RESPONSES OF THE MALARIA VECTOR, ANOPHELES GAMBIAE, TO PLANT- AND MAMMALIAN- DERIVED ODORS

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Responses of the malaria vector, *Anopheles gambiae*, to plant- and mammalian- derived odors

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DECLARATION

This is my original work and has not been presented for a degree in any other University or any other award

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DEDICATION

To my beloved daughters, Precious Muthoni and Gloriah Wanjiru.

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ABBREVIATIONS AND ACRONYMS

IRS	Indoor Residual Spraying
LLINS	Long Lasting Insecticide treated Nets
WHO	World Health Organization
SRC	Scientific Review Committee
KEMRI	Kenya Medical Research Institute
ERC	Ethical Review Committee
CDC	Centers for Disease Control and Prevention
BG	Biogent
CO_2	Carbon dioxide
ITNs	Insecticide Treated Nets
PCR	Polymerase Chain Reaction
HRM	High Resolution Melting
MMX	Magnetic Mosquito-X

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ABSTRACT

Several studies have shown that odors of plant and animal origin can be developed into lures for use in surveillance of mosquito vectors of infectious diseases. However, the effect of combining plant- and mammalian-derived odors into an improved lure for monitoring both nectar- and blood-seeking mosquito populations in traps is yet to be explored. Laboratory dual choice olfactometer and field assays were used to investigate responses of the malaria vector, *Anopheles gambiae*, to plant- and mammalian-derived compounds and a combined blend derived from these two odor sources. Using subtractive bioassays in dual choice olfactometer, it was shown that a 3-component terpenoid plant-derived blend comprising (*E*)-linalool oxide, β-pinene, β-ocimene was more attractive to female *An. gambiae* than (*E*)-linalool oxide only (previously found to be attractive) and addition of limonene to this blend antagonized its attractiveness. However antagonistic effect of limonene was not exhibited in field trials in malaria endemic areas probably due to species specificity in odorant perception by different malaria vectors. Likewise, a mammalian-derived lure comprising the aldehydes heptanal, octanal, nonanal and decanal, was more preferred than (*E*)-linalool oxide. Surprisingly, combining the plant-derived 3-component blend of (*E*)-linalool oxide, β-pinene, β-ocimene with the mammalian derived 4-component blend attracted fewer females of *An. gambiae* than the individual blends in laboratory assays. However, this pattern was not replicated in field trials, where a dose-dependent effect on trap catches while combining both blends with significantly improved trap catches at higher doses was observed. This indicates the significance of ratio and concentration in formulation of odorant blends for outdoor biting malaria vectors. Therefore, field evaluation of odorant compounds is paramount in the design of vector control strategies involving kairomones from plant- and mammalian-basedsources.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria poses both health and economic burden in sub-Saharan Africa. Despite concerted efforts for control involving vector management and preventive chemotherapy, recent reports indicate that transmission of the parasite persists with about 90% of the global malaria morbidities and mortalities still occurring in the sub-Saharan Africa region (WHO, 2017). Major emphasis has been on vector control which is one of the cheapest and historically most successful approaches to fight vector-borne diseases (WHO, 2017). Vector management efforts focus on insecticide-based strategies mainly long-lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS). However, widespread use of LLINs and ITS has led to selection pressure and emergence and spread of insecticide resistance among key vectors and changes in species composition and feeding behaviour (emergence of early biting and outdoor biting species). Together, these highlighted challenges undermine sustainability of these current strategies (Bayoh et al., 2010; Toé et al., 2014; Reddy et al., 2011, Mwangangi et al., 2013).

Vector control measures targeting their life cycle and behavior (sugar and blood feeding) offer novel approaches for integration with existing insecticide – based control strategies (Russell et al., 2013). The malaria vector, *Anopheles gambiae*, uses odorant cues emitted by blood and sugar hosts (animals and plants, respectively) to locate and exploit resources from these hosts (blood and sugar), important for its survival and reproduction (Takken & Knols, 1999). Therefore, there is increasing recognition to exploit such odorant cues involved in host finding of this vector as potential control targets. To date, several odorants and the specific receptors that contribute to attractive and aversive behaviors have been identified for adult female mosquitoes (Carey et al.,

2010; Nyasembe & Torto, 2014; Ray, 2015). While the mode of detection of these odorant cues has allowed for insights into their degree of activation and specificity in the mosquito, for practical and control perspectives, detailed behavioral assessment of potential odorants in a field setting is required. This is imperative for predicting their effects on natural populations and to gain an understanding of heterogeneities which normally influence the dynamics of natural systems. Studies have shown that although naturally-occurring odorants from plant and mammalian hosts are complex, mosquitoes generally detect and respond to specific compounds often in certain doses and ratios (Bruce et al., 2005; Bruce & Pickett, 2011; Mukabana et al., 2012; Nyasembe & Torto, 2014; Syed & Leal, 2009).

In a previous study on mosquito-host plant interaction, six compounds, namely (*E*)-linalool oxide, β-pinene, β-ocimene, (*E*)-β farnesene, limonene and hexanal, derived from the host plant -*Parthenium hysterophorus*- were identified as eliciting activity in antennae of female *An. gambiae* (Nyasembe et al., 2012). In behavioral assays, a blend of these six compounds was more attractive to female *An. gambiae* than to the odors of a preferred host plant, *P. hysterophorus*. However, in field trials, this blend underperformed in capturing this vector compared to (*E*)-linalool oxide alone, a constituent of the blend (Nyasembe et al., 2014). On the other hand, when (*E*)-linalool oxide was combined with CO_2, this blend was found to be as attractive as odors emanating from worn socks, representing human foot odors. Furthermore, in the absence of CO_2, (*E*)-linalool oxide performed better than worn socks in capturing female *An. gambiae*. Human foot odors trapped on worn socks have been shown to be highly attractive to anthropophilic mosquitoes such as *An. gambiae* and *Aedes aegypti* (Njiru et al., 2006; Owino et al., 2015; Schmied et al., 2008; Tchouassi et al., 2013).

In a similar study, a four component blend of the aldehydes; heptanal, octanal, nonanal and decanal developed for Rift Valley fever virus mosquito vectors from five mammalian hosts- sheep, goat, donkey, cattle and human- (Tchouassi et al., 2013), was assessed in field trials for attractiveness to *An. gambiae*. This blend was better than

control solvent with or without CO_2 and comparable to odors from worn socks in trapping *An. gambiae* (Nyasembe et al., 2014). This finding was however not surprising given that aldehydes are the major compounds in human foot odors (Owino et al., 2015; Tchouassi et al., 2013). Further, possible antagonism was suspected when the blend comprising these mammalian-based compounds was combined with (*E*)-linalool oxide and reduced trap captures of *An. gambiae s.l* and *Anopheles funestus* group were noted (Nyasembe et al., 2014). As such, further elucidation of the observed effect encompassing detailed laboratory and field assessments was imperative to ascertain whether the effect was limited to a particular attractive compound or a blend of plant compounds. Such investigation would aid in the design and development of potent lures seeking to combine diverse cues from both plant and mammalian sources. Such lures would be used to target females of different physiological needs (sugar and blood) and males which are exclusive nectar feeders to reduce the chances of female mating hence a non-viable future generation (Foster, 1995; Nyasembe & Torto, 2014). In addition, the vectors use an integrated multisensory mechanism of host odor (McMeniman et al., 2013) for improved signal detection and efficient host seeking in a cluttered sensory environment.

This study was designed, to investigate whether blends of odorants from plant and mammalian origin in varying ratios and doses can be exploited together for development of improved lures for surveillance and control of malaria vectors. The hypothesis that reduced captures of *An. gambiae* by the six-component plant-based blend is associated with antagonism by certain constituents in the blend by identifying these possible antagonist(s) was further tested.

1.2 Statement of the problem

Malaria endemicity in sub-Saharan Africa remains a challenge despite concerted efforts mainly focusing on indoor vector management through insecticide based tools such as mass coverage with long lasting insecticide treated nets (LLINS) and indoor residual

spraying (IRS) (WHO, 2017). This has been attributed to insecticide resistance, change in vector feeding behavior from endophagic to exophagic and change in vector populations to an increase in outdoor biting fractions (Riehle et al., 2011; Russell et al., 2011; Tchouassi et al., 2012). In addition, IRS is very expensive for mass coverage. Therefore, there is a need for development of more tools for surveillance and control to target other vector behavior such as sugar feeding, and blood feeding. These life style behaviors are discriminative and guided by odorant compounds from suitable hosts (plants and mammals) (Russell et al., 2013). Such odors could be used in lure and kill technique by incorporating contact toxins, entomopathogenic fungi and viruses. In addition, odorant cues derived from blood and sugar hosts together with available traps such as Center for Diseases Control and Protection light traps, offer novel approaches in surveillance and control of malaria vectors in endemic areas.

1.3 Justification

An earlier study demonstrated antennal activity of six plant derived volatiles (hexanal, β-pinene, β-ocimene, limonene, (*E*)-linalool oxide and (*E*)-β-farnesene) to the malaria vector, *An. gambiae*. In behavioral assays, a blend of these six compounds was more attractive to females of *An. gambiae* than to the odors of a preferred host plant, *P. hysterophorous* L., from which these odors were derived (Nyasembe et al., 2012). However, in field trials, a blend of the six compounds underperformed in capturing this vector compared to (*E*)-linalool oxide alone, a constituent of the blend. While odorant blends are thought to define more the attractiveness of vectors to a particular host, the reason(s) for underperformance of the six component blend compared (*E*)-linalool oxide alone (Nyasembe et al., 2014) was unclear suggesting possible antagonism by some blend constituent(s). Further, possible antagonism was suspected when the blend comprising four mammalian-based compounds- heptanal, octanal, nonanal and decanal- was combined with (*E*)-linalool oxide and reduced trap captures of *An. gambiae sensu lato* and *An. funestus* group were noted. As such, further elucidation of the observed effect encompassing detailed laboratory and field assessments is imperative to ascertain

whether the effect is limited to a particular attractive compound or a blend of plant compounds.

1.4 Hypothesis of the study

Interaction of odorant compounds impacts blend effectiveness for surveillance and control of *Anopheles gambiae.*

1.5 Objectives

1.5.1 General objective

To evaluate plant and mammalian-derived odorant compounds for development of potent lures for *An. gambiae sensu lato* control.

1.5.2 Specific objectives:

1. To identify the odorant compound(s) with possible antagonistic or synergistic effects to *An. gambiae sensu stricto* (*s.s*) (here and after as *An. gambiae*) in the laboratory.
2. To evaluate the responses of *An. gambiae* to different blends in the laboratory.
3. To assess the attractiveness of the formulated blend(s) to populations of *An. gambiae* in the field.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria vectors in sub-Saharan Africa

Mosquitoes of the genus *Anopheles* (Diptera: Culicidae) are documented to have 465 species globally most of which occur as complexes (Sinka et al., 2012) and new ones continue to be reported. Of these, 70 species are capable of transmitting human malaria but only 41 are considered dominant vector species (Sinka et al., 2012) as they not only have the ability to transmit a majority of the human malaria parasites but also exhibit high propensity to feed on humans, are long lived and abundant (Takken & Lindsay, 2003). In sub-Saharan Africa, the major malaria vectors are of the *Anopheles gambiae* complex (*An. arabiensis, An. gambiae s.s and An. coluzzii*) and *Anopheles funestus* complex (*Anopheles funestus s.s* (here and after as *An. funestus*)). *An. gambiae* and *An. funestus* are the most important due to their high susceptibility to *Plasmodium* parasites, preference for human hosts (anthropophagy) as well as the characteristic indoor feeding (endophagy) and resting behavior (endophily) (Coetzee et al., 2000; Coetzee & Fontenille, 2004, Coetzee et al., 2013). In recent studies by Mwangangi et al. (2013), other anopheline species such as *Anopheles coustani* has been implicated in malaria transmissions both indoors and outdoors. Other secondary and zoophilic vectors include west African *Anopheles melas,* east African *Anopheles merus, Anopheles nili, Anopheles moucheti* and some species in the *Anopheles funestus* group such as *Anopheles parensis, Anopheles rivulorum, Anopheles veneedeni* and *Anopheles aruni* (Coetzee et al., 2000; Coetzee & Fontenille, 2004; Sinka et al., 2012).

2.2 Current vector control strategies and drawbacks

Malaria is an important parasitic disease that poses both economic and health burden in the sub-Saharan Africa. Despite concerted efforts for vector control, chemotherapy and

environmental management, recent reports showed that 90% of the morbidities and mortalities by malaria occur in the sub-Saharan Africa (WHO, 2017). Vector management has been the heart of malaria control with main focus based on conventional insecticide strategies such as IRS, use of LLINs and larval source management (WHO, 2017). However, widespread use of insecticide based control measures has led to insecticide resistance and change in vector feeding behavior (emergence of early biting and outdoor biting fractions) which together subvert the effectiveness of the current strategies (Haji et al., 2013; Toé et al., 2014; Ochomo et al., 2013). For example, *An. funestus, An. farauti* and a cryptic sub group of *An. gambiae s.s* have changed their feeding strategy to outdoor biting at dawn and dusk (Reddy et al., 2011; Riehle et al., 2011; Russell et al., 2013, 2011). Also, massive coverage by ITNs has led to a decrease in the endophagic vectors (*An. gambiae s.s*) and an increase in exophagic vectors (*An. arabiensis* and *An. coustani*) which are not targeted by conventional control tools (Bayoh et al., 2010; Mwangangi et al., 2013a, 2013b). As such, new measures targeting other ecological aspects of the vector behavior such as sugar feeding, blood feeding and oviposition all of which are guided by odorant cues should be considered for integration with existing vector control strategies for complete elimination of malaria transmission (Russell et al., 2013).

2.3 The role of semiochemicals in malaria vector behavior

Success of mosquito's survival and reproduction is characterized by parameters such as oviposition, blood feeding and sugar feeding. Each of these behaviors is discriminative and mediated by odorant cues (semiochemicals) emanating from preferred oviposition sites, blood and plant hosts (Bruce et al., 2005; Foster, 2008; Navarro-Silva et al., 2009; Nyasembe & Torto, 2014). In this regard, semiochemicals play a crucial role in ensuring success of different mosquito species and this chapter reviews such cues in relation to different behaviors.

2.3.1 Role of semiochemicals in mosquito host seeking

2.3.1.1 Blood feeding

Host seeking for a blood meal required for egg development by mosquito vectors is mediated by odorant cues released by host animals/human and these cues are perceived through olfactory receptors located in the antennae, maxillary palpi and labellum of the vectors (Lu et al., 2007, Takken & Knols, 1999). As such, due to differential odorant profiles, some blood hosts are highly preferred for biting to others (Dormont et al., 2013; Mukabana et al., 2012; Okumu et al., 2010; Takken, 1999). It is worth noting that skin micro flora, diet or disease contributes to differences in odor profiles of individuals. This may contribute to making some individuals more attractive to malaria vectors than others (Dormont et al., 2013; Verhulst et al., 2011). For example, studies have shown that infections with transmissible gametocyte parasite stage (Busula et al., 2017) and alcohol consumption increases human attractiveness to malaria vectors due to high release of certain attractants such as octen-3 -ol, heptanal, nonanal, octanal, (E)-2-octanal, 2-octanone, (E)-2-decenal and carbon dioxide (Lefèvre et al., 2010; Lacroix et al., 2005, Robinson et al., 2017). Also, individuals with a high abundance of bacterial species such as *Staphylococcus* spp. have been shown to be more attractive to *An. gambiae* than individuals with *Pseudomonas* spp. due to odorants associated with such microbes (Verhulst et al., 2010, 2011).

Human/animal based kairomones such as carbon dioxide, carboxylic acids, ammonia, aldehydes, alcohols and lactic acid among others, have been shown to elicit either attractive or repellent activity to malaria vectors and other mosquito species of medical significance in the laboratory and field trials when dispensed singly or as blends (Bernier et al., 2003; Okumu et al., 2010; Owino et al., 2015, 2014; Smallegange et al., 2005). For example, studies by Tchouassi et al. (2013) led to identification of four aldehyde blend from mammalian host (human, cattle, sheep, goat and donkey) skin odors (heptanal, octanal, nonanal and decanal) used by Rift Valley fever vectors as olfactory cues for host location. A blend of ammonia, (S)-lactic acid, tetradecanoic acid, carbon

dioxide and 3-methyl-1-butanol was shown to be attractive to malaria vectors and other mosquito species of medical importance in field studies (Mukabana et al., 2012). In addition, hexanoic acid alone and a binary blend of octanal and nonanal showed significant attractiveness while a blend of the three components led to reduced trap captures of the dengue and chikungunya virus vector, *Ae. aegypti* in field experiments (Owino et al., 2015).

It is worth noting, mammalian-derived odorant cues are emitted in a complex mix in different ratios and doses which impact on host recognition by the vectors. Some blends of the odorant compounds have been shown to elicit more attraction than single compounds. For example, a blend of ammonia, 12 carboxylic acids and lactic acid was highly attractive to *An. gambiae* than ammonia alone, while lactic acid alone was repellent (Smallegange et al., 2005). In another study, Smallegange et al. (2012) showed that when some compounds such as 4,5-dimethylthiazole was added to a blend of ammonia, lactic acid and tetradecanoic acid at a high concentration, there was a significant reduction in the attraction of *An. gambiae* while when added to the blend at a lower dose the blend was synergized. This indicates that odorant cues can be perceived as host or non- host based on the concentrations. Also, it has been shown that the presence of one component might antagonize or synergize the activity of odorant blends. For instance, when 3-methyl-1-butanol was added to a blend of ammonia, (S)-lactic acid, tetradecanoic acid and carbon dioxide, there was a marked increase in attractiveness of the blend to *An. gambiae* while addition of isovaleric acid and 4, 5 dimethyl-thiazole to the blend led to a diminished attractiveness of *An. gambiae* (Mukabana et al., 2012). In a similar study by Verhulst et al. (2011), synergism between 3-methyl-1-butanol and a tripartite blend of ammonia, (S)-lactic acid and tetradecanoic acid was observed resulting to a marked increase in attractiveness of *An. gambiae* while 2-phenylethanol was antagonistic to the blend.

There is, therefore, a need to understand the interactions among blend components for development of potent lures and repellents for use in malaria vector surveillance and

control. In addition, most of these compounds or blends only target blood seeking female vectors, have not been evaluated for their effectiveness in the field and are not very effective as lures on their own in the absence of CO_2 which acts as an activator and a long range attractant (Foster, 2008; Okumu et al., 2010; Tchouassi et al., 2013). As such, more research on optimization of the identified cues for mosquito vector surveillance is needed.

2.3.1.2 Sugar feeding

Plant foraging by mosquitoes forms a part of their diet from which carbohydrates in form of sugars and other metabolites are obtained. Sugar is a crucial dietary requirement for most mosquito species and it has been shown that some malaria vectors such as *An. gambiae* have a strong preference for honey odors than worn socks during the first four days after emergence (Foster & Takken, 2004) and lack of sugar meals led to a reduced vectorial capacity, survival and longer gonotrophic cycles. As such, sugar is needed to sustain survival, vectorial capacity, fecundity and other metabolic processes and it is the only nutritional source for male mosquitoes (Foster, 1995; Gary et al., 2009; Manda et al., 2007; Okech et al., 2003; Gu et al., 2011).

Interestingly, mosquito plant foraging does not happen indiscriminately and hence, *An. gambiae* and other mosquitoes species have a strong preference for certain plants compared to others owing to odorant blends emitted by these plants (Nyasembe et al., 2012, 2014; Takken & Knols, 1999), nectar quantity, seasonal availability, abundance, floral structure of the plant and the physical fitness conferred by feeding on certain plant hosts and not others (Gouagna et al., 2010; Manda et al., 2007; Müller et al., 2010). It is worth noting, use of odors for plant host source location is not only limited to mosquitoes but is also used by other hematophagous disease vectors for resource location. For example, octen-3-ol and beta caryophyllene elicited strong electroantenographic responses and a binary blend showed significant attraction to tsetse in wind tunnel studies. These plant volatiles are used in nature by *Glossina* spp.to locate their most preferred sheltering plant, *Lantana camara* (Syed & Guerin, 2004). Further,

electrophysiological responses to a floral based compound-acetophenone have been documented in *Simulium* species (Young et al., 2015). Additionally, studies by Machado et al. (2015) showed that plant based saturated primary alcohols such as hexanol and octanol and a blend of heptanol, octanol and nonanol showed a marked activation and attraction of *Nyssomyia neivai*, a vector of American cutaneous leishmaniasis, in olfactometer studies

Several plant based compounds in the phenolic, terpenoid, ketone, aldehyde and alcohol classes have been shown to elicit responses to different mosquito species (Nyasembe & Torto, 2014). For example, earlier studies by Jepson & Healy (1988) showed a significant preference for floral odors of *Ligustrum vulgare* by *Ae. aegypti* even in absence of visual cues. In other studies, *An. arabiensis* was shown to have strong preference for floral odorants from *Achillea millefolium* (Healy & Jepson, 1988). Also, studies by Nyasembe et al. (2012, 2014) showed significant preference and attraction to volatiles mainly terpenes such as ocimene, pinene and the green leaf aldehyde, hexanal, among others, derived from *P. hysterophorus*, in olfactometer assays and field experiments by female *An. gambiae.* In other studies, *Ae. aegypti* an important vector of dengue and chikungunya viruses was significantly attracted to a floral derived volatile component (acetophenone) in olfactometer flight preference assays (Von Oppen et al., 2015). Floral compounds of *Asclepias syriaca* (benzaldehyde, (E)-β-ocimene, phenyl acetaldehyde, benzyl alcohol, nonanal, and (E)-2-nonenal) elicited significant orientation from both male and female *Cx. pipiens* in laboratory olfactometer studies. When blended together, they elicited a response comparable to the extract while a three-component blend consisting of benzaldehyde, phenyl acetaldehyde, and (E)-2-nonenal was as attractive as the full blend (Otienoburu et al., 2012). In addition, studies by Jhumur et al. (2008, 2007) showed electrophysiological responses of *Ae. aegypti* and *Cx. pipiens Molestus* to floral odorants of *Silene otites* such as (Z)-3-hexenyl acetate, hexanol, linalool oxide (furanoid) and acetophenone among others while in olfactometer studies acetophenone, linalool oxide (pyranoid), phenyl acetaldehyde, phenyl ethyl alcohol was the most attractive to *Cx. pipiens*.

Plant odors are emitted as a complex mix of compounds in different ratios and concentrations (Bruce & Pickett, 2011; Nyasembe et al., 2012) and presence of some compounds might antagonize or synergize the attractiveness of an odorant blend. For example, reduced mosquito attraction by a blend of six compounds namely, (*E*)-linalool oxide, β-pinene, β-ocimene, (*E*)-β farnesene, limonene and hexanal compared to a single compound, (*E*)-linalool oxide, in field tests have been observed suggesting attraction may be mediated by a single compound (Nyasembe et al., 2014). As such, the overall host-seeking process is mediated by a complex interaction of chemicals occurring in different ratios and concentration that attract and mask the host from mosquitoes (Bruce & Pickett, 2011; Bruce et al., 2005). Therefore, identification of such compounds that result in repellency within an odor blend (that can reduce the host finding ability) can serve as personal protection tools thereby preventing mosquitoes from locating attractive hosts and prevent disease transmission.

Plant based compounds can be exploited for field trapping of malaria vectors and other mosquito species. For instance, studies by Nyasembe et al. (2012, 2014) showed significant attraction of *An. gambiae s.s* to six plant based terpenes and a green leaf aldehyde (ocimene, pinene, farnesene, limonene, linalool oxide and hexanal) in olfactometer studies and in field trials, one of the compounds, linalool oxide, was shown to be as good as odors emanating from worn socks when combined with CO_2 and even better than socks when not combined with CO_2 in trapping *An. gambiae s.l.* Therefore, use of plant based lures/repellents in malaria vector surveillance/control could eliminate the need for CO_2 which is cumbersome and expensive especially in remote areas (Foster, 2008). Moreover, sugar is a basic requirement for both male and female mosquitoes of all ages and gonotrophic states, unlike blood only needed during egg development by female mosquitoes (Foster, 2008, 1995) hence plant based control tools such as incorporation of toxins, entomopathogenic fungi and viruses in attractive lures could target all adult populations of mosquitoes.

Also, based on studies which showed increased probing activity of most preferred plants by *Plasmodium falciparum* positive mosquitoes, plant based lures could be used to target infected malaria vectors hence minimize contact with humans (Nyasembe et al., 2014). In addition, due to increasing malaria transmissions by outdoor fractions of malaria vectors which are not targeted by current indoor control measures, plant- based lures could be a potential tool in management of such vectors. In conclusion, it would be interesting to combine plant and animal based odours to target sugar and blood questing vectors.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

Experimental research design was used for both laboratory dual choice olfactometer assays and field evaluation of the blends. In dual choice assays, the optimal dose for the positive control was first established. At the optimal dose, the positive control was further used in formulating binary and ternary blends which were evaluated for their attractiveness to *Anopheles gambaie sensu stricto*. The most attractive and antagonistic blends derived from plant and mammalian hosts were further evaluated in field trials in Ahero and Marigat for 12 days whereby 7 traps/day (number of traps was equivalent to number of treatments) were set as shown in figure 3.1. For randomization of treatments in the field, Latin square block design was used whereby each treatment was evaluated in each of the selected study block point to minimize on positional biasness.

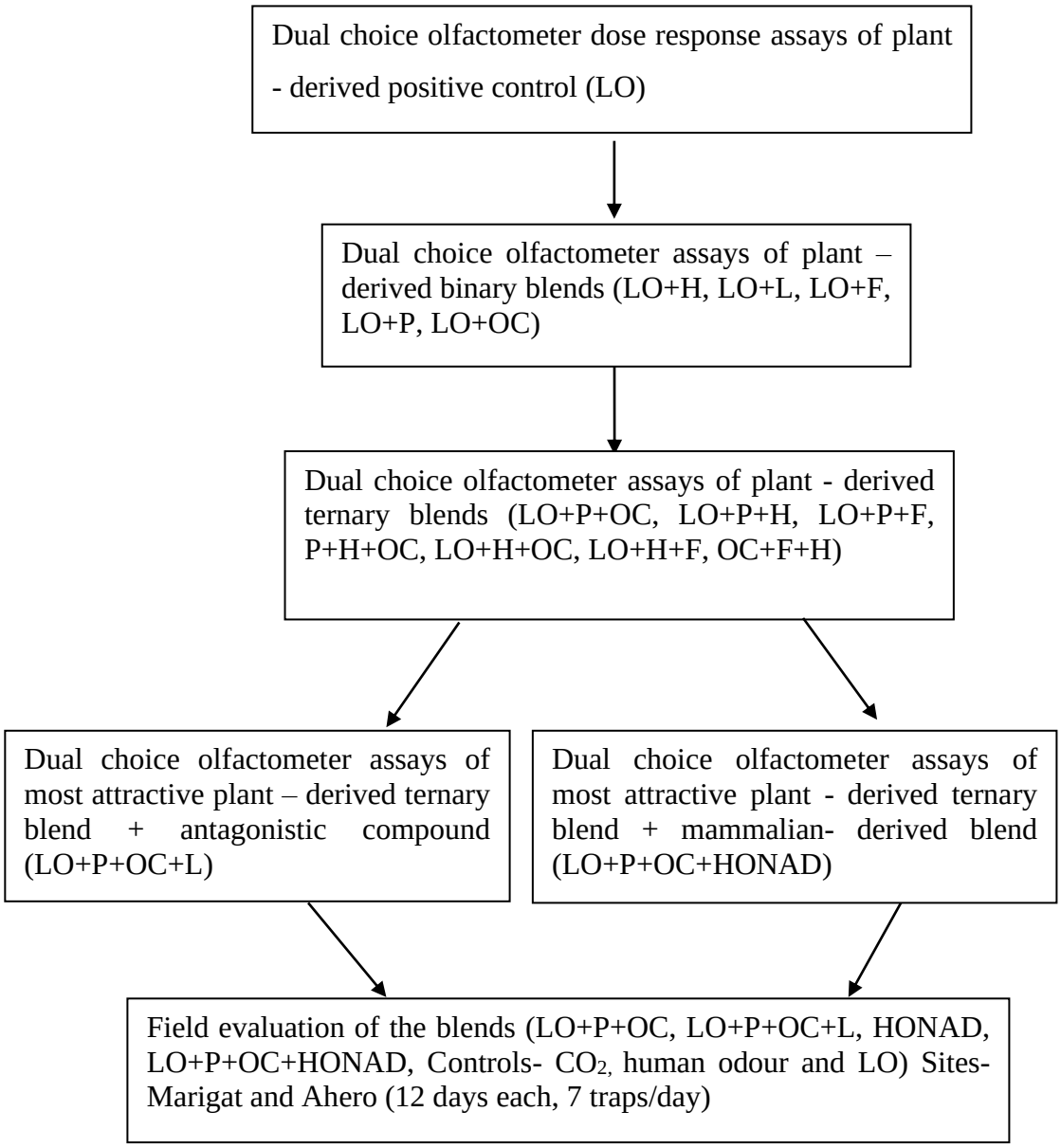

Figure 3.1: A flow chart showing the study design for laboratory and field trials

3.2 Laboratory rearing conditions for mosquitoes used in dual choice assays

Female *Anopheles gambiae sensu stricto* (hereafter as *An. gambiae*) used for dual choice olfactometer studies were obtained from a colony established in 2001 and constantly infused with field collected gravid female mosquitoes to minimize on genetic variability,

at the insectary of the International Centre of Insect Physiology and Ecology (*icipe*) Duduville Campus, Nairobi, Kenya. The rearing conditions were maintained at a mean temperature of 31°C and relative humidity (RH) of 52% during the day while at night the mean temperature and relative humidity were 24°C and 72% RH, respectively. The photophase and scotophase period was maintained at 12 hr light and 12 hr darkness respectively. Adult mosquitoes were maintained on a diet of 6% glucose solution (ad libitum) (Sigma®) and human blood thrice a week. Filter paper lined oviposition cups (4 cm diameter, 2 cm depth) were placed in cages for gravid females to lay their eggs after which they were transferred into 25 cm L × 20 cm W × 14 cm H plastic trays filled with distilled water up to a depth of 8cm. Hatched larvae were reared (density of 100-150/tray) in these trays and fed three times a day on fish food (Tetramin®) i.e. 0.3 g tetramin/100 larvae/day. Emerging pupae were transferred into mesh-covered cages measuring 30 L × 30 W × 30 H cm after which newly emerged 2-3-day old females were transferred into 15 L × 15 W × 15 H cm mesh-covered cages. The experimental adults were maintained on 6% glucose solution only while 6 h prior to the experiments, they were starved of glucose and given distilled water on cotton wool.

3.3 Synthetic chemicals used in developing test blends

The synthetic chemicals formulated were previously identified from *Parthenium hysterophorus*, a suitable host plant for *An. gambiae s. s* (Nyasembe et al., 2012) and mammalian hosts for primary vectors of Rift Valley fever (Tchouassi et al., 2013). The synthetic standards constituted a previously formulated blend C (Nyasembe et al., 2012) which included hexanal (Aldrich, 98%), β-pinene (Chemika, 99.5%), β-ocimene (Chemika, (Z)-β-ocimene =27%, (*E*)-β-ocimene = 67% and allo-ocimene = 6%), limonene (Sigma), (*E*)-linalool oxide (Aldrich), and (*E*)-β farnesene (Bedoukian Research, CT, USA). The mammalian-based blend of aldehydes found to be major components of animal skin (sheep, goat, cow and donkey) and human foot odors (Tchouassi et al., 2013) comprising heptanal, octanal, nonanal and decanal, all from Aldrich, 98%, is hereafter referred to as HONAD.

3.4 Design of laboratory dual choice olfactometer assays

Bioassays were carried out using a dual choice olfactometer (Figure 3.2) similar to that described by Nyasembe et al. (2012). Compressed air from a cylinder was purified by passing it through activated charcoal and afterwards humidified by passing it through distilled water. For test and control chambers (ARS, Gainesville, FL, USA) on either side of the olfactometer (30 L × 30 W × 100 H cm) enclosing the treatment and control compounds, a steady air at a flow rate of 350 ml/min was passed through them into the olfactometer with temperature and humidity in the bioassay room maintained at 24°C and 72% RH, respectively. A vacuum created by a fan at the center of the olfactometer ensured continuous pulling of air at a rate of 700 ml/min thereby preventing a build-up of the odors.

Two red fluorescent bulb (40-Watt) placed above the center of the olfactometer were used to illuminate the test arena. Female *An. gambiae* aged 3-4 days were assayed for responses to different blends . A 100 µl of each treatment blend dissolved in pentane was dispensed on 100 mg of Luna dental roll (Roeko®, Langenau, Germany) and left for 30 min at room temperature to allow for solvent evaporation before experimental use. Similar procedure was applied for the positive ((*E*)-linalool oxide) and negative (pentane) controls. (*E*)-Linalool oxide, was used as a positive control because it had been shown to be highly attractive to malaria vectors and comparable to human foot odors, known to be highly attractive to these vectors (Nyasembe, Tchouassi, et al., 2014). Mosquitoes were first starved of glucose solution for 6 hrs. prior to the choice assays. Each experiment comprised release of mosquitoes at the center of the olfactometer in batches of 10 and then allowed 10 min to make a choice between the treatment and control. All experiments were conducted at the same time between 1400hr-1800hr and for each compound or blend. This was replicated 10 times. Randomization of the test blends and the control in the olfactometer arms was done between the runs to minimize positional bias. Mosquitoes landing in zone A and D within 25 cm from either ends of the olfactometer as shown in Figure 1, were considered

as positive responses to either the control or test blends. On the other hand, mosquitoes staying between zones B and C, 25 cm from the release point on either side, were considered non-respondents. The number of mosquitoes responding to the test and control odor sources were counted and recorded in each run.

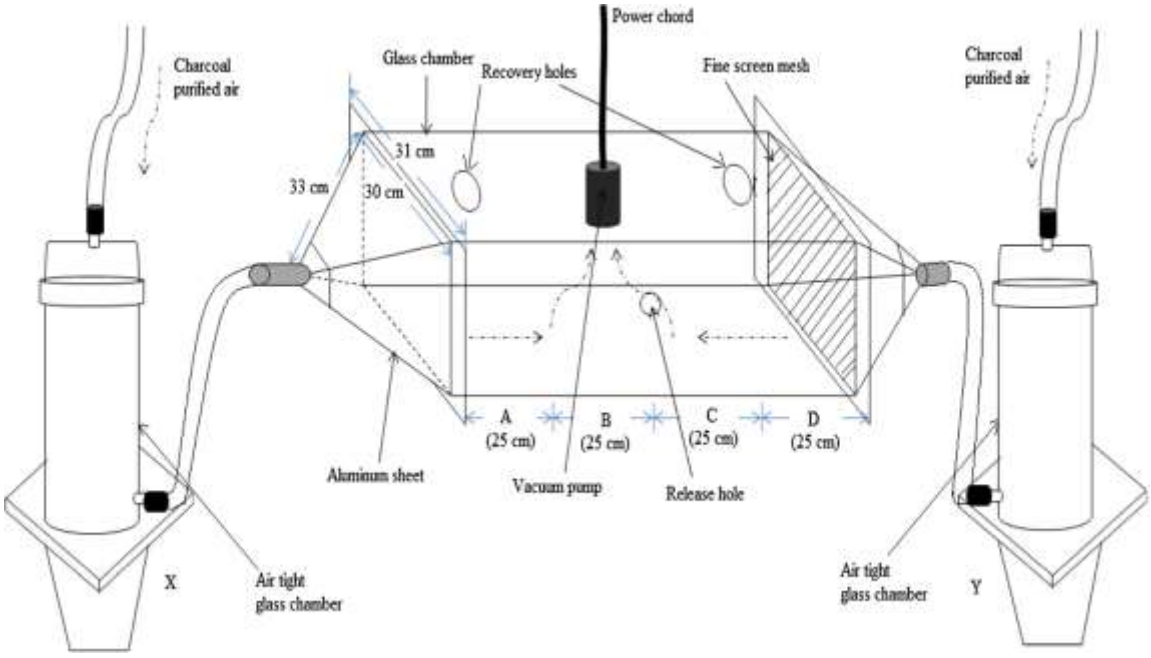

Figure 3.2: Schematic diagram of a dual choice olfactometer (Source: Nyasembe et al., 2012)

3.5 Laboratory dual choice bioassays with chemicals

(*E*)-linalool oxide (hereafter, LO) was first evaluated over a range of doses by arbitrarily halving and doubling the optimal dose of 0.2 ng/µl as previously established by Nyasembe et al. (2012). A lower dose of 0.1 ng/ul was first evaluated followed by a consecutive two-fold increase in the dose, up to 0.8 ng/ul against a control solvent to establish the optimal attractive dose. Binary blends of LO at its optimal dose of 0.4 ng/ul

with other blend C components including, hexanal, β-pinene, β-ocimene, limonene, and (*E*)-β farnesene, at three different doses each viz 0.1 ng/ul, 0.2 ng/ul, 0.4 ng/ul as previously established (Nyasembe et al., 2012) were developed and evaluated against control solvent (pentane) for their attractiveness to female *An. gambiae*. Compound/s found to antagonize the response of LO was further subtracted from blend C to develop blends for further evaluation. Potential antagonist(s) was added to any blend found to be more attractive than LO, to further confirm its effect on female response. This was followed by formulating ternary blends based on a 3-component selection of all possible combinations of blend C components and evaluation against LO for their attractiveness to *An. gambiae s.s*. The mosquito's response to the mammalian-based blend, HONAD, when combined with the most attractive blend of plant compounds observed was equally evaluated (Table 3.1).

Table 3.1: Binary and ternary blends used in dual choice assays

Blend type	Blend composition	Abbreviations	Doses (ng/µl)
Binary	(*E*)-linalool oxide+ β-pinene	LO + P	0.4+ (0.1, 0.2, 0.4)
	(*E*)-linalool oxide+ β-ocimene	LO + OC	0.4+ (0.1, 0.2, 0.4)
	(*E*)-linalool oxide+(*E*)-β farnesene	LO + F	0.4+ (0.1, 0.2, 0.4)
	(*E*)-linalool oxide + hexanal	LO + H	0.4+ (0.1, 0.2, 0.4)
	(*E*)-linalool oxide + limonene	LO + L	0.4+ (0.1, 0.2, 0.4)
Ternary	(*E*)-linalool oxide+ β-pinene+ β-ocimene	LO+P+OC	0.4+0.4+0.1
	(*E*)-linalool oxide+ β-pinene+ hexanal	LO+P+H	0.4+0.4+0.4
	(*E*)-linalool oxide+ β-pinene+(*E*)-β farnesene	LO+P+F	0.4+0.4+0.2
	β-pinene+ hexanal+ β-ocimene	P+H+OC	0.4+0.4+0.1
	(*E*)-linalool oxide+ hexanal+ β-ocimene	LO+H+OC	0.4+0.4+0.1
	(*E*)-linalool oxide+ hexanal+(*E*)-β farnesene	LO+H+F	0.4+0.4+0.2
	β-pinene+ β-ocimene+(*E*)-β farnesene	P+OC+F	0.4+0.1+0.2
	β-ocimene+(*E*)-β farnesene+ hexanal	OC+F+H	0.1+0.2+0.4
	heptanal + octanal + nonanal + decanal	HONAD	0.4+0.4+0.2+0.1

3.6 Field evaluation of developed blends

3.6.1 Field study sites

Field trials were conducted in Ahero and Marigat both endemic areas for malaria in Kenya. Ahero is situated approximately 24 km south east of Kisumu, western Kenya. It has an annual rainfall of 1000-1800 mm, a temperature range of 17-32°C and relative humidity of 65% due to its closeness to Lake Victoria and River Nyando (Atieli *et al.*, 2009). In terms of malaria transmission, it is holoendemic with entomological inoculation rates of 0.4-17 infective bites/person/year with major malaria vectors being

An. gambiae s.l. and *An. funestus* group (Ndenga et al., 2006; Zhou et al., 2011). Traps were set in Kigoche village (S00°09.206 E034°55.904) located near the Nyando River.

Marigat district is in the Rift Valley Province of Kenya, 250 km North West of Nairobi. The mean temperature ranges between 30-35°C and annual rainfall of 300-700 mm (Tchouassi et al., 2012). In terms of malaria endemicity, it is a low transmission area with major vector being *Anopheles gambiae sensu lato* (Mala et al., 2011, Omondi et al., 2017). The traps were set in Kapkuikui village (N00°22.359 E036°02.616) located near Lake Bogoria (Figure 3.3). Therefore, the two sites with varying malaria endemicity were selected for comparison of performance of the odorant compounds.

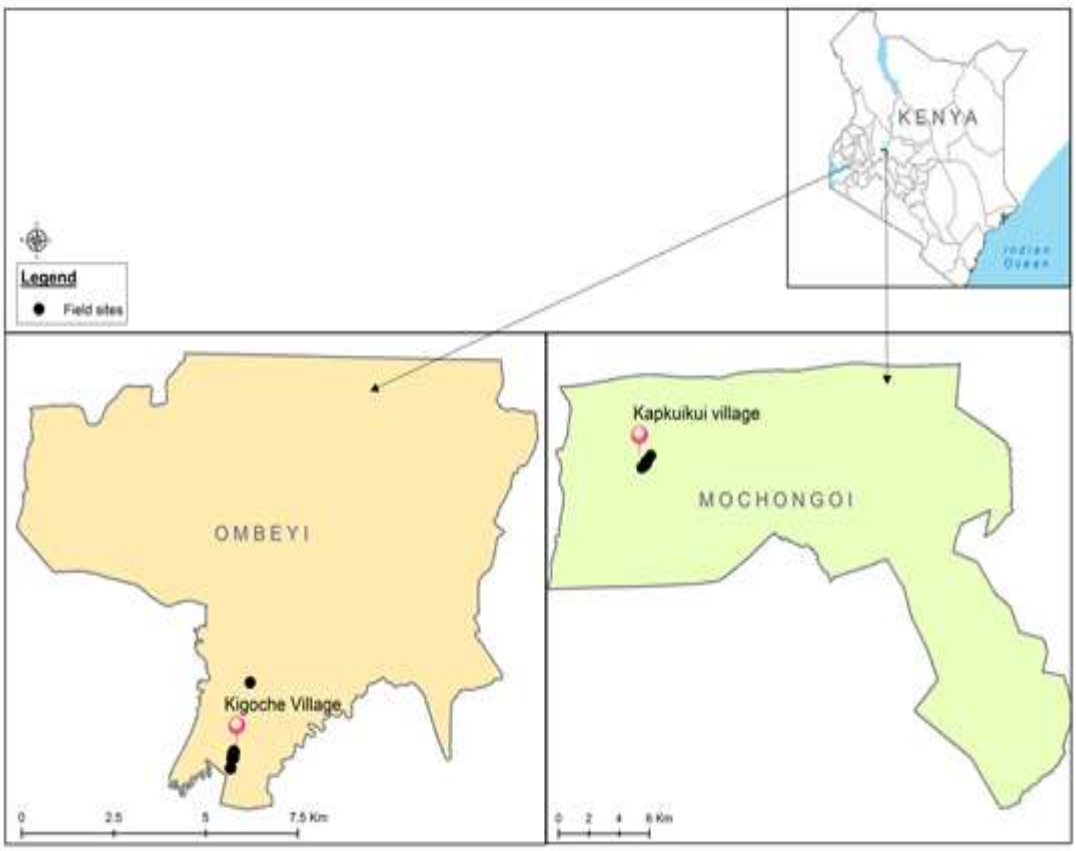

Figure 3.3: Map showing location of the study sites for field trials in Kigoche village, Ahero (bottom left panel) and Kapkuikui village, Marigat (bottom right panel), Kenya (Source: Jackson Kimani)

3.6.2 Feld study design

Field studies were conducted during the rainy seasons when the malaria vector population in both sites was high. The study was carried out in November and February 2016 in Ahero and Marigat, respectively, for at least eleven days as replicates per site. Solutions of treatment blends in pentane were each dispensed in rubber septa (Figure 3.4) and evaluated on mosquito catches using Magnetic mosquito–X (MMX) traps (Figure 3.5).

Figure 3.4: Rubber septa used for dispensing odors in field trials (Source: Juliah Wanjiru)

The rubber septa were replaced daily for each treatment. The superior performance of MMX trap over the CDC trap in trapping the malaria vectors has been reported (Nyasembe et al., 2014). Based on their high attractiveness/repellency to *An. gambiae* in dual choice assays relative to LO, four blends (blend LO+P+OC, blend LO+P+OC+L, HONAD and HONAD + blend LO+P+OC) were selected for further field trials. Also included, were three controls of carbon dioxide (CO_2), human foot odors from worn socks and (*E*)-linalool oxide. CO_2 in the form of dry ice was dispensed at a release rate of approximately 41±2.3 g/h (Nyasembe et al., 2014) by placing 2 kg in 2L Igloo

thermos containers (John W Hock, Gainesville, FL) with a 13-mm hole in the bottom center and delivered to the top of the Magnetic mosquito–X trap through a Tygon tubbing.

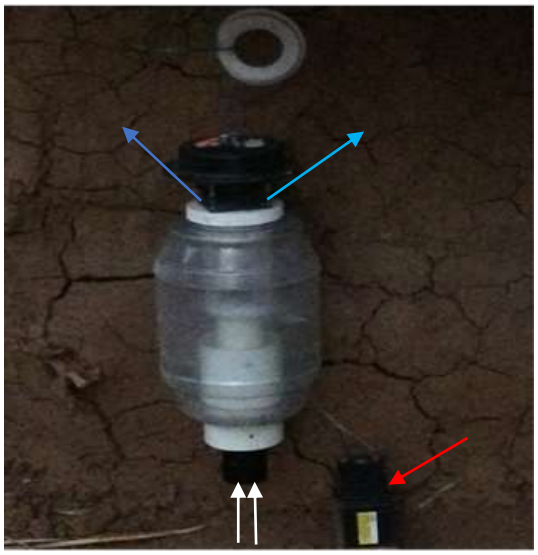

Figure 1.5: Magnetic Mosquito-X (MMX) trap powered by 12volt portable battery (red arrow) with a fan at the top to dispense the odors (blue arrows) and at the bottom to pull in the mosquitoes (white arrows) (Source: Juliah Wanjiru)

Traps (treatments) were deployed daily at 1800hrs and set approximately 30m apart and 15 cm above the ground near homesteads and left overnight till 0600hrs the following morning. Randomization of the different trap treatments was done using the Latin square block design in 12-days as replicates. For this study design, different points, equivalent to the number of treatments, in a transect were selected and each treatment was rotated around the points.

3.6.3 Optimization of blends in the field

Dose optimization on blends was carried out in Ahero. This was to suit field conditions which unlike controlled laboratory conditions, attractiveness of test compounds varies

based on other factors such as background odors, temperatures, wind and humidity. Arbitrarily, 100-fold higher concentration of the optimal dose for each blend established in the laboratory was selected for field trials. From these, two 10-fold higher doses were included, and mosquito catches were monitored based on evaluation of these respective doses for each blend. The doses used for further field trials for blend LO+P+OC, HONAD and blend LO+P+OC+L were 0.1 mg/ml, 1 mg/ml and 0.01 mg/ml, respectively, as these doses recorded the highest mean number of *Anopheles* captures in a 3-day replicate trial. Based on a previous study (Nyasembe et al., 2014), (*E*)-linalool oxide was used at a dose of 40 ng/µl as the reference (positive control).

3.7 Molecular identification of *Anopheles gambiae s.l* member species

A subset of 200 field trapped *Anopheles gambiae sensu lato* samples from Ahero and 205 from Marigat were randomly selected from all the trap treatments and subjected to molecular analysis for member species identification in the complex. High resolution melting (HRM) analyses, a post-polymerase chain reaction (PCR) method using primer pairs targeting the ribosomal internal transcribed spacer (ITS2) gene to discriminate *An. gambiae s.s* and *An. arabiensis* (Zianni et al., 2013) which are the major Anopheline species reported in Ahero and Marigat was used (Bayoh et al., 2010; Mala et al., 2011; Nyasembe et al., 2014). Genomic DNA was extracted from individual whole mosquitoes using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, GmbH-Hilden, Germany) as per the manufacturer's instructions. DNA amplification was conducted using the Solis Biodyne kit (Solis BioDyne, Tartu, Estonia), using established universal primers: forward 5′-GTGAAGCTTGGTGCGTGCT-3′ and reverse 5′-GCACGCCGACAAGCTCA-3 (Zianni et al., 2013). The PCR mix contained 2 µl of 5X Hot Firepol EvaGreen HRM Mix, 0.5 µM of each primer, 1 µl of DNA template and 6µl of PCR water in a final volume of 10 µl. A negative control comprising of PCR grade water and positive controls comprising laboratory colonies of *An. gambiae s.s.* and *An. arabiensis* were included. Thermal cycling conditions involved an initial denaturation for 1 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds,

annealing at 57°C for 30 seconds, and extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. Without stopping the reaction, the PCR amplicons were denatured at 95°C for 1 minute, held for another minute at 40°C and melted by gradually raising the temperature from 70°C to 95°C by 0.1°C in 2 second steps, waiting for 90 seconds of pre-melt conditioning on first step and 2 seconds with gradual temperature increase till 95°C. The outcome was automatically plotted on a connected computer and visually observed and analysed using the Rotor-Gene Q Series software v2.1.

3.8 Ethical considerations

Permission to set traps in homesteads was sought orally from village elders and homeowners at both field sites prior to the experiments. Insectary rearing of mosquitoes followed institutional standard operating procedures to ensure good laboratory practice. Mosquitoes were arm-fed by insectary personnel only (with written consent) with approval from the Scientific Ethics Review Unit (SERU), at the Kenya Medical Research Institute (KEMRI) under Protocol number 391 renewed annually. Arm-feeding is done to ensure that laboratory reared mosquitoes are behaviorally responsive to human odors which is vital for behavioral studies.

3.9 Statistical analyses

For laboratory dual choice assays, the number of mosquitoes responding to the treatment and controls was recorded. The data was further analysed by subjecting to a generalized linear model (GLM) with binomial error structure and/or quasibinomial error structure in case of over/under dispersion and logit in R 3.2.1 software (R development core team, 2010). The incidence rate ratio (IRR) and corresponding confidence interval (CI) that mosquitoes prefer other treatments relative to the control were estimated.

Field collected mosquitoes were first identified to species level using existing morphological keys (Gillies and De Meillon., 1968; Gillies and Coetzee., 1987. All analyses were implemented in R version 3.3.1 at 95% significance level. Pair-wise

comparison in performance between the treatments was performed by Tukey's HSD test. Each treatment was compared to the control ((*E*)-linalool oxide) as the reference and incidence rate ratio (IRR) estimated, as a likelihood measure that mosquitoes chose other treatments other than the control. For the control, the IRR is 1 with values above this indicative of treatments with better performance and values below underperformance relative to the control (Tchouassi et al., 2012).

CHAPTER FOUR

RESULTS

4.1 Dual choice olfactory bioassays

Anopheles gambiae responded in a dose dependent manner to all the four doses (0.1, 0.2, 0.4 and 0.8 ng/µl) of (*E*)-linalool oxide (LO) tested. The dose of 0.4 ng/µl was optimal and the most attractive relative to control solvent (pentane) (Figure 4.1).

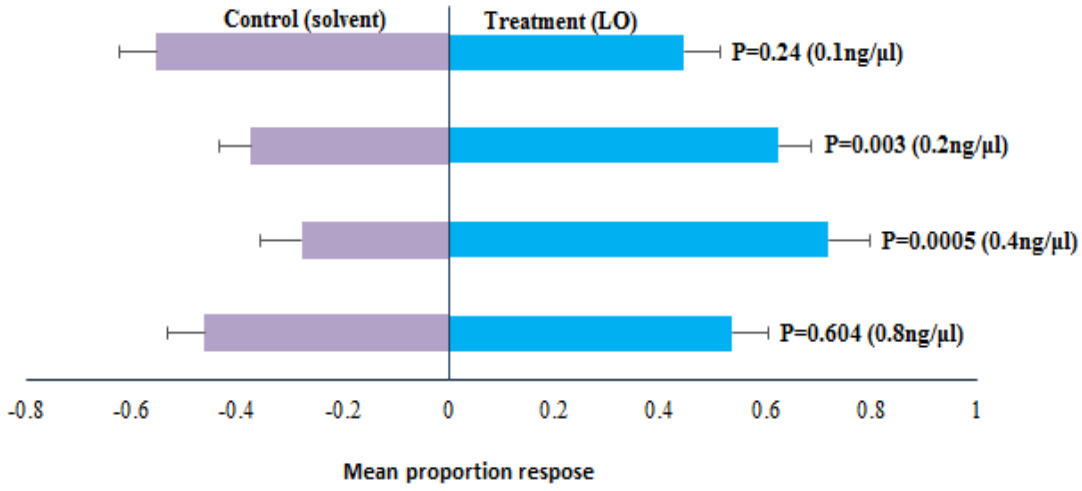

Figure 4.1: Dual choice olfactometer dose response assay of female *Anopheles gambiae sensu stricto* to (E)-linalool oxide against control solvent (pentane)

Mean proportion represents the number of mosquitoes responding to either treatment or control in 10 replicates. P-values indicate levels of significance between LO at the different doses and the control whereby P>0.05 indicates no significant difference between treatments and P<0.05 indicates a significant difference between treatments.

Each pair of evaluation used a total of 100 mosquitoes released in batches of 10 in ten replicate trials for each experiment.

The binary blends of LO + β-ocimene, LO + β-pinene, LO + (*E*)-β farnesene and LO + hexanal at all the doses tested (0.1, 0.2 and 0.4 ng/µl) were more attractive than the control solvent, but none of the binary blends performed better than LO alone (Figure 4.2). However, a binary blend of LO and limonene was antagonistic at all doses of limonene tested (0.1, 0.2 and 0.4 ng/µl) and was not significantly different from the control (Figure 4.2).

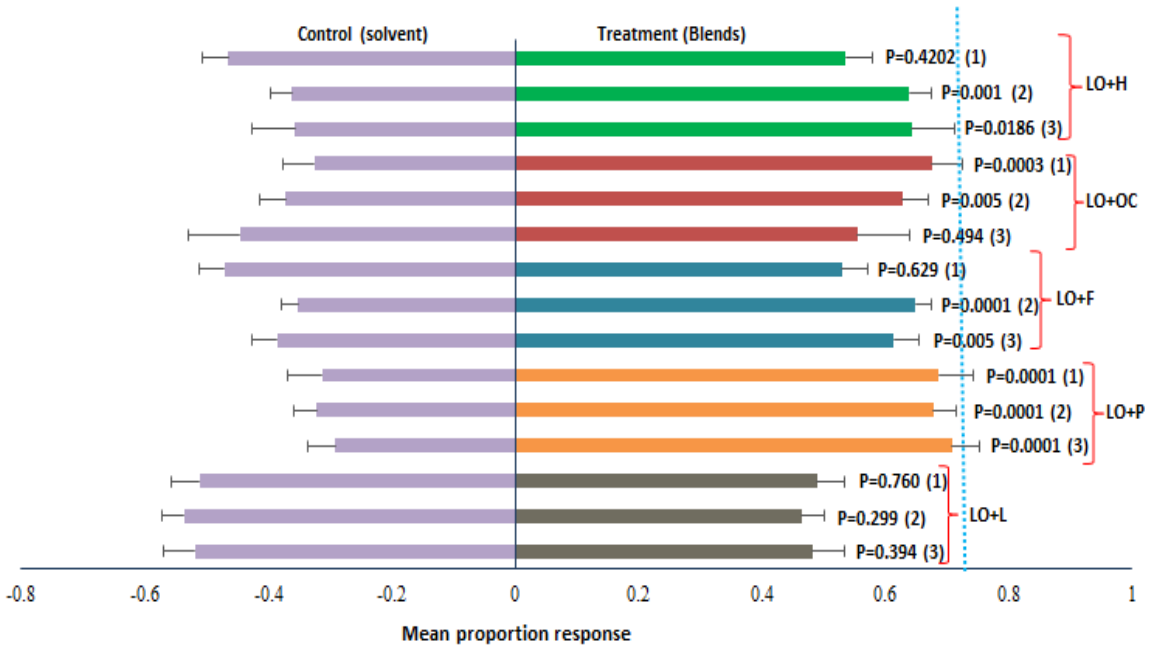

Figure 4.2: Dual choice olfactometer response of female *Anopheles gambiae sensu stricto* to binary blends at different doses relative to control solvent

Mean proportion represents the number of mosquitoes responding to either treatment or control in 10 replicates. H-hexanal, P- β-pinene, OC- β-ocimene, L-limonene, F-(E)-β farnesene. The numbers 1, 2 and 3 represent doses of the compounds at 0.1 ng/µl, 0.2 ng/µl and 0.4 ng/µl, respectively. P-values indicate levels of significance between each

treatment and control whereby P>0.05 indicates no significant difference between treatments and P<0.05 indicates a significant difference between treatments. Each pair of experimental evaluation used a total of 100 mosquitoes released in batches of 10 in ten replicate trials. The blue line indicates mean proportion response of LO [(E)-linalool oxide] at optimal dose.

Further, of the eight ternary blends evaluated against LO, only a blend of LO (0.4 ng/µl) + β-pinene (0.4 ng/µl) + β-ocimene (0.1 ng/µl) was significantly preferred relative to LO with about a 2-fold greater preference [IRR=2.3; 95% CI (1.3-4.4)] (Figure 4.3).

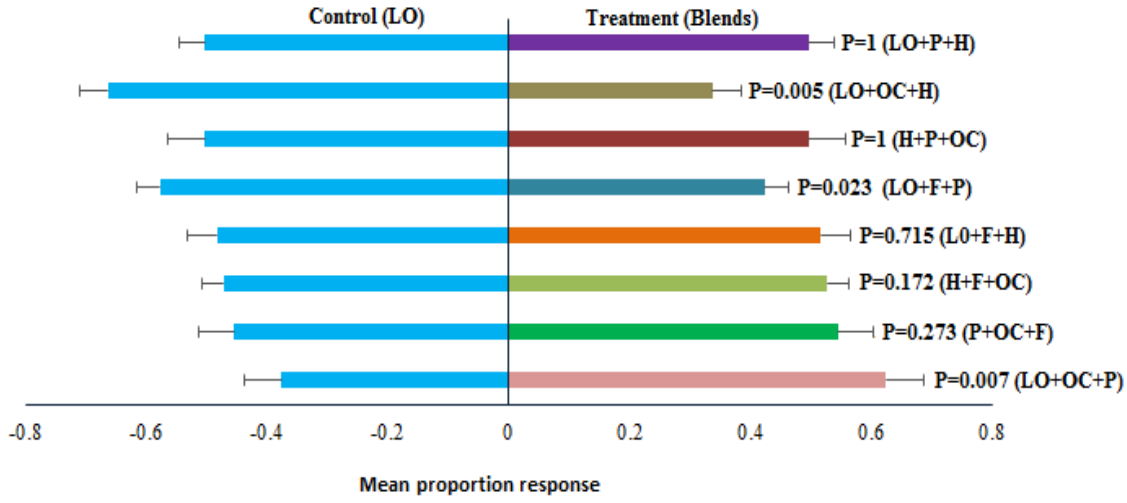

Figure 4.3: Dual choice olfactometer response of female *Anopheles gambiae sensu stricto* to ternary blends against (E)-linalool oxide

Mean proportion represents the number of mosquitoes responding to either treatment or control in 10 replicates. H-hexanal, P- β-pinene, OC- β-ocimene, L-limonene, F-(E)-β farnesene. P-values indicate the levels of significance for the pair of treatments whereby P>0.05 indicates no significant difference between treatments and P<0.05 indicates a significant difference between treatments. Each pair of experimental evaluation used a total of 100 mosquitoes released in batches of 10 in ten replicate trials.

Addition of limonene (previously found to reduce attractiveness of LO (Figure 4.2)) to blend LO+P+OC (which exhibited higher attraction than any other blend and better than LO (Figure 4.3), to form the blend LO+P+OC+L, there was a 2-fold decrease in attractiveness of this blend relative to LO [IRR=2.3; 95% CI (1.4-3.8)] (Figure 4.4). Also, the effect of combining animal-based odor from a known blend of aldehydes (HONAD) and a blend of LO+P+OC (representing the most attractive plant-based blend) was evaluated. There was a reduced attractiveness of female *An. gambiae* to blend LO+P+OC+HONAD, compared to the attractiveness recorded to the individual plant- [LO+P+OC [IRR=2.3; 95% CI (1.3-4.4)] and animal-based HONAD [IRR=2.9; 95% CI (0.2-0.7)] blends (Figure 4.4).

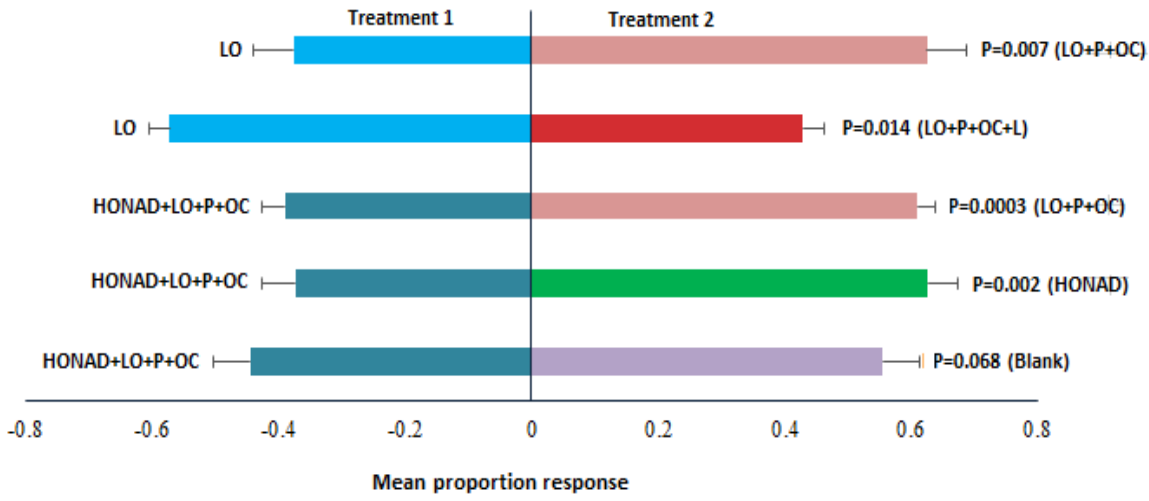

Figure 4.4: Dual choice olfactometer response of female *Anopheles gambiae sensu stricto* to different blends against control

Mean proportion represents the number of mosquitoes responding to either treatments in 10 replicates. HONAD (heptanal + octanal + nonanal + decanal), P- β-pinene, OC- β-ocimene, and L-limonene. P-values indicate the levels of significance for each pair of treatments compared whereby P>0.05 indicates no significance difference between

treatments and P<0.05 indicates a significant difference between treatments. Each pair of evaluation used a total of 100 mosquitoes released in batches of 10 in ten replicate trials.

4.2 Field evaluation of the blends

4.2.1 Evaluation of odorant blends in Ahero

Initial field trials to optimize doses for the different blends were carried out. For blend, LO+P+OC the highest mean *Anopheles* captures was recorded for the concentration of 0.1 mg/ml while for blend LO+P+OC+L, a concentration of 0.01 mg/ml had the highest mean captures. The aldehyde blend (HONAD) was found to be most attractive at a concentration of 1 mg/ml. A combination of the plant- and mammalian- based compounds (blend LO+P+OC+HONAD) was at the optimal concentrations of the respective individual blends (Figure 4.5).

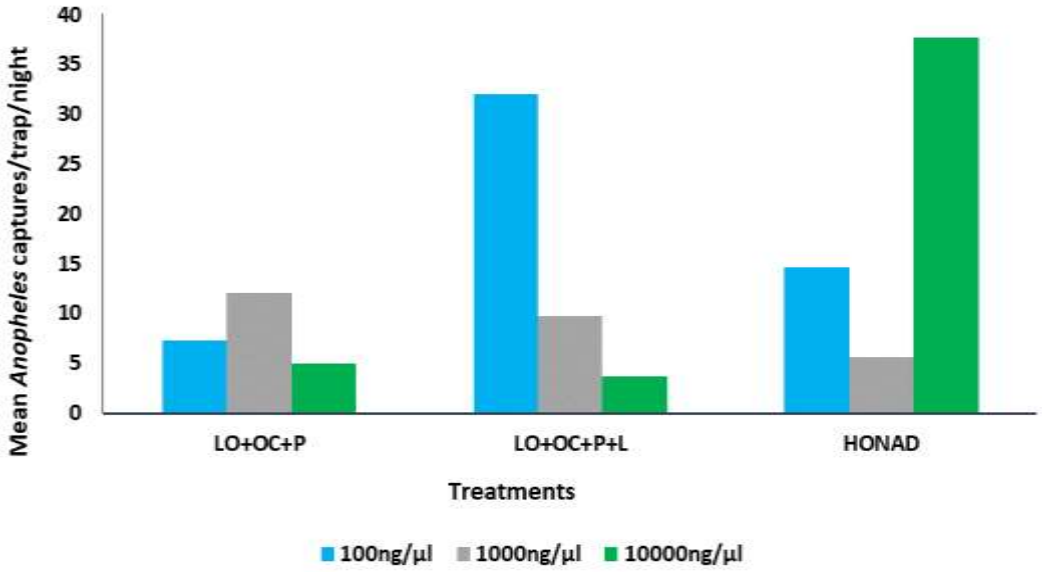

Figure 4.5: Mean daily catches of female *Anopheles* at different doses for different treatments.

P- β-pinene, OC- β-ocimene, L-limonene, HONAD [heptanal + octanal + nonanal + decanal].

A total of 764 anophelines from all the treatments following field evaluation in Ahero over a period of ten days was recorded. Out of these, *An. gambiae s.l* and *An. funestus* were the major species. The total catch for *An. gambiae s.l.* was 329, out of which 69 were engorged and 39 males. Molecular speciation of 200 randomly selected *An. gambiae s.l* from this site were all identified as *An. arabiensis*. A representative HRM profile to discriminate the two species is shown in figure 4.6.

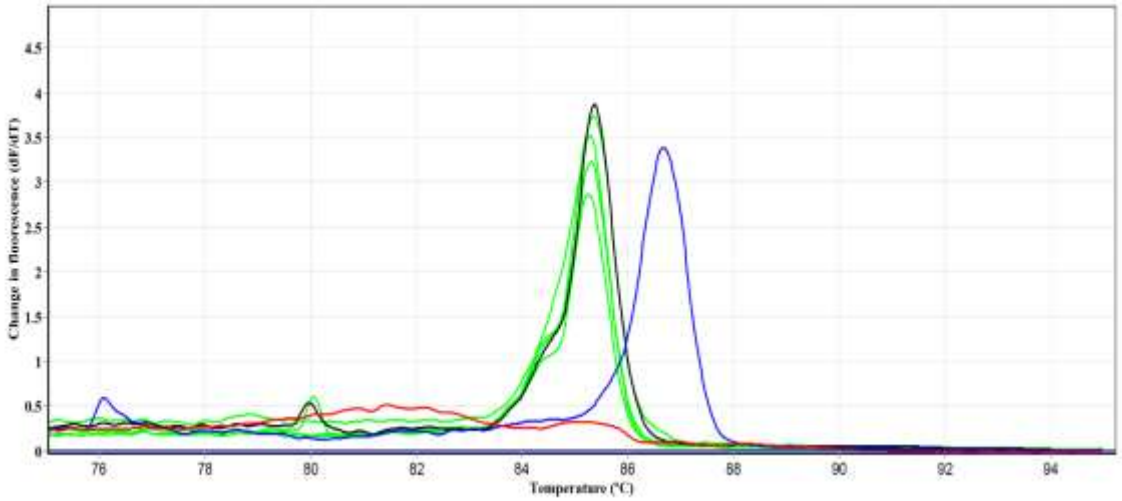

Figure 4.6: Representative HRM profile to discriminate *Anopheles gambiae sensu stricto* and *Anopheles arabiensis*

Melt curves in blue- laboratory colony of An. gambiae s.s; black- laboratory colony of An. arabiensis; green- field samples; red- negative control (non-template). The Y-axis shows a change in fluorescence and the X-axis, melting temperatures.

Total catch for *An. funestus* was 373 out of which 17 were engorged and 34 males. Minor anopheline species (present in low numbers) were *An. ziemanni* (n=11) and *An. coustani* (n=51). Other mosquito species such as *Culex* spp. (n=1756), *Aedes* spp.

(n=22), and *Mansonia* spp. (n=99) were also recorded. In Ahero, comparison of mosquito trap catches across the treatments was limited to female anophelines of the species, *An. gambiae s.l.* and *An. funestus* group.

Only traps baited with CO_2 attracted more *An. arabiensis* [IRR=3.9; 95% CI (1.9-8.2)] than LO (Table 4.1). Trap captures of *An. arabiensis* recorded for human odors from worn socks, blend LO+P+OC+L, LO+P+OC, HONAD and LO+P+OC+HONAD were comparable to LO. For *An. funestus* group, none of the treatments was better than LO (Figure 4.7, Table 4.1).

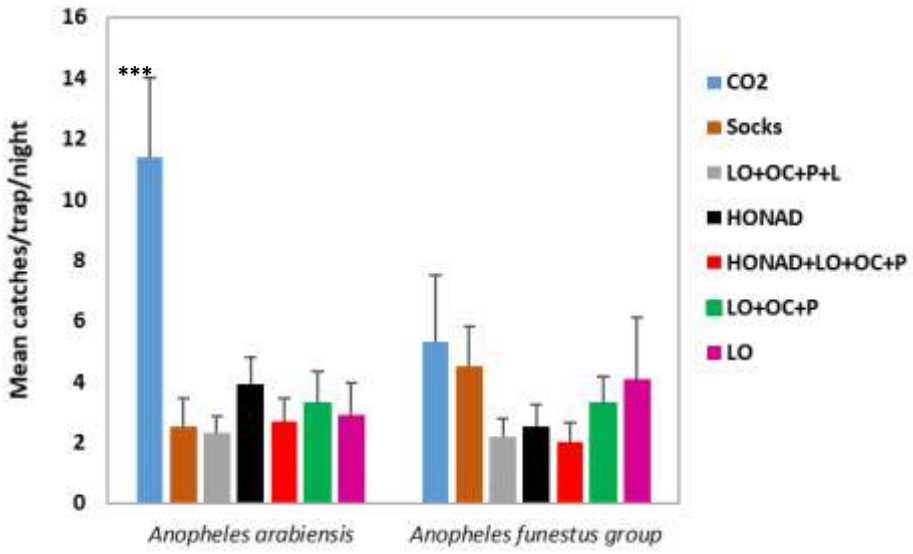

Figure 4.7: Mean daily mosquito catches ((±SEM) recorded for different treatments in Ahero, Kenya, 10 replicate trials.

P- β-pinene, OC- β-ocimene, L- limonene, HONAD [Heptanal+Octanal+Nonanal+Decanal], CO_2 (carbon dioxide), worn socks and LO [(E)-linalool oxide] as the control. Asterisks indicate levels of significance for each treatment compared to the control at P<0.001 (***).

Table 4.1: Comparison of female anophelines captures in the different treatments relative to (E)-linalool oxide (control) from Ahero, Kenya

Site	Species	Treatment	Number of captures (n)	IRR(CI)	*P-value*
Ahero	*An. arabiensis*	CO2[a]	114	3.9(1.9-8.2)	<0.001***
		LO [b]	29	1(reference)	
		Socks [b]	25	0.9(0.4-1.9)	0.719
		LO+P+OC [b]	33	1.1(0.5-2.5)	0.478
		LO+P+OC+L [b]	23	0.8(0.3-1.8)	0.759
		HONAD [b]	39	1.3(0.6-2.9)	0.454
		LO+P+OC+HONAD[b]	27	0.9(0.4-2.1)	0.862
	An. funestus group	CO2 [a]	53	1.3(0.5-3.1)	0.561
		LO [a]	41	1(reference)	
		Socks [a]	45	1.1(0.5-2.6)	0.835
		LO+P+OC [a]	33	0.8(0.3-2.0)	0.633
		LO+P+OC+L [a]	22	0.5(0.2-1.4)	0.186
		HONAD [a]	25	0.6(0.2-1.5)	0.288
		LO+P+OC+HONAD[a]	20	0.5(0.2-1.2)	0.131

Number of replicate trials: 10. IRR, incidence rate ratios; CI, confidence interval at α=0.05 level of significance. Treatments followed by different alphabetical letters are

significantly different for each species. Asterisks indicate levels of significance for each treatment compared to the control at P<0.001 (***).

4.2.2 Evaluation of odorant blends in Marigat

Optimized doses for the blends used in Ahero were also evaluated in Marigat using the Latin square design with each treatment compared to (*E*)-linalool oxide (LO). A total of 2454 anophelines was captured by all the treatments over a period of twelve days. The predominant species was *Anopheles pharoensis* with total captures of 1852 out of which 414 were engorged, followed by *An. gambiae s.l* (with total captures of 583 out of which 155 were engorged and 71 males). Randomly selected 205 *An. gambiae s.l* were all identified as *An. arabiensis* after molecular processing (Figure 4.6). Other anophelines trapped but in reduced numbers included *An. funestus* (n=17), and *Anopheles coustani* (n=2). Other mosquito species captured included *Culex* spp. (n=690), *Aedes* spp. (n=13), *Mansonia* spp. (n=137) and *Coquilettidia* spp. (n=27).

For Marigat data, only captures of the major female anophelines viz: *An. arabiensis* and *An. pharoensis*, were compared across the different treatments. As found in Ahero, relative to LO, only CO_2 significantly captured a higher number of *An. arabiensis* [IRR=3.1; 95% CI (1.4-6.7), while catches for the other treatments were as found for LO. For *An. pharoensis*, significant higher catches were observed only for CO_2 [IRR=4.2; 95% CI (1.2-14.4)] relative to LO. Although blend LO+P+OC+L showed better performance, the difference was not significant compared to LO [IRR=3.2; 95% CI (0.9-11.2)]. (Figure 4.8, Table 4.2).

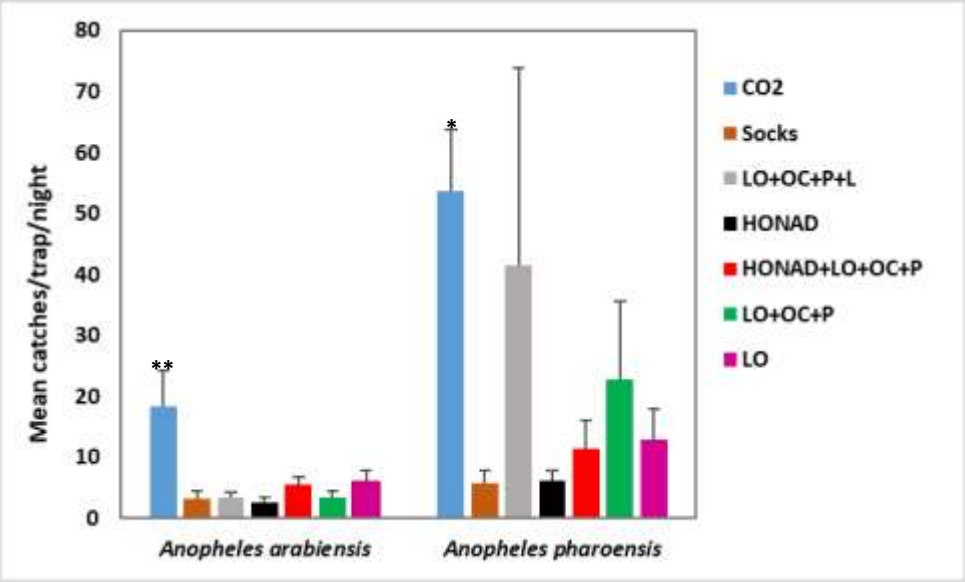

Figure 4.8: Mean daily catches (±SEM) of *Anopheles gambiae sensu lato* and *Anopheles pharoensis* recorded in the different treatments in Marigat, Kenya, 12 replicate trials

P- β-pinene, OC- β-ocimene, L-limonene, HONAD [heptanal+ octanal + nonanal+ decanal], CO_2 (carbon dioxide), worn socks and LO [(E)-linalool oxide] as the control. Asterisks indicate levels of significance for each treatment compared to the control at $P<0.05$ (*) and $P<0.01$ (**).

Table 4.2: Comparison of female anophelines captures in the different treatments relative to (E)-linalool oxide (control) from Marigat, Kenya

site	Species	Treatment	Number of captures (n)	IRR(CI)	*P-value*
Marigat	*An. arabiensis*	CO2 [a]	221	3.1(1.4-6.7)	0.004**
		LO [b]	72	1(reference)	
		Socks [b]	38	0.5(0.2-1.2)	0.128
		LO+P+OC [b]	42	0.6(0.3-1.3)	0.196
		LO+P+OC+L [b]	42	0.6(0.3-1.3)	0.196
		HONAD [b]	32	0.4(0.2-1.0)	0.057
		LO+P+OC+HONAD[b]	65	0.9(0.4-2.0)	0.801
	An. pharoensis	CO2 [a]	645	4.2(1.2-14.4)	0.02*
		LO [b]	154	1(reference)	
		Socks [b]	70	0.5(0.1-1.6)	0.207

	LO+P+OC [b]	272	1.8(0.5-6.1)	0.356
	LO+P+OC+L [b]	498	3.2(0.9-11.2)	0.056
	HONAD [b]	75	0.5(0.1-1.7)	0.249
	LO+P+OC+HONAD[b]	138	0.9(0.3-3.1)	0.859

Number of replicate trials: 12. IRR, incidence rate ratios; CI, confidence interval at α=0.05 level of significance. Treatments followed by different alphabetical letters are significantly different for each species. Asterisks indicate levels of significance for each treatment compared to the control (LO) at P<0.05 (*) and P<0.01 (**)

4.2.3 Evaluation of different doses for odorant blends in Ahero

For logistical reasons, dose response evaluation was only carried out in Ahero.

Since mosquitoes' response to a given compound may vary in a dose-dependent manner, different doses of the original blends of HONAD and LO+P+OC in varied combinations were developed and further tested. The doses of HONAD comprised the original (optimal) dose (B), half the optimal dose (B1) and double the optimal dose (B2). Similarly, for the plant based-blend, LO+P+OC, original (optimal) dose of the blend (A), half the optimal dose (A1), double the optimal dose (A2) were prepared and evaluated against LO as the control (Table 4.3). This evaluation recorded 803 *An. gambiae s.l* (20 males and 783 females), 612 *An. funestus* group (97 males and 515 females), 293 *An. coustani* (1 male and 292 females) and 159 *An. ziemanni* females in all the treatments over a 9-day replicate trial. Trap captures were compared between the treatments focusing on high captures of *An. arabiensis* previously established as the dominant specie of the *An. gambiae s.l* complex in this region and *An. funestus* group.

For *An. arabiensis*, significant and about 2-fold higher catches were observed for only one of the blends A2+B2 [IRR=2.3; 95% CI (1.0-5.0)] relative to LO. In fact, this blend combines higher doses of the individual mammalian- and plant-based blends. Pairwise comparison between the treatments revealed a significant difference in trap catches of blend A2+B2 and A1+B [IRR=2.9; 95% CI (1.3-6.1)] (Table 4.3).

None of the new blends performed better than LO in capturing *An. funestus* group (Table 4.3). In fact, when compared to LO, significantly lower catches for *An. funestus* group were observed for blend A1+B1 [IRR=0.5; 95% CI (0.3-0.9)]. The blends did not differ in their performance in trapping this species (Figure 4.9, Table 4.3).

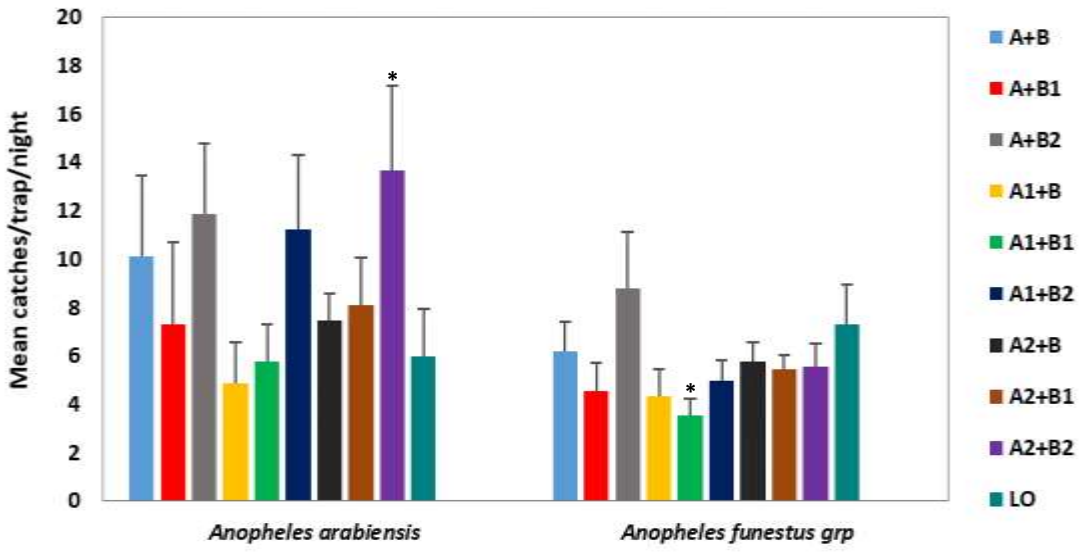

Figure 4.9: Mean daily catches (±SEM) of *Anopheles arabiensis* and *Anopheles funestus* group recorded in the different treatments in Ahero, Kenya in 9 replicate trials

A (Optimal dose of blend LO+P+OC), A1 (half optimal dose of blend LO+P+OC), A2 (double optimal dose of blend LO+P+OC), B (optimal dose of HONAD), B1 (half optimal dose of HONAD) and B2 (double optimal dose of HONAD). P- β-pinene, OC- β-ocimene, L- limonene, HONAD [Heptanal+Octanal+Nonanal+Decanal], LO [(E)-

linalool oxide] as the control. Asterisks indicate levels of significance for each treatment compared to the control at P<0.05 (*).

Table 2.3: Comparison in the captures of female anophelines at different treatment doses from Ahero, Kenya in October 2017.

Site	Species	Treatment (dose)	Number of captures (n)	IRR(95%CI)	*P-value*
Ahero	*Anopheles arabiensis*	LO [a]	54	1 (reference)	
		A+B [ab]	92	1.7(0.8-3.7)	0.191
		A+B1 [ab]	66	1.2(0.6-2.7)	0.620
		A+B2 [ab]	107	2.0(0.9-4.3)	0.085
		A1+B [ac]	44	0.8(0.4-1.8)	0.620
		A1+B1 [ab]	52	1.0(0.4-2.2)	0.927
		A1+B2 [ab]	103	1.9(0.9-4.1)	0.115
		A2+B [ab]	69	1.2(0.6-2.8)	0.593
		A2+B1 [ab]	73	1.4(0.6-3.0)	0.454
		A2+B2 [b]	123	2.3(1.0-5.0)	0.04*
	Anopheles funestus group	LO [a]	66	1 (reference)	
		A+B [ab]	58	0.8(0.5-1.4)	0.537
		A+B1[ab]	41	0.6(0.4-1.1)	0.087
		A+B2 [ab]	79	1.2(0.7-2.0)	0.483
		A1+B [ab]	39	0.6(0.3-1.0)	0.061
		A1+B1 [b]	32	0.5(0.3-0.9)	0.01*
		A1+B2 [ab]	45	0.7(0.4-1.2)	0.163
		A2+B [ab]	54	0.9(0.5-1.3)	0.375
		A2+B1 [ab]	51	0.7(0.4-1.3)	0.272
		A2+B2 [ab]	50	0.8(0.4-1.3)	0.304

Number of replicate trials: 9; IRR, incidence rate ratios; CI, confidence interval at α=0.05 level of significance. Treatments followed by different alphabetical letters are significantly different for each species. Asterisk indicate levels of significance for each treatment compared to the control (LO) at P<0.05 (*).

CHAPTER FIVE

DISCUSSION

The results from laboratory studies identified limonene as the possible antagonistic constituent to *An. gambiae* in the 6-component plant-derived blend of (*E*)-linalool oxide, β-pinene, β-ocimene, (*E*)-β farnesene, limonene and hexanal, identified in a previous study as moderately attractive to this mosquito species compared to linalool oxide alone (Nyasembe et al., 2012). This pattern was however, not replicated in field experiments since the blend containing limonene (LO+P+OC+L) recorded comparable captures of *An. arabiensis* relative to those without. Such disparities are not unique as this could have been due to blend components competing with background odors from the vegetation in the environment hence masking the antagonistic effect of the blend. In addition, the differences in odor response observed between laboratory and field settings could be related to the species used and their physiological needs. In the laboratory, *An. gambiae s.s.* of a known physiological age was used while the dominant species collected from the field was *An. arabiensis.* This is supported by molecular speciation data where all the *An. gambiae s.l.* specimens analyzed were found to be *An. arabiensis*. This is in line with previous studies, reporting this species as the main member in the *An. gambaie* complex in these areas (Bayoh et al., 2010; Mala et al., 2011; Nyasembe et al., 2014). These findings suggest that the precise odors utilized in plant and mammalian host location might vary for mosquitoes in the *An. gambiae s.l.* as well as their behavioral responses to odor blends. In addition, in the field setting, mosquito responses may vary depending upon their physiological needs such as sugar-, blood- or egg-laying-seeking which would impact on the overall trap captures.

In this study, it had been hypothesized that improved lures can be developed by combining kairomones from plant- and mammalian-based sources to target sugar- and blood-seeking adult females. In laboratory assays reduced responses of *An. gambiae s.s* to a combined blend of HONAD and LO+P+OC compared to those of the respective

individual blends was found. This finding suggested the possibility of antagonistic effect when compounds from both mammalian and plant sources were combined. However, the reduced effect of the blend combination was not observed in field experimental captures of *An. arabiensis* and *An. funestus* group at both study sites, possibly relating to the doses evaluated.

The response of an insect to a given compound is known to occur in a dose dependent manner whereby at certain doses it is attractive and at others repellent. This led to further evaluation of varying combined blends of the mammalian- and plant-derived blends. Interestingly, one of the blends (A2+B2) significantly recorded improved catches of *An. arabiensis* more than 2-fold compared to LO and the blend A1+B1.

Human odors from worn socks were similarly as attractive as the four-component aldehyde blend (HONAD). This was not surprising as aldehydes are among the major components of human foot odors and secondary metabolites of human skin microflora reported to be attractive to this mosquito and other species (Owino et al., 2015; Tchouassi et al., 2013; Verhulst et al., 2010). The aldehyde blend appeared to impact on increased *An. arabiensis* and *An. funestus* group collections with increasing doses when added to the plant-derived blend. This was not surprising as highest catches of *An. arabiensis* but not *An. funestus* group was observed for this blend when highest dose of each of the blends was combined.

Host odors are usually not very attractive on their own without the use of CO_2 which is a universal activator and attractant for most disease vectors (Gillies, 1980; Tchouassi et al., 2012). In fact, CO_2 baited traps had the highest overall captures of female anopheline species supporting its invaluable role in augmenting the efficacy of most host associated lures in increasing captures of disease vectors. It has been demonstrated that CO_2 can gate attractiveness of other host cues and mutation of *Aedes aegypti* CO_2 receptor desensitized the mosquito to some host cues and human odors (Corfas et al., 2013). Notwithstanding, field evaluation allowed for assessment of the attractiveness treatments alone. The number of daily mosquito catches recorded by the treatments especially blend

A2+B2 compared well and even better than reported in previous studies deploying odorant attractants together with carbon dioxide (Mukabana et al., 2012). Clearly, the improved blend holds promise in monitoring mosquito populations especially in remote areas where CO2 may be logistically challenging to deploy.

Although odorant blends tested in this study were developed for *An. gambiae s.s.* in the laboratory, other anophelines known to be malaria vectors or potentially so such as *An. funestus*, *An. pharoensis*, *An. coustani* and *An. ziemannii* (Antonio-Nkondjio et al., 2006; Kamau et al., 2006; Mukiama & Mwangi, 1989; Tchouassi et al., 2012) were captured in field trials. Nonetheless, a disparity in the performance of the odorants among *An. gambiae s.l* and other anophelines was observed, possibly attributed to the fundamental difference in the biology among the species suggesting that specific optimization is necessary for maximal attraction targeting individual species. For instance, the performance of blend LO+P+OC+L in trapping *An. pharoensis* suggests high sensitivity for this species to this blend, however, it appears that the detection threshold of this blend may vary with species. Also, (*E*)-linalool oxide was the most effective in trapping *An. funestus* group just as CO_2 compared to other treatments. A recent study reported the potential of LO as a generalist plant-based lure (Nyasembe et al., 2015) however, its importance in the sensory physiology and ecology of this species is worthy of further investigation. The overall pattern among these species may reflect differences in detection thresholds and degree of utilization of mammals and plants as important resources. Taken together, these findings show that by combining mammalian- and plant-based compounds improved lures can be developed for use in the surveillance and control targeting these species especially against exophilic fractions that elude the current indoor control measures.

Furthermore, although plant-based lures have been thought to better target males considering their exclusive herbivory behavior (Foster, 1995; Gary et al., 2009) very low captures were found in all traps. This could have been due to placement of the traps near homesteads where females seem to dominate. Better trap placements exploiting their

resting sites may be the key to enhancing their captures using such lures. Some of the treatments evaluated were found to be attractive to other mosquito species such as *Aedes, Culex* and *Mansonia,* some of which are known vectors of diseases such as Rift valley fever, chikungunya, dengue fever, west Nile virus, and filariasis among others (Owino et al., 2015; Sang et al., 2010; Snow & Michael, 2002; Tchouassi et al., 2013). Such attractive plant-based treatments could be optimized as lures in endemic areas also surveillance of these mosquito vectors.

5.1 Conclusion and Recommendations

The current insecticide-based malaria vector control strategies only target indoor biting fractions. Therefore, residual malaria transmission by outdoor vectors remains a challenge. As such, novel surveillance, and control tools such as use of odorant baits from vector hosts are paramount for malaria control. Malaria vectors of all sexes and different physiological state are known to forage on plants for metabolic energy and other nutritional benefits while females, in addition, visit vertebrates for a blood meal. This feeding behavior is discriminative and guided by odorant cues from preferred hosts. Therefore, understanding how odorant cues from preferred hosts interact is crucial for development of lures for surveillance and control of malaria vectors.

In this study, limonene was identified as a possible antagonist to linalool oxide and the most attractive blend of linalool oxide, pinene and ocimene in laboratory assays on *An. gambiae*. The antagonistic effect of this compound was not exhibited in field trials in malaria endemic areas. In addition, combining plant- and mammalian- derived odorant compounds antagonized the attractiveness of the individual blends in laboratory assays. However, in field trials this combination either reduced or improved mosquito trap catches depending on the dose utilized.

The study highlighted the gross disparity in the results evaluating mosquito odorant responses between laboratory and field settings. Such laboratory evaluations of chemical attractants must be validated in field trails. *An. gambiae* was used in laboratory

evaluation and was not represented in the field captures. Potential differences in the response profile to these odorants between the species could have contributed to the observed pattern. An understanding of the mechanism of coding of these odors by different vectors at the level of olfactory receptors could shed light on their specificity to guide formulation of better blends to maximize their trap collections. In line with the study hypothesis, if properly formulated, combining constituents from both plant- and mammalian-based sources can result to improved lures for surveillance of outdoor biting malaria vectors. Linalool oxide (LO) stood out as the most important plant-derived attractant for the malaria vectors encountered. This compound consists of a mixture of stereoisomers of the furanoid (trans (2R, 5S), cis (2S,5R)) and pyranoid (trans (2R,5S), cis (2S,5S)) forms; such studies will be required to define the exact stereoisomer accounting for behavioral activity which so far is unknown.

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