dependent on olfactory cues from plant and microbial origin (Bentley and Day, 1989; Afify and Galizia, 2015; Lindh *et al*., 2015). Although some of the compounds released by these sources have been identified for *Aedes* and *Culex* mosquitoes (Bentley and Day, 1989; Afify and Galizia, 2015), we are only now starting to understand the complexity of chemical cues regulating oviposition site selection in *Anopheles* mosquitoes (Lindh *et al*., 2015; Chapter III of this thesis). To date, synthetic compounds that attract and stimulate oviposition in gravid *Anopheles gambiae* and *An. arabiensis* have been identified from emergent vegetation, such as rice, as well as microbes and associated microbial activity in the breeding water (Takken and Knols, 1999; Sumba *et al*., 2004; Chapter III of this thesis). In this study, we analysed the behavioural response of gravid *An. arabiensis* to volatile headspace collections from maize pollen collected from two varieties, BH-660 and ZM-521. We specifically selected the high-yield and late pollen-shedding variety, BH-660, the cultivation of which has been shown to correlate with higher malaria transmission than other cultivated varieties (Kebede *et al*., 2005). We showed that volatiles released by pollen of both varieties

elicited attraction and stimulated oviposition, although the volatiles of BH-660 pollen were preferred when tested in two-port olfactometer and oviposition assays. Subsequent gas chromatography and electroantennographic detection analysis, in combination with subtractive behavioural assays, allowed us to identify a synthetic odour blend, which elicited the full oviposition behavioural repertoire of the gravid mosquitoes.

4.2. Methods and Materials

4.2.1. Experimental mosquitoes

Anopheles arabiensis, Nazareth and Dongola strains were used for behavioural and electrophysiological analyses in Ethiopia and Sweden, respectively. The colonies were maintained at 27±2°C, 75±5% relative humidity and under a 12 h light/12 h dark cycle. Briefly, the aquatic stages of the mosquitoes were reared in distilled water, and fed Faffa food (Ethiopia) or Tetramin® fish food (Sweden). Pupae were transferred from the rearing trays in 100 ml polypropylene cups (Qingdao Ori-Color Industry and Commerce Co., Ltd., China), containing distilled water, to cages (30 cm × 30 cm × 30 cm; custom-made or Bugdorm, MegaView Science, Taiwan). The emerging adults were supplied with 10% sucrose solution *ad libitum*. Five days after emergence, female mosquitoes were offered a rabbit (Ethiopia) or sheep blood (Sweden) from an artificial feeder (Hemotek, Discovery Workshops, Accrington, UK) over the course of two days, 3 h per day. For all experiments, gravid females, 3 days post-blood feeding, were selected by visually inspecting the pale white abdomen and used for bioassays.

4.2.2. Headspace volatile collection

The headspace of BH-660 and ZM-521 maize pollen was collected at Melkassa Agricultural Research Center, in East Oromia region of Ethiopia. In addition, headspace was collected from the water of a major malaria mosquito breeding site at the shore of lake Ziway, Ethiopia. The inflorescence of a fully mature male flower (45 replicates) which will be called pollen from hereby was enclosed in a polyacetate bag (Toppits, Cofresco, Germany), and a charcoal-filtered continuous airstream (1 l min^{-1}) was drawn by a Personal Air Sampler (PAS-500, Spectrex, Redwood City, CA, USA) over the tassel, onto an aeration column for 3 h. Alternatively, for collecting the headspace from breeding water (35 replicates), 1 l was poured into a polyacetate bag, after which the headspace was collected for 3 h using a diaphragm vacuum pump (12 V, KNF-Neuberger, Freiburg, Germany), using charcoal-filtered air as described above. The aeration columns were made of Teflon tubing (6 cm x 3 mm i.d.), filled with 35 mg Super Q (80/100 mesh; Alltech, Deerfield, IL, USA) between polypropylene wool plugs and nylon stoppers. The aeration columns were cleaned with 1 ml *n*-hexane (LabScan, Malmö, Sweden), re-distilled before use. Adsorbed volatiles were eluted with 300 µl re-distilled *n*-hexane. Headspace volatile extracts were stored in glass vials at -20°C until used for behavioural and electrophysiological analyses.

4.2.3. Two-port olfactometer procedures

A two-port olfactometer, which is described above (Chapter III, Section 3.2.3.) was used to test the attraction preference of the mosquitoes for the headspace volatiles collected from the BH-660 and ZM-521 pollen, and from natural breeding water. Attraction preference of the mosquitoes to the following treatments were analysed: a) headspace volatiles of breeding water *vs.* hexane

control, b) headspace volatiles of BH-660 or ZM-521 pollen *vs.* hexane, c) headspace volatiles of BH-660 or ZM-521 pollen *vs.* headspace volatiles of breeding water, and d) headspace volatiles of BH-660 *vs.* headspace volatiles ZM-521 pollen. For all treatments, a hexane *vs.* hexane control was performed. The behavioural response to the volatile headspace of the two maize pollen varieties was analysed to increasing amounts of headspace extract from the two pollen varieties. After five minutes, the behavioural responses of the mosquitoes were scored by counting the number of mosquitoes in each arm. Ten replicates *per* treatment and *per* dose were performed.

4.2.4. Oviposition bioassay

The oviposition preference of gravid mosquitoes was analysed in a two-choice assay (Chapter III, Section, 3.2.4.). Treatment cups were conditioned by dosing the oviposition substrate with the same range of doses of headspace volatile extracts of BH-660 and ZM-521 pollen in hexane, as described above. An equivalent amount of hexane was used as a control. In addition, a hexane *vs*. hexane control was performed for all treatments. Gravid mosquitoes were transferred from the maintenance cages at dusk (18:00), and on the following day (09:00) the numbers of eggs in the two cups were counted.

4.2.5. Electrophysiological analysis

Antennal responses of gravid female *An. arabiensis* to the headspace extract of BH-660 pollen were analysed using GC-EAD. For this analysis similar techniques were followed as it was mentioned in above (Chapter III, Section 3.2.5.).

4.2.6. Chemical analysis

The BH-660 pollen headspace extract was injected (2 µl) and analysed on a combined gas chromatograph and mass spectrometer (Chapter III, Section 3.2.6.). Compounds were identified according to retention times (Kovat's indices) and mass spectra, in comparison with custom made and NIST05 libraries (Agilent), and confirmed by co-injection of authentic standards: (±)-*α*-pinene (Cas no. 7785-70-8; Aldrich, 98%), (±)-limonene (Cas no. 5989-27-5; Sigma, 97%), nonanal (Cas no. 124-19-6; Aldrich, 95%), benzaldehyde (Cas no. 100-52-7; Aldrich, 99%), *p*-cymene (Cas no. 99-87-6; Aldrich, 97%). For quantification, 100 ng of heptyl acetate (99.8% chemical purity; Aldrich) was added as an internal standard.

4.2.7. Bioassay with synthetic blend

The assays were carried out in the same two-port olfactometer and oviposition bioassay that were used for the natural headspace extract experiments. The synthetic blend mimicked the composition and ratio of compounds in the headspace collected from BH-660 pollen. Synthetic blends were prepared at seven different doses between 1 and 1000 ng in pentane dispensed from dental-wick and in distilled water for the attraction and oviposition assays, respectively. Thereafter, subtractive blends, in which single compounds of the full blend were removed, were tested against the full blend (240 ng).

4.2.8. Statistical analysis

A preference index was calculated, (T-C)/(T+C), for both attraction preference (AP) and oviposition (OP) preference; where T is the number of mosquitoes or eggs associated with the test odours, and C the number of mosquitoes or eggs associated with the control odours. The

behavioural responses of gravid *An. arabiensis* in the two-port olfactometer and oviposition bioassay were analysed using a nominal logistic fit model, in which choice was the dependent variable, weighted by the number of 1) mosquitoes in the attraction assays and 2) eggs laid in the oviposition assays, with dose as the independent fixed effect and replicate as a random effect (JMP® Pro 12.0.1. SAS Institute Inc., Cary, NC, USA). Here, we report the χ^2 and *p*-value from the Likelihood Ratio Test. The generation of the good fit logistic models were made by omitting the highest dose, which in most cases was shown to cause either a neutral or avoidance behaviour.

4.3. Results

4.3.1. Maize pollen volatiles attract gravid mosquitoes

Gravid *An. arabiensis* were significantly attracted, over a range of doses, to the headspace collections of pollen collected from the ZM-521 and BH-660 maize varieties in a two-port olfactometer, when compared to the hexane control (ZM-521: χ^2=9.647, P=0.0019; BH-660: χ^2=8.976, P=0.0027; Fig. 4.1a, c) and the headspace of breeding water (ZM-521: χ^2=10.66, P=0.0011; BH-660: χ^2=16.77, P<0.0001; Fig. 4.1b, d). Comparison between the two varieties showed a significantly higher attraction to the headspace of BH-660 pollen by gravid *An. arabiensis* than to that of ZM-521 (χ^2=9.648, P=0.0019; Fig. 4.1e). No significant difference was observed in the attraction to the hexane control and the headspace of breeding water when tested in the same assay (χ^2=0.5968, P=0.4398; data not shown).

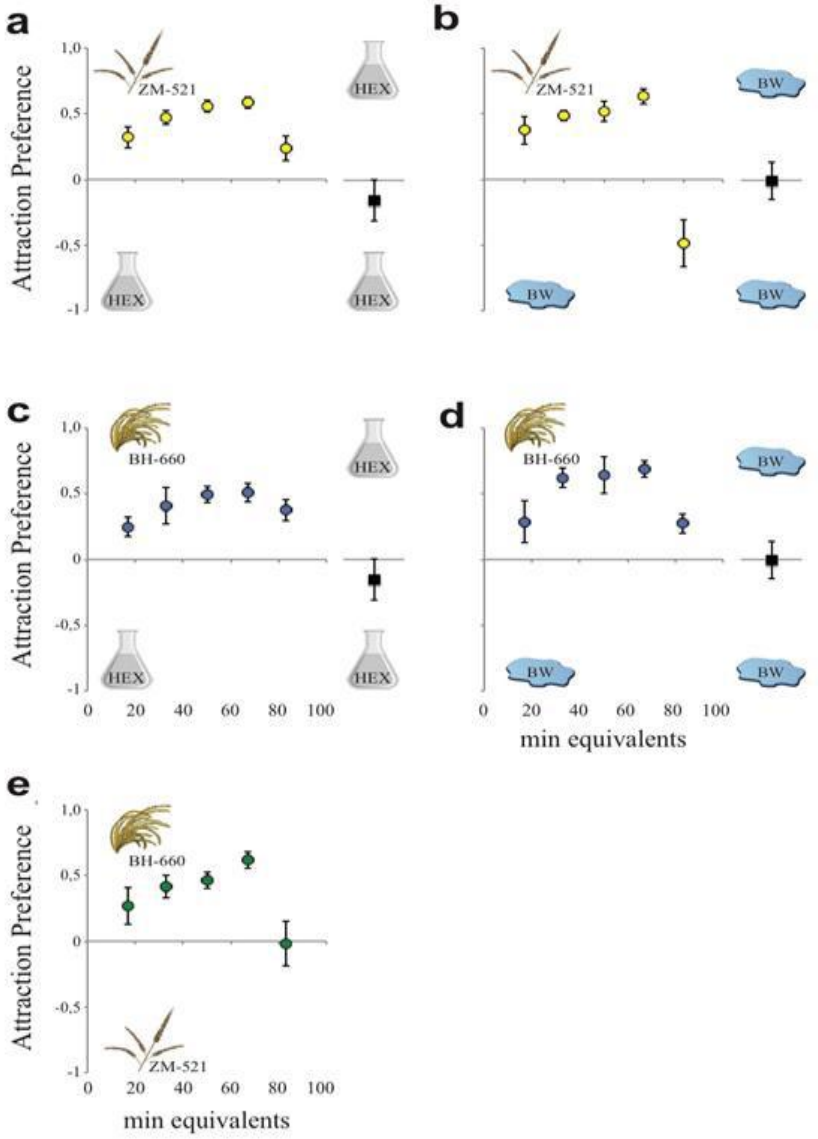

Figure 4.1 Headspace volatiles of ZM-521 and BH-660 maize pollen attracts gravid *Anopheles arabiensis*. Attraction preference of mosquitoes to headspace volatiles of the ZM-521 (a, b) and BH-660 (c, d) maize pollen compared to the controls, hexane (H, left) and headspace of breeding water (BW, right), in the two-port olfactometer. The headspace of the BH-660 maize pollen was significantly preferred over that of the ZM-521 (e). Controls (hexane *vs.* hexane) are denoted by

black squares (a-d). Different lowercase letters indicate significant differences. Error bars denote standard error of the mean.

4.3.2. Maize pollen volatiles stimulate oviposition in gravid mosquitoes

Gravid females preferred to lay their eggs in both distilled and breeding water conditioned with the headspace of ZM-521 and BH-660 maize pollen, over water with hexane added as a control (ZM-521 *vs*. distilled water: χ^2=4.405, P=0.0358; BH-660 *vs*. distilled water: χ^2=7.887, P=0.0050; ZM-521 *vs*. breeding water: χ^2=8.980, P=0.0027; BH-660 *vs*. breeding water: χ^2=6.812, P=0.0091; Fig. 2a-d). Comparison between the headspace of the two varieties demonstrated a significantly higher number of eggs laid by *An. arabiensis* in response to the headspace of BH-660 pollen than to that of ZM-521, irrespective of the oviposition substrate (distilled water: χ^2=11.71, P=0.0006; Fig. 2e; breeding water: χ^2=8.492, P=0.0036; Fig. 2f). The number of eggs laid by *An. arabiensis* was not significantly different between the two controls (χ^2=0.1959, P=0.6581; data not shown).

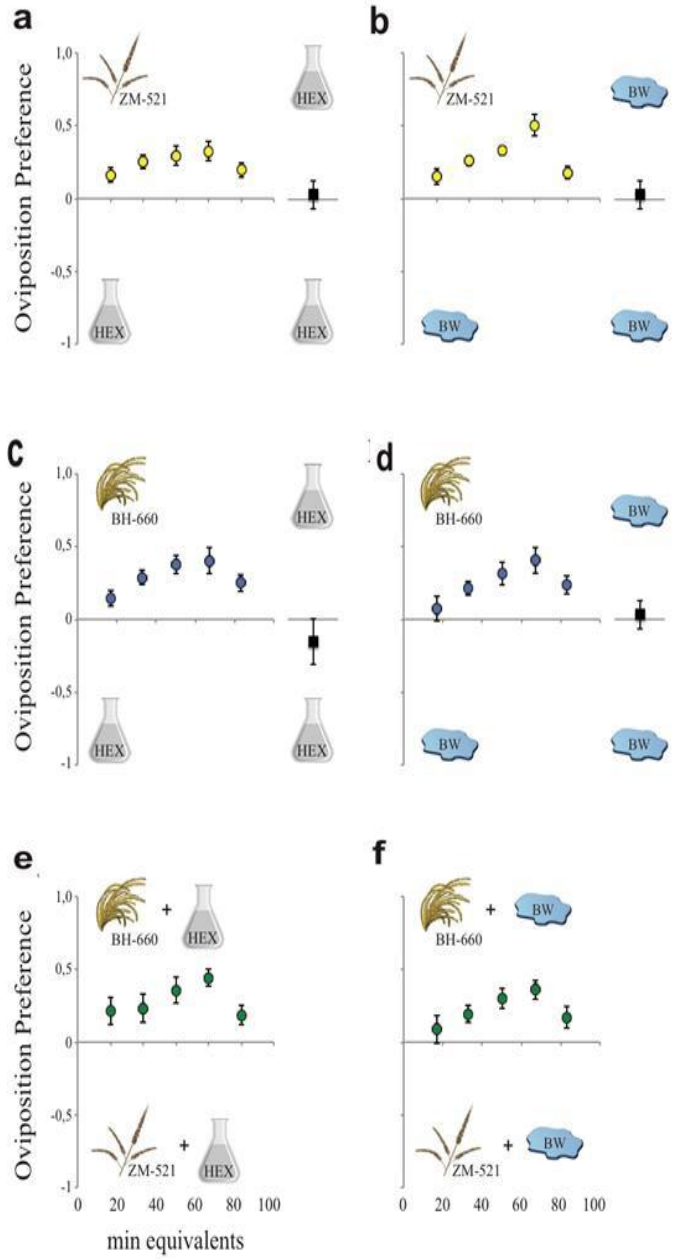

Figure 4.2 Headspace volatiles of BH-660 and ZM-521 maize pollen stimulates oviposition by gravid *Anopheles arabiensis*. Oviposition preference of mosquitoes to headspace volatiles of the ZM-521 (a, b) and BH-660 (c, d) maize pollen compared to controls, distilled (left) and breeding

(right) water, dosed with hexane. In the two-choice oviposition assay, the headspace of the BH-660 maize pollen was significantly preferred over that of the ZM-521 in both distilled (e) and breeding (f) water. Controls (hexane *vs.* hexane) are denoted by black squares (a-d). An oviposition index of zero indicates preference to neither treatment nor control. Different lowercase letters indicate significant differences. Error bars denote standard error of the mean.

4.3.3. Identification of bioactive compounds in maize pollen headspace

The GC-EAD and GC-MS analyses identified five bioactive compounds in the headspace of the more attractive BH-660 pollen: limonene, nonanal, *α*-pinene, benzaldehyde and *p*-cymene. The overall volatile release rate was 100 ng min^{-1} with limonene as the most abundant compound followed by nonanal and *α*-pinene (Fig. 4.3).

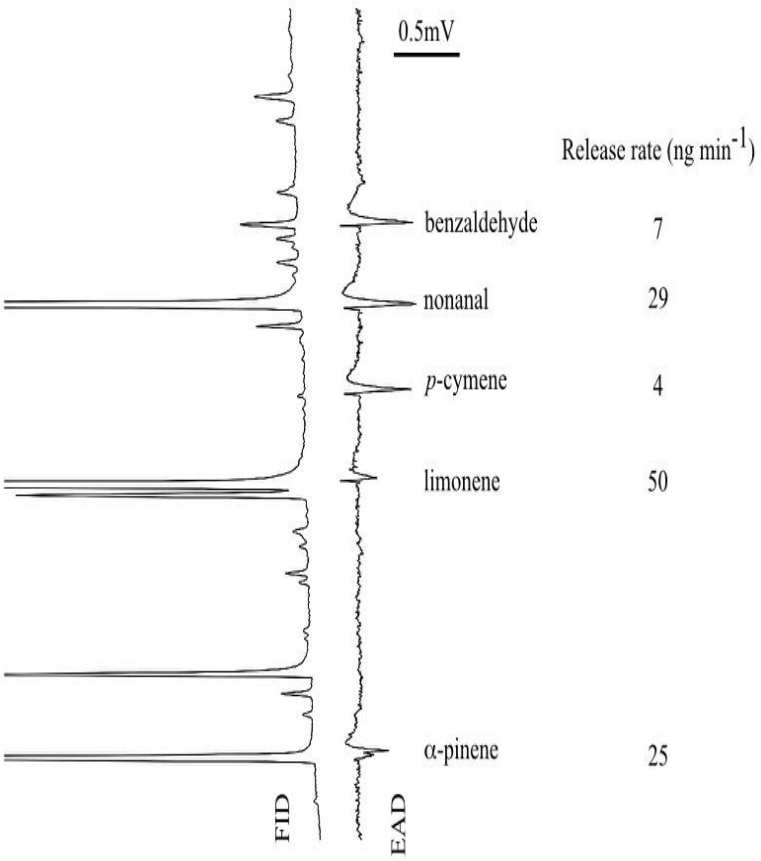

Figure 4.3 Antennal responses of gravid *Anopheles arabiensis* to BH-660 pollen volatile compounds. Electroanntenographic detection (EAD) trace depicts voltage changes (mV) in response to the bioactive compounds in the headspace of BH-660 pollen eluting from the gas chromatograph and detected by the flame ionisation detector (FID). The identity and release rate (ng min^{-1}) of the bioactive compounds are shown at the right.

4.3.4. Behavioural response to synthetic maize pollen odour

A synthetic blend containing all five GC-EAD-active compounds, approximating their natural ratio 10:5:5:1:1 (limonene : nonanal : *α*-pinene : benzaldehyde : *p*-cymene), elicited short-range attraction and stimulated oviposition in gravid *An. arabiensis* over a range of doses (χ^2=9.581,

P=0.0020; χ^2=8.125, P=0.0044, respectively) (Fig. 4). The release rate that elicited the optimal behavioural responses was found to be similar to the natural release rate (Fig. 4). To assess the role of each individual component in the blend, five subtractive blends, from which single compounds were removed, were evaluated against the full blend in both behavioural assays (Fig. 5). All subtractive blends were found to be less attractive (χ^2=4.02, P<0.0449) and females laid fewer eggs in the subtractive blends (χ^2=5.13, P<0.0236) than in the full blend (Fig. 5).

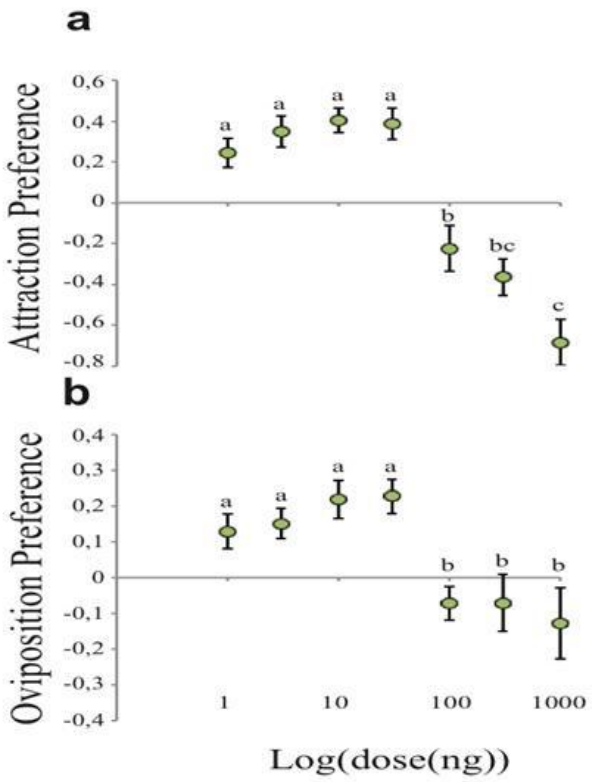

Figure 4.4 Synthetic maize pollen blend attracts and stimulates oviposition in gravid *Anopheles arabiensis*. Attraction (a) and oviposition (b) preference of gravid mosquitoes to the full five component synthetic blend presented at various doses. An attraction or oviposition index of zero indicates preference to neither treatment nor control. Different lowercase letters indicate significant differences. Error bars denote standard error of the mean.

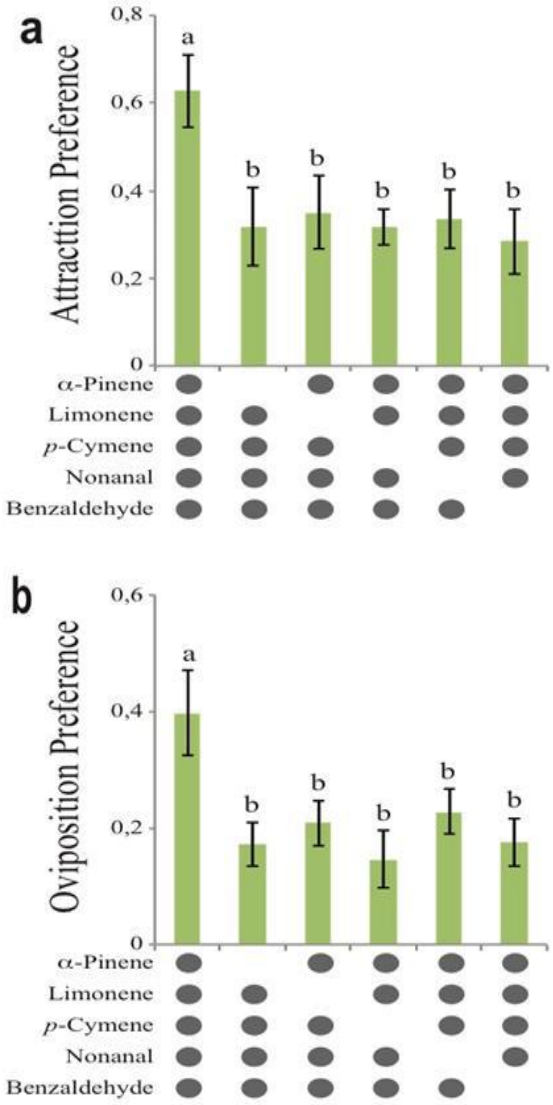

Figure 4.5 Behavioural responses of gravid *Anopheles arabiensis* to subtractive blends of compounds from BH-660 maize pollen. Attraction (a) and oviposition (b) preference of gravid mosquitoes to the subtractive blends. Significant difference * P<0.05, ** P<0.01 and *** P<0.001). Error bars denote standard error of the mean.

4.4. Discussion

Intensification of maize cultivation in Sub-saharan Africa contributes to the propagation of vector mosquitoes, and the expansion or exacerbation of malaria transmission (Kebede *et al*., 2005). One key link in this interaction is the presence of maize pollen in the breeding habitats of *Anopheles* mosquitoes, created by existing irrigation schemes (Ye-ebiyo *et al*., 2000; Kebede *et al*., 2005). Here, we have shown that gravid *An. arabiensis* are attracted to volatiles released by maize pollen, and identified a synthetic maize pollen odour blend that attracts and stimulates oviposition. The identification of habitat odour cues from oviposition sites, preferentially selected by *Anopheles* mosquitoes, provides important substrates for mosquito control measures that target gravid malaria mosquitoes.

The five-component blend identified from BH-660 maize pollen constitutes the second synthetic blend identified from a cereal crop, shown to attract and stimulate oviposition in gravid *An. arabiensis*. Three components identified from the headspace of the MR3 rice cultivar (Chapter III), which are limonene, *α*-pinene and nonanal, are shared between the synthetic maize pollen blend and the eight-component blend. For both blends, the full component blends are required to elicit the full behavioural repertoire of the gravid mosquitoes, which is in line with studies on herbivorous insects showing that blend perception is critical for host plant recognition and behavioural responses (Bruce and Pickett, 2011).

Although the odour space of mosquitoes (Carey *et al*., 2010; Wang *et al*., 2010) has yet to be definitively defined, it is interesting that volatile compounds identified as oviposition cues, also appear as salient features of other essential resources used during the gonotrophic cycle of the

female. Of the five blend components identified in this study, limonene, *α*-pinene and benzaldehyde are among the most common floral odour constituents (Knudsen *et al*., 2006), and are known to attract nectar-seeking mosquitoes (Bowen, 1992; Jhumur *et al*., 2007, 2008; Nyasembe *et al*., 2012; Otienoburu *et al*., 2012). The other two components of the blend, *ρ*-cymene and nonanal, are less abundant floral constituents (Knudsen *et al*., 2006; Otienoburu *et al*., 2012), however, the behavioural significance of these compounds as floral attractants for mosquitoes is yet to be revealed (Bowen *et al*., 1992; Otienoburu *et al*., 2012). Benzaldehyde, *α*-pinene and nonanal have also been shown to be present in human odour headspace (Bernier *et al*., 2000; Curran *et al*., 2005), and have been shown to play a role in host attraction of mosquitoes (Puri *et al*., 2006; Logan *et al*., 2008; Syed and Leal, 2009; Tchouassi *et al*., 2013). As the perception of odour blends changes with the physiological state of the female (Takken *et al*., 2001; Rinker *et al*., 2013), it is likely that the importance of individual compounds will depend on the context in which they are detected.

For the selection of oviposition site by mosquitoes, the ten components identified from cereal crops, along with the soil component cedrol **(**Lindh *et al*., 2015), are to our knowledge, the only known oviposition attractants for *Anopheles* mosquitoes. Considering the generally non-overlapping oviposition habitat choices of *Anopheles, Aedes* and *Culex* mosquitoes (Bentley and Day, 1989; Merritt *et al*., 1992), it is interesting that *Aedes* and *Culex* mosquitoes generally are attracted to a different subset of volatile compounds than *Anopheles* (Du *et al*., 1999; Waliwitiya *et al*., 2009; Afify and Galizia, 2015). This separation of odour space and habitat choice may be related to the inherent competition between sympatric species.

The ability to interrupt the oviposition site selection behaviour of malaria mosquitoes provides a needed additional target to be exploited in the development of novel control methods. Combined with existing intervention strategies (indoor residual spray and long-lasting insecticide treated nets), odour baited gravid traps could alleviate the increasing problem of outdoor malaria transmission.

4.5. Conclusions

We have shown that gravid malaria mosquitoes can be attracted to an affordable volatile synthetic blend, providing concrete evidence of a substrate that can be used as a lure in a gravid trap. Ongoing semi-field and field trials are aimed at validating the efficacy of the synthetic lure. At the same time we are evaluating chimeric blends, based on the bioactive volatile compounds identified in other cereal grasses.

Chapter V

Response of gravid *Anopheles arabiensis* to volatile compounds of sugarcane pollen

5.1. Introduction

The presence of emergent grasses correlates with high densities of the malaria vectors of the *Anopheles gamibae sensu lato*, which increases risk of malaria infection for people in the surrounding areas (Ijumba *et al*., 2002; Kebede *et al*., 2005; Muturi *et al*., 2008). While natural larval habitats are commonly associated with wild grasses of the family Poaceae (Torres-Estrada *et al*., 2005; Asmare *et al*., unpublished; Albicócco *et al*., 2011), these habitats have expanded into agricultural areas due to human cultivation practices, mainly the domestication of Poaceae grasses such as maize, rice and sugarcane (Kebede *et al*., 2005; Mwangangi *et al*., 2006; Jaleta *et al*., 2013). Such association between vector and crop suggests a selective evolutionary adaptability of mosquitoes to these domesticated grasses despite many environmental challenges.

The ability of gravid anophelines to select such domesticated grasses as larval habitats has likely evolved over time as a result of the enhanced fitness of the offspring and the ability to pass the preference from generation to generation. The abundance and distribution of mosquitoes among suitable breeding sites are regulated by several biotic and abiotic factors and their interactions. Grasses provide the larvae with shelter from abiotic and biotic threats as well as nutrients, directly from shed pollen, and indirectly from the rhizosphere supporting etable microorganisms (Merritt *et al*., 1992; Ye-Ebiyo *et al*., 2000). The degree of larval habitat suitability is the

principal factor influencing the performance of the emerging adult mosquito, such as flight endurance, survival, longevity and reproductive fitness (Ye-ebiyo *et al*., 2000; Okech *et al*., 2007; Araujo *et al*., 2012). Moreover, the larval habitats influence the selection of oviposition site by gravid mosquitoes (Minakawa *et al*., 2006; Ndenga *et al*., 2011; Himeidan *et al*., 2013; Afify and Galizia, 2015). For larval habitat selection, gravid mosquitoes mainly rely on olfactory cues emanating from the habitats, including organic material from water bodies, and the surrounding and emerging vegetation (Bentley and Day, 1989; Himeidan *et al*., 2013; Afify and Galizia, 2015; Lindh *et al*., 2015).

The abundance of *Anopheles* mosquitoes in relation with domestic and wild grasses could be due to the ovipositional choice of the gravid mosquitoes, the main driver of which is olfaction (Asmare *et al*., unpublished; Chapter III and IV of this thesis). In Chapter III and IV of this thesis, different semiochemicals with the potential to attract *Anopheles* mosquitoes have been identified from two domesticated grasses, maize and rice, two of which are shared between them. The attraction to grass-associated volatiles is not limited to malaria vectors, as volatiles emitted from the fermenting infusion of various grasses attract various species of gravid Culicine mosquitoes (Afify and Galizia, 2015). The only compound identified in domesticated grasses so far that has also been identified from the fermented grass infustions is nonanal (Chapters III and IV of this thesis; Du and Millar, 1999). These could indicate the importance of this grass family; including rice, maize and sugarcane for mosquito larvae growth and development since it provides food security and ideal larval habitat (Ye-Ebiyo *et al*., 2000; Mwangangi *et al*., 2006). However, this puts the people nearby these grasses habitats at high risk of the disease malaria. In the present study, the role of chemical cues from domesticated grasses by determining the role of sugarcane

pollen in the decision of gravid *Anopheles* mosquitoes to choose an oviposition site. The outcome from the present study, together with synthetic lures identified from maize and rice, could be used to create a super lure eliciting an enhanced attraction of malaria vectors. The combination of a super blend with a trap or contamination station has the potential to be incorporated as a component of integrated vector management for future sustainable management of the vector.

5.2. Materials and methods

5.2.1. Mosquitoes rearing

The experimental mosquitoes used on the behavioural study were obtained from established laboratory-reared colonies of *An. arabiensis* (Mbita strain) at the International Centre of Insect Physiology and Ecology (ICIPE) Duduville campus, Nairobi, Kenya. For electrophysiological bioassay, *An. arabiensis* (Dongola strain) were reared at Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden. The aquatic stages (egg, larvae, pupae) of the mosquitoes were kept in plastic rearing trays (15-39 cm × 30-28 cm × 3-14 cm) filled with distilled water to a depth of 4-5 cm. The larvae were provided with Tetramin® fish food (Tetra, Melle, Germany) and the water were changed every other day. Once the pupae emerged it is transferred to cups (20-30 cm × 30 cm D:H) in cage (30 cm × 30 cm × 30 cm; custom made or Bugdorm, MegaView Science, Taiwan) until adults emerged. When the adults emerged, 10% honey solution or sucrose solution was provided *ad libitum* and the adults were maintained under standard conditions 27±2°C, 75±5% relative humidity under a 12 h light/12 h dark cycle. Female *An. arabiensis* (5-days post-emergence) were provided with defibrinated sheep or rat blood, or the arm of a volunteer for two days, 15-30 min *per* day. For both behavioural and electrophysiological bioassay 3-days post-blood feeding *An. arabiensis* were used.

5.2.2. Headspace volatile collections

For headspace volatile collections, Coll-48 and EAK 71-402 sugarcane cultivars were obtained from Mombasa located, 500 km south east of Nairobi, Kenya's capital. The volatiles released by Coll-48 and EAK 71-402 sugarcane pollen cultivars were collected in an air-tight glass chamber rafter the pollen was collected from the detached tassels (40 replicates per cultivar). Air at a flow rate of 1 L min^{-1} was filtered through activated charcoal and humidified by passing through double distilled water. The humidified air was pushed through multiple ports to which odour sources enclosed in glass jars were connected in parallel. The volatiles from the cultivars were adsorbed onto Super-Q traps (30 mg, Analytical Research System, Gainesville, Florida, USA). Aerations were run for 5 h and each adsorbent was eluted with 600 µl GC/GC-MS-grade dichloromethane (DCM) (Burdick and Jackson, Muskegon, Michigan, USA) and was kept at -80 °C until used.

5.2.3. Two-port olfactometer procedures

The choice experiments were conducted in previously established two-port bioassays (Chapter III Section 3.2.3.). Three different experiments were conducted in this two-port olfactometer. The first experiment compared the response of gravid females to the headspace aeration extracts of each of the Coll-48 and EAK 71-402 sugarcane pollen *versus* DCM, whereas the second experiment compared compared the response of gravid females to the headspace aeration extract of Coll-48 *vs.* EAK 71-402. The behavioural responses were analysed to increasing amounts of the aeration extracts corresponding to the amount of volatiles released during 16 to 80 min from the Coll-48 and EAK 71-402 sugarcane pollen. The third experiment, tested the response of the gravid females to full synthetic blends based on the GC-EAD and GC-MS analyses of the pooled

aeration extract of Coll-48 and EAK 71-402 sugarcane pollen (5.4.5-7) at different release rates (20-20 000 ng min^{-1}) *vs.* DCM. Each experiment was replicated ten times and the mosquitoes that landed in either of the arm were recorded after five minutes. All assays were conducted between 18:00 and 21:00 under 27±2°C, 75±5% relative humidity conditions.

5.2.4. Oviposition bioassay

The oviposition stimulation of *An. arabiensis* to the headspace aeration sugarcane pollen volatiles and synthetic compounds described above were tested in (30 cm × 30 cm × 30 cm) cages covered with white nylon mosquito netting (Chapter III, Section 3.2.4). Two oviposition cups were prepared for each assay and placed in opposite corners of the cage, 4 cm from each wall. Each cup filled with distilled water as an oviposition substrate, was then loaded with different treatment doses as described above.

5.2.5. Electrophysiological bioassay

The antennal response of the experimental mosquitoes to pooled aeration headspace extract of Coll-48 and EAK 71-402 sugarcane pollen cultivars was recorded using GC-EAD (Chapter III, Section 3.2.5.)

5.2.6. Chemical identification

Biologically active compounds in the pooled headspace extract of sugarcane pollen cultivars from GC-EAD were identified using GC-MS both as single analysis, and with Kovats-mix (C6-C25 n-alkanes) and 100 ng of heptyl acetate (99.8% chemical purity; Aldrich) in a con-injection, and with other standards: *α*-pinene and nonanal (Chapter III, Section 3.2.6.).

5.2.7. Chemicals analysis

Active chemical compounds identified from GC-MS (5.2.6); *o*-xylene (Cas no. 95-47-6; Sigma-Aldrich, 98%), styrene (Cas no. 100-42-5; Aldrich, 99.5%), 1,8-cineole (Cas no. 470-82-6; Sigma, 99%), undecane (Cas no. 1120-21-4; Aldrich, 99%), N-ethyl benzenamine, dibutyl phthate (Sigma, 99%). (±)-α-pinene (Cas no. 7785-70-8; Sigma, 98%), nonanal (Cas no. 124-19-6; Sigma, 95%), benzaldehyde (Cas no. 100-52-7; Sigma, 99%), *p*-cymene (Cas no. 99-87-6; Aldrich, 97%).

5.2.8. Statistical analysis

The behavioural response of gravid *An. arabiensis* in two-port olfactometer and oviposition bioassay were analysed using binary logistic regression in SPSS Statistics for Windows, Version 20 (Armonk, NY: IBM Corp). An attraction preference (AP) as well as oviposition preference (OP) were determined using; AP = T-C/T+C and OP = T-C/T+C, where T is the number of mosquitoes or eggs that responded test odours and C the number of mosquitoes or eggs that responded control odours.

5.3. Results

5.3.1. Attraction and oviposition stimulation of gravid *Anopheles arabiensis* to sugarcane pollen volatiles

The behavioural response of gravid *An. arabiensis* to headspace volatiles of Coll 48 and EAK 71-402 sugarcane pollen were tested in a two-port olfactometer and an oviposition bioassay. Gravid mosquitoes were attracted and stimulated to lower doses of Coll 48 headspace over its solvent DCM (Wald (χ^2=0.687, 95% Confidence Interval (CI): 0.482-0.977, P<0.037; χ^2=0.991, 95% CI: 0.986-0.995, P<0.0001; Fig 5.1a,b, respectively), and a loss of attraction and stimulation at higher doses of Coll 48 extracts were observed. However, the attraction and stimulation of the gravid mosquito increases to higher doses of EAK 71-402 headspace extracts compared to control (DCM) (χ^2 =0.462, 95% CI: 0.318-0.669, P<0.001; (χ^2=0.987, 95% CI: 0.982-0.993, P<0.0001; Figs 5.1b,d, respectively).

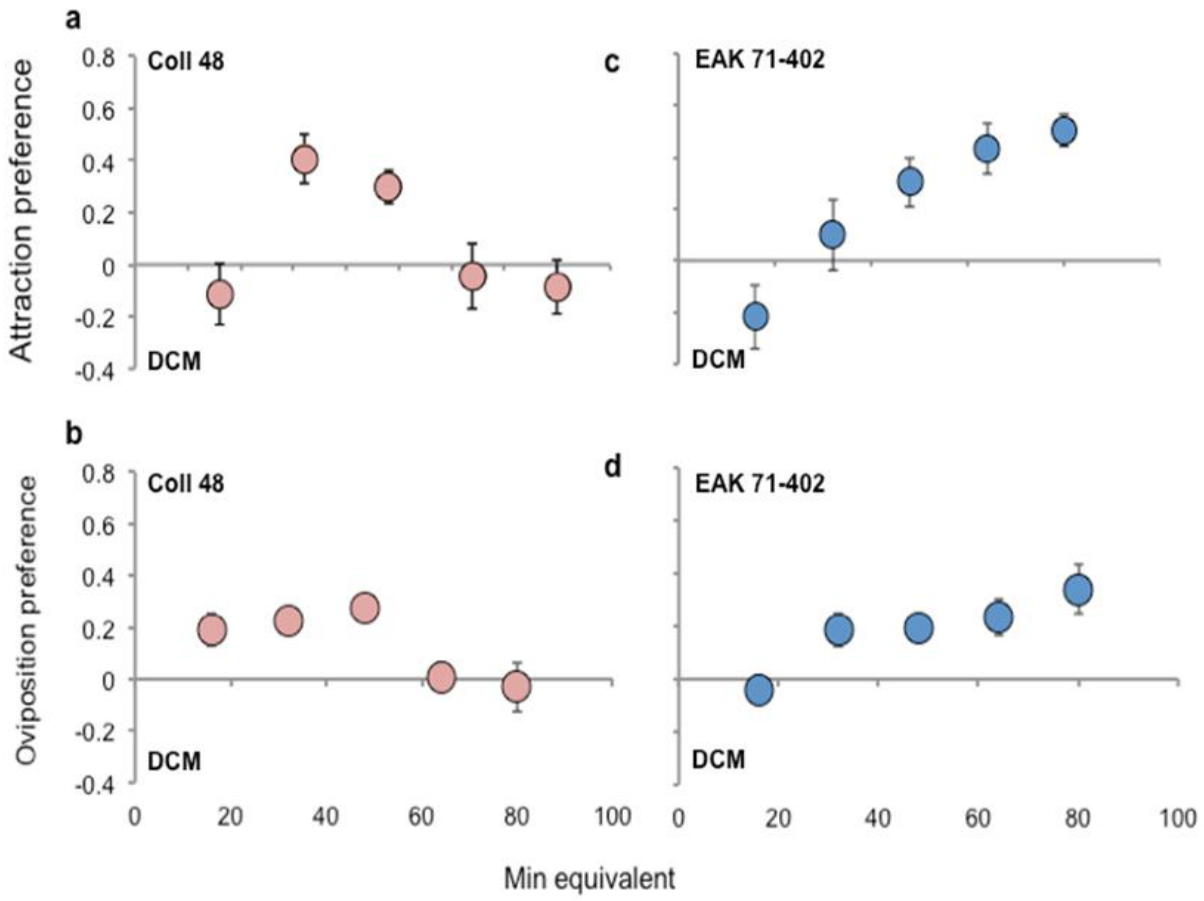

Figure 5.1 Gravid *Anopheles arabiensis* behavioural responses to headspace volatiles of Coll 48 (a, b) and EAK 71-402 (c, d) sugarcane pollen compared with the solvent dichlormethane (DCM). Attraction preference of mosquitoes to headspace volatiles from sugarcane pollen of the Coll 48 (a) and EAK 71-402 (c) cultivars was compared to DCM. Oviposition preference of mosquitoes to the headspace volatiles from sugarcane pollen of the Coll 48 (b) and EAK 71-402 (d) was compared to DCM. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

Gravid *An. arabiensis* were more attracted to, and stimulated by, the lower doses of Coll 48 headspace extracts, while higher doses of EAK 71-402 were more attractive and stimulatory (Fig

5.2). The range of sensitivity of the mosquitoes to lower doses of Coll 48 and higher doses of EAK 71-402 headspaces extracts was supported when compared to the response to the solvent in (Fig 5.1). However, there is no overall significant difference in attraction (χ^2=1.059, 95% CI: 0.760-1.477, P=0.735; Fig 5.2a) and oviposition stimulation (χ^2=0.999, 95% CI: 0.996-1.003, P=0.690; Fig 5.2b) between the headspace volatiles of the cultivars.

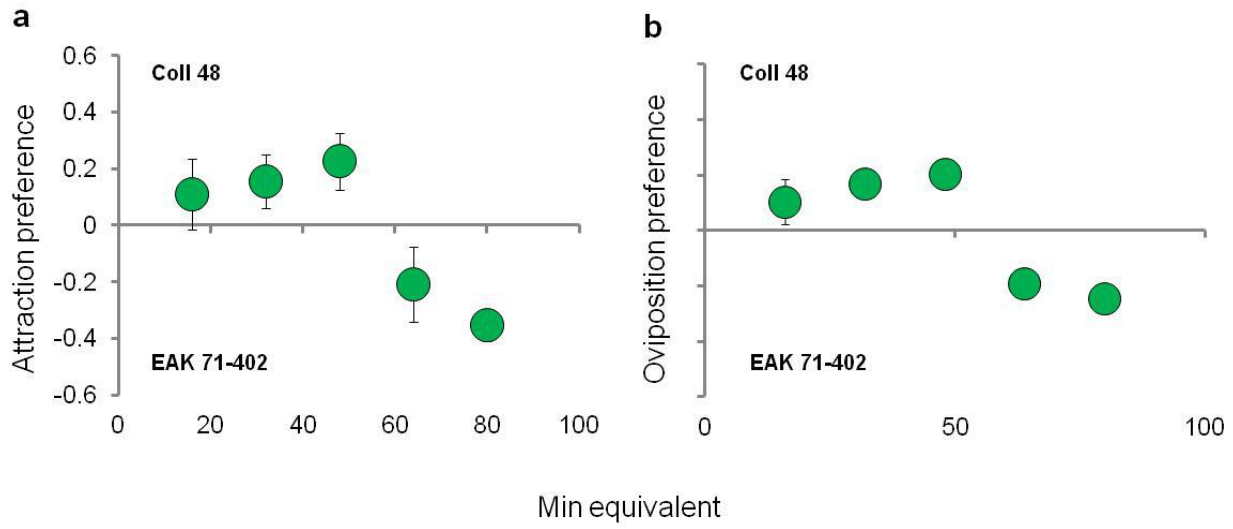

Figure 5.2 Behavioural responses, attraction (a) and oviposition stimulation (b), of gravid *Anopheles arabiensis* to Coll 48 *versus* EAK 71-402 sugarcane pollen headspace extracts. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

5.3.2. Identification of bioactive compounds in sugarcane pollen volatiles

The antennae of gravid *An. arabiensis* were exposed to pooled headspace volatiles of Coll 48 and EAK 71-402 sugarcane pollen (Fig 5.3). The antennae responded consistently to 17 compounds in sugarcane pollen headspace extracts: *o*-xylene, styrene, α-pinene, benzaldehyde, 1,8-cineole,

undecane, nonanal, *p*-cymene, N-ethyl benzenamine, dibutyl phthate, eicosane, C5-benzene, C11-benzene, C12-benzene, *p*-xylene, nonane, 3-octanone, of which 11 were confirmed to be bioactive and used for behavioural assays (Table 5.1). The total release rate of the compounds was 200 ng min^{-1} (Table 5.1). The most abundant compounds from the identified bioactive compounds are *o*-xylene and benzaldehyde.

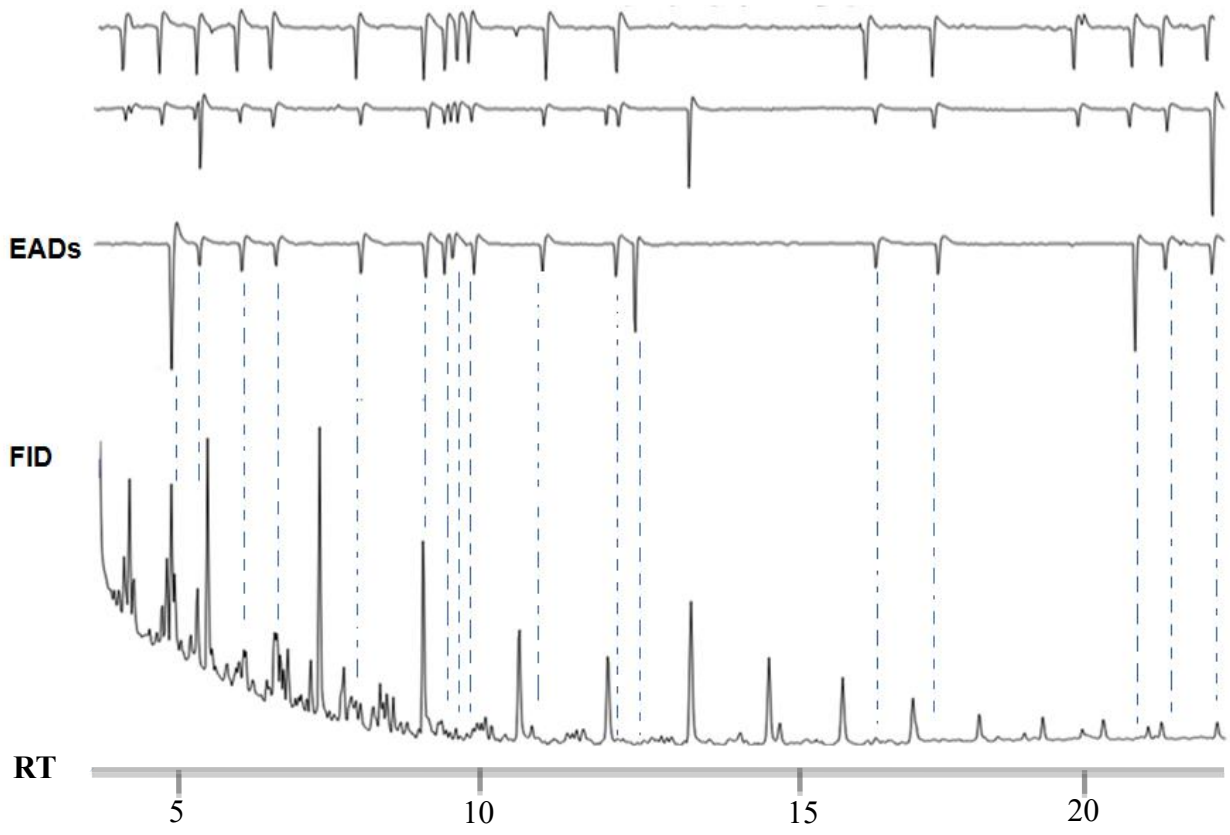

Figure 5.3 Combined gas chromatographic and electroantennographic responses of gravid *Anopheles arabiensis* antennae to pooled headspace volatiles of Coll 48 and EAK 71-402 sugarcane pollen. Electroanntenographic detection (EAD) trace, flame ionisation detector (FID), retention time (RT).

Table 5.1 Electrophysiologically active compounds identified pooled of headspace extracts of Coll-48 and EAK 71-402 sugarcane pollen.

Compounds	Retention time (min)	Release rate (ng min^{-1})
o-Xylene	7.87	80.919
Styrene	8.296	3.671
α-Pinene	9.199	16.770
Benzaldhyde	9.726	46.502
1,8-Cineole	11.196	2.478
Undecane	12.54	4.149
Nonanal	12.54	4.149
p-cymene	12.893	10.729
N-Ethyl Benzenamine	13.152	2.492
Dibuty phthate	25.981	8.666
Eicosane	28.773	5.369

5.5.3. Behavioual response of gravid *Anopheles arabiensis* to synthetic blend of sugarcane pollen

The synthetic blend was made from commercially available compounds of the identified bioactive sugarcane pollen volatiles by mimicking the proportion of the natural volatile

compounds (Table 5.1). The behavioural responses of gravid *An. arabiensis* to different release rates (20-20 000 ng min^{-1}) of the full synthetic blend in both two-port olfactometer and oviposition bioassays demonstrated an effect of release rate on attraction (χ^2 =0.619, 95% CI: 0.455-0.891), P<0.002; Fig 5.4a) and oviposition stimulation (χ^2 = 0.997, 95% CI: 0.994-1.000; P<0.022; Fig 5.4b) of gravid mosquitoes to full synthetic blend over the solvent DCM.

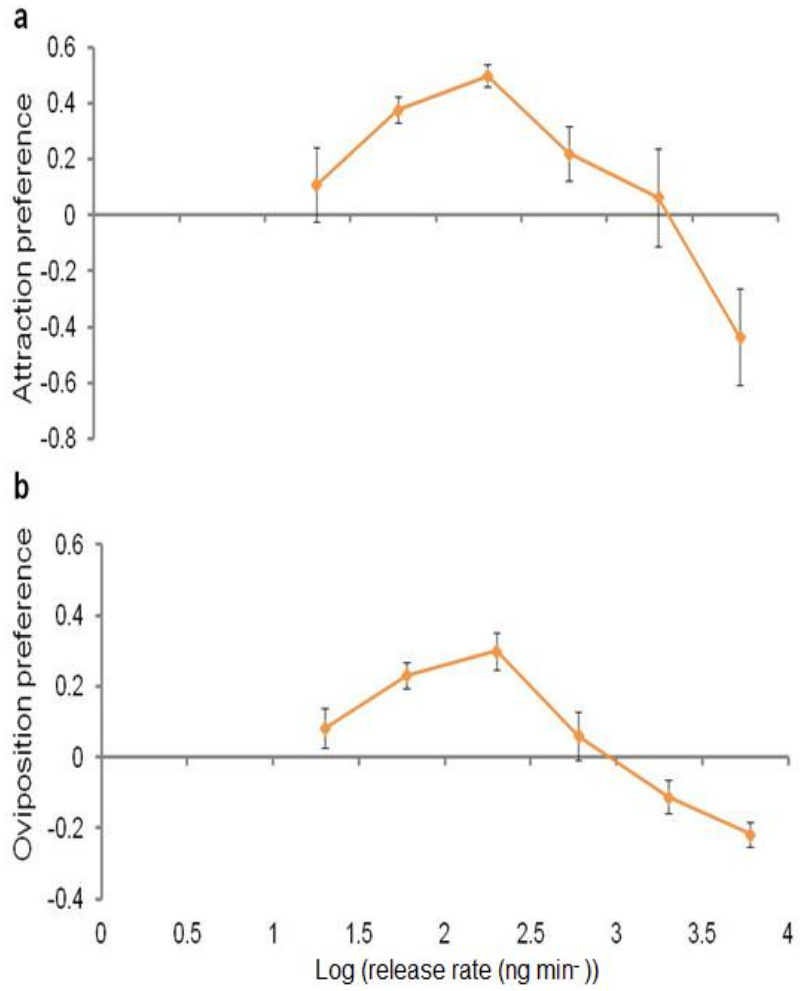

Figure 5.4 Attraction (a) and oviposition stimulation (b) of gravid *Anopheles arabiensis* to various release rates (ng min^{-1}) of the full seventeen component synthetic blend of sugarcane pollen volatiles. Different letters denote significant differences between doses. Error bars represent standard errors of the mean.

5.4. Discussion

Olfactory cues emanating from sugarcane pollen play a role in mediating oviposition behaviour of the malaria vector, *An. arabiensis*. Volatiles from sugarcane pollen, like those of maize pollen (Chapter IV) and rice plants (Chapter III), attract gravid *An. arabiensis* and stimulate oviposition. Taken together, this demonstrates the significant role of olfaction in the selection of domesticated grasses as preferred breeding sites for this malaria vector (Chapters III, IV, and this Chapter). Of the bioactive compounds identified from sugarcane pollen volatiles α-pinene and nonanal are shared among the domesticated grasses studied. The responses of *An. arabiensis* to similar grass-borne volatiles suggest an evolutionary relationship between grasses and anopheline mosquito larval habitat choice.

Some of the volatiles of domisticated grasses (nonanal and α-pinene) are also found in grass infusions used as lures for gravid culicine mosquitoes (Du and Millar, 1999). This appears to demonstrate the innate preference of gravid mosquitoes to grasses, particularly from the family Poaceae. The innate selection by gravid mosquitoes of emergent grass habitats may indicate the potential of the site to be able to host mosquito larvae through to emergence, including habitat characteristics such as nutrients (*e.g.* pollen and microorganisms) for immature mosquitoes (Ye-ebiyo *et al*., 2003a; Ijumba *et al*., 2002; Mwangangi *et al*., 2010). The location oviposition sites by gravid anophelines through the use of volatiles emanating from domesticated and wild grasses, as well as microbes, associated with the larval habitats (Sumba *et al*., 2004; Torres-Estrada *et al*., 2005; Lindh *et al*., 2015), may indicate an evolutionary history to the preference of gravid anophelines for grasses in the Poaceae family.

This study demonstrated the role of sugarcane volatiles as attractants for gravid malaria vectors. Previous studies have mainly focused on olfaction behaviour of blood-seeking adult mosquitoes. As a consequence, it is well known that blood-seeking mosquitoes are attracted to volatiles emanating from animal hosts, such as (*R*)-1-octen-3-ol and CO_2 (Takken and Verhulst, 2013; Webster *et al*., 2015). These compounds are being used as lures for predominantly indoor blood-seeking mosquitoes. Volatile compounds from oviposition sites, such as those identified in this study, have the advantage of attracting gravid mosquitoes, regardless of where these mosquitoes have rested and fed. Hence, a gravid lure derived from this grass may be integrated to other control methodologies with advantages of higher accuracy in the monitoring and capture of the targeted vector species, by providing better estimates of the number of egg-laying females, as well as the potential of a more dynamic control of mosquito populations regardless of the vectors' insecticide or behavioural resistance.

5.5. Conclusions

Gravid malaria vectors need to find suitable breeding sites for their aquatic life stages to develop. Olfactory cues are used for of gravid mosquitoes to orient towards and select oviposition sites (Himeidan *et al*., 2013; Afify and Galizia, 2015). This study demonstrates that gravid *An. arabiensis* are attracted to headspace extracts of sugarcane pollen and to its synthetic blend under laboratory conditions. The potential lure developed in the present study can be further tested in the field to determine how best to integrate it with other control strategies for sustainable management of anopheline mosquitoes.

Chapter VI

General Discussion, Conclusions and Recommendations

6.1. Discussion

The development and survival of immature stages of mosquitoes depends on resources available at oviposition sites. Thus, to increase her fitness, a gravid female (Bentley and Day, 1989) needs to carefully search and select sites with abundant sources of nutrients, low competition and no predators. To do so, female mosquitoes mainly use olfactory cues (Bentley and Day, 1989; Himeidan *et al*., 2013; Afify and Galizia, 2015). Much research has been done on the role of olfactory cues in the oviposition behaviour of culicine mosquitoes, where severeal potential semiochemicals have been identified (Himeidan *et al*., 2013; Afify and Galizia, 2015). In contrast, for *Anopheles* mosquitoes not much is known about what lures gravid mosquitoes towards suitable oviposition sites. In this thesis, the olfactory cues from cultivated grasses play an important role in attracting oviposition seeking *An. arabiensis*.

In this study, three crop grasses, including maize, rice and sugarcane, were selected since the cultivation of these crops are often associated with a high density of vector mosquitoes (Kebede *et al*., 2005; Mwangangi *et al*., 2010; Jaleta *et al*., 2013). These crops are cultivated through irrigation, creating suitable breeding grounds for many species of mosquitoes, including *Anopheles* (Lacey and Lacey, 1990; Kebede *et al*., 2005; Mwangangi *et al*., 2010; Jaleta *et al*., 2013). The change and expansion of agricultural practices, such as irrigation, is considered to be

among the primary factors driving the increase in the global malaria risk. However, the increase in mosquito density does not always mean there will be high disease transmission, a phenomenon referred to as the "paddies paradox" (Ijumba and Lindsay, 2001).

Irrigated rice, maize and sugarcane cultivations provide oviposition sites for gravid mosquitoes, and shelter and nutrients for their offspring (Lacey and Lacey, 1990; Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a; Mwangangi *et al*., 2010). The pollen of maize, in particular, is an important food source for *An. arabiensis* larvae even compared to filamentous algae (Ye-ebiyo *et al*., 2000; Ye-ebiyo *et al*., 2003a). The adults developing from feeding on maize pollen are bigger in size, long-lived and resistant to insecticides (*e.g.* DDT) (Ye-ebiyo *et al*., 2003a; Oliver and Brooke, 2013). In this thesis, I showed that volatiles released by maize and sugarcane pollen differentially attract gravid *An. arabiensis* mosquitoes (Chapter IV). Among the maize cultivars, the volatiles released by pollen of BH-660 were significantly more attractive to gravid *An. arabiensis* than that of ZM-521 (Chapter IV). This could be a contributing factor resulting in the high transmission of malaria vector after the introduction of BH-660 maize cultivar in Ethiopia (Kebede *et al*., 2005). For sugarcane pollen, gravid *An. arabiensis* were equally attracted to the two tested cultivars, Coll-48 and EAK 71-402. This kind of association has previously been observed in culicine mosquitoes, in which larval food such as plant detritus, microbes and their metabolites act as ovipositional attractants and/or stimulants for gravid females (Bentley and Day, 1989; Afify and Galizia, 2015). For malaria vectors, this is the first study showing the attraction of gravid *An. arabiensis* mosquitoes to its larval food.

Gravid *An. arabiensis* were also attracted to volatile compounds released by rice plants, irrespective of phenological (tillering, booting and flowering) stage (Chapter III). Moreover, female mosquitoes were more attracted and preferred to lay their eggs in response to volatiles released by one of the tested rice cultivars (MR3) than the other (MR1) (Chapter III). These selective ovipostional choices of female mosquitoes may be a leading cause of the vector distribution in different habitats (Sattler *et al*., 2005).

Anopheles arabiensis are highly associated with irrigated crops such as rice, maize and sugarcane. In this thesis, I showed that gravid *An. arabiensis* are attracted to volatiles from all of the three crop grasses tested. Although a number of volatile compounds have previously been proposed as oviposition semiochemicals for malaria vectors, none of these have been shown to cause attraction over longer distances, except one, cedrol, which was identified from soil infusions (Blackwell and Johnson, 2000; Lindh *et al*., 2008; Rinker *et al*., 2013; Lindh *et al*., 2015).

In this study, *An. arabiensis* were attracted to synthetic blends of volatile compounds identified from rice, maize and sugarcane cultivars. The identified blends, composed of 5-17 compounds, only shared two compounds. Subtractive blends were inferior to the full blend tested suggesting that the full blend is required to elicit long- and medium range attraction, as well as short-range attraction and acceptance of oviposition substrates. Whether or not individual components in the blend may play key roles in either long- or short-range stimulation require further studies (Baker *et al*., 1981; Linn *et al.,* 1986; Bruce and Pickett, 2011).

The synthetic attractive volatile blends identified in these studies are novel, and offer an exciting possibility for an eco-friendly, sustainable complementary strategy for monitoring and controlling disease vectors. The studies also offer a way to close the gap towards developing plant-based oviposition attractive odours as a new approach to the management of malaria vectors which can be integrated with other components of IVM for more fruitful and maintainable control.

6.2. Conclusions

- The studies emphasised the role of olfactory cues in orienting gravid *An. arabiensis* towards potential oviposition sites of domesticated grasses.
- The headspace volatile compounds emitted from maize, rice and sugarcane cultivars are attractive to gravid *An. arabiensis.*
- Gravid *An. arabiensis* prefer volatiles released by maize pollen of BH-660 over ZM-521 cultivar.
- Gravid *An. arabiensis* were attracted to volatile compounds of tillering, booting and flowering stages of rice of the two cultivars (MR1 and MR3) of rice.
- MR3 rice volatile compounds attracted significantly higher number of gravid mosquitoes compared to MR1 rice volatile compounds.
- Volatile compounds of Coll-48 and EAK 71-402 sugarcane pollen cultivars were found to be equally attractive to gravid *An. arabiensis*
- Compounds identified in these studies, when tested behaviourally, are more active as complex blends rather than as single molecules.

- The increased preference for the grass based blends shows the potential for exploitation of phytochemical attractants in surveillance and control of malaria vectors.
- The study is the first of its kind to identify and develop novel plant based oviposition attractants for gravid *Anopheles* mosquitoes and is an important breakthrough in developing 'attract and kill' strategies against the vector.

6.3. Recommendations

- Further trials with synthetic blends of headspace extracts of BH-660 and sugarcane headspace extracts under semifield and field conditions are recommended.
- The synthetic lure of MR3 rice should be evaluated under field conditions.
- The synthetic blends should be evaluated using various tools/traps.
- The blends identified from the studies could also be tested for other malaria vectors.
- The synthetic blends could be used together with other components of IVM for sustainable management of vector mosquitoes.

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