Nectar feeding and volatile chemical signatures influencing host plant selection in major Afrotropical mosquito disease vectors

By

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Declaration

I, Vincent Odhiambo Nyasembe declare that the thesis/dissertation, which I hereby submit for the degree Doctor of Philosophy (Entomology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Disclaimer

This thesis consists of a series of chapters that have been prepared as stand-alone papers already published or manuscripts for different scientific journals. Consequently, unavoidable overlaps and/or repetitions may occur and the reference style and format may differ between chapters. In addition, although the chapters have been organised in paper format with each paper having a number of authors, I conducted all the experiments with guidance from all the supervisors.

Thesis summary

Although it is widely accepted that plant resources play an important role in the biology of mosquito species, the preferred mosquito host plants in the natural habitats remain largely unknown. The persistence of mosquito-borne diseases and the re-emergence of others such as Zika have created the need for novel control strategies with plant feeding becoming a new focus for such strategies. Effective deployment of these new tools requires an accurate identification of preferred host plant species that are attractive to mosquitoes. Chapter two of this study explored the potential of a single component plant-based lure, linalool oxide (LO), previously developed for malaria vectors in trapping other mosquito species important in the transmission of Rift Valley fever (RVF) and dengue diseases. LO performed as well as human-based BioGent (BG) lure and animal-based HONAD in trapping female RVF vectors, *Aedes mcintoshi* and *Ae. ochraceus* when combined with CO_2 but performed poorly in the absence of carbon dioxide. On the other hand, LO was as good as BG lure in trapping female *Ae. aegypti* and was better than the BG lure in trapping males of this species both in the presence and absence of CO_2. To improve on the performance of this single-component blend, chapter three sought to identify the natural plant species utilised by malaria vectors *Anopheles gambiae* s.l., dengue vectors *Aedes aegypti* and Rift Valley fever vector *Ae. mcintoshi* and *Ae. ochraceus.* Three plant-specific gene targets were used to amplify and identify the preferred plant DNA from the mid-guts of these species. A total of three plant species were identified as host plants for malaria vectors, one for dengue vectors and two for RVF vectors. These host plants were further evaluated for their nutritional value using first generation field collected *Ae. aegypti* as a model through a series of survival, fecundity and egg hatchability assays as well as chemical analyses. Differential survival rates of *Ae. aegypti* on the five plants was observed, partly explained by qualitative and

quantitative differences in sugar reward from the plants. Differences in oviposition rates and egg hatchability were also observed which was in tandem with amino acid reward obtained from these sources. In chapter four, the study sought to develop a potent plant-based lure by exploiting chemical cues utilised by different Afrotropical mosquito species to locate their preferred natural host plants. The study revealed a clear difference in the classes of volatile organic compounds that dominate the fragrance of different host plants. Electrophysiological assays revealed chemical convergence in detection of hexanal, hexenol isomers, ocimene, linalool oxide and decanal for both *Ae. Aegypti* and *Anopheles gambiae* s.l. A three-component lure was developed and field evaluated against *Ae. aegypti*. This lure showed a superior performance to both linalool oxide and commercial BG lure in trapping male and female *Ae. aegypti*. Together, these findings indicate the potential of exploiting plant feeding targeted strategies in the management of Afrotropical mosquito species.

Dedication

To my loving family and in memory of my late mother, Dourine Akoth Obonyo,

may her soul rest in eternal peace.

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List of abbreviations

RVF - Rift Valley fever

LO – Linalool oxide

BG – BioGent

HONAD – Heptanal, Octanal, Nonanal and Decanal

KEMRI – Kenya Medical Research Institute

icipe – International Centre of Insect Physiology and Ecology

GLM – Generalized Linear Model

SIDA – Swedish International Development Cooperation Authority

ITS – Internal transcribed subunit

matK – maturase K

ATSB – Attractive Toxic Sugar Bait

GC/EAD – Gas chromatography/electroantennoghic detection

EAG – electroantennograph

GC/MS – Gas chromatography/mass spectrophotometry

FID – flame ionisation detector

Presentations and publications from this work

Presentations

International Congress of Entomology XXV (25-30th September, 2016): Oral presentation. Title: **Exploiting plant DNA barcoding to determine nectar sources of Rift Valley fever, dengue and malaria mosquito vectors**. Orlando, Florida, United States of America.

The 32nd Annual meeting of the International Society of Chemical Ecology (ISCE) and the 4th Congress of the Latin America Association of Chemical Ecology (ALAEQ)-first joint meeting (4-8th July 2016): Oral presentation. Title: **Nutritional value informs mosquito-plant interactions in malaria disease vectors**. Iguazu Falls, Brazil.

Graduate course in Insect Chemical Ecology (ICE-15), (8-19th June 2015): Poster presentation. Title: **Attractive plant-based lure for the management of mosquito disease vectors**. Alnarp, Sweden.

icipe-Rothamsted Workshop, Identifying opportunities for collaboration (25-27th November 2015): Oral presentation. Title: **Nectar feeding: Opportunities for exploitation in mosquito disease vector control**. Nairobi, Kenya.

Gordon Research Conference: Exploring the Plant Headspace: Functional Analysis and Emerging Applications (26-31st January 2014): Poster presentation. Title: **Plant Scent: Potent New Target for Control of Malaria Vectors**. Ventura, USA.

Publication

Nyasembe V. O., Tchouassi D. P., Mbogo C. M., Sole C. L., Pirk C. and Torto B. (2015**).** **Linalool oxide: generalist plant based lure for mosquito disease vectors**. *Parasites and Vectors* 8: 581.

CHAPTER ONE

General Introduction, Rationale of the study and Key questions

General introduction

Vector-borne diseases constitute the greatest health burden throughout the world, with more than half of the world population estimated to be at risk of these diseases (WHO 2014). Least developed countries and the poorest segment of the society are the most vulnerable, with countries in sub-Sahara Africa bearing the heaviest brunt (WHO 2014). A number of mosquito species are implicated in the transmission of important pathogenic diseases, some of which results in devastating illnesses, morbidity and mortality (WHO 2014). In sub-Saharan Africa, important mosquito species include various *Anopheles* species (principally *Anopheles gambiae* and *An. funestus* complexes) which transmit malaria and filariasis (White 1974), *Aedes aegypti* which transmit dengue, chikungunya and yellow fever (WHO 2014), and flood-water *Aedes* species (mainly *Aedes mcintoshi* and *Ae. ochraceus*) which are key primary vectors of Rift Valley fever (Linthicum et al. 1985, Sang et al. 2010). Important to note is the fact that *Ae. aegypti* has been implicated in a number of re-emerging infectious diseases including Zika virus in the Americas and elsewhere (Li et al. 2012, Chouin-Carneiro et al. 2016), a disease which was first detected in Uganda in the early 1900 (Kirya et al. 1977). Save for malaria, most of these mosquito-borne diseases have neither treatment nor an effective vaccine. Even in the case of malaria for which artemisinin combination therapy is currently recommended as a first line of treatment, resistance to these drugs has been reported in a number of countries (Ashley et al. 2014). Vector control therefore constitutes the most viable and effective control strategy for these diseases. Some of the control strategies that have been employed with considerable success

include insecticide treated nets (ITNs), indoor residual sprays (IRS), outdoor sprays, environmental management practices aimed at reducing breeding sites and proper housing designs among others (Organization 2014). However, the risks posed by these diseases still persist, facilitated by the spread of insecticide resistance among various mosquito species. In fact the spread of some of the diseases such as dengue, chikungunya and Zika to new geographical regions has raised major concerns (Roth et al. 2014, Musso and Gubler 2015). This has created the need for a more concerted vector control effort accompanied by invention of more vibrant, environmentally-friendly control tools.

Understanding the vector biology and ecology is pivotal to generating new control tools against these mosquito-borne diseases. Mosquitoes are known to forage for plant sugar from floral and extrafloral nectars, honey dew or exudates from damaged leaves and fruits, which contribute to their energy reserves as well as provide ready energy for flight and other metabolic functions (Nayar and Sauerman 1971a, Magnarelli 1977, Nyasembe and Torto 2014). Males depend solely on nectar as an energy source, while females of most mosquito species feed intermittently on plant sugars between or before blood meals (Nayar and Sauerman 1971b, Foster 1995). In disease vectoring female mosquitoes, plant sugars have been touted to play an important role in their vectoral capacity by affecting their survival, reproductive fitness and vector competence (Stone and Foster 2013). The availability of plant sugar sources could enhance chances of successful disease transmission by extending the survival of disease vectors, sustaining high vector density and promoting extrinsic development of the pathogen (Gu et al. 2011, Nyasembe et al. 2014). On the other hand, plant feeding could suppress disease transmission by reducing vertebrate host biting rate, altering the reproductive fitness of disease vector and suppressing the development of the pathogens in their host vectors (Schlein and Jacobson 1994, Stone and Foster

2013). Either way, the role of plant nectar in disease transmission dynamic is important, but a paucity of information on this aspect of vector ecology still persists.

Few direct field observations of nectar feeding mosquitoes have been made (Sandholm and Price 1962, Magnarelli 1977, 1979, 1983, Andersson and Jaenson 1987, Smith and Gadawski 1994), but this has not been easy due to nocturnal nature of most mosquito species and difficulty in deciphering actual plant feeding from such observations. This coupled with the fact that there are a myriad of plant species which vary from one ecological setting to another (Schlein and Muller 1995, Manda et al. 2007b, Gouagna et al. 2010, Gu et al. 2011), has made it difficult to pinpoint the preferred nectar sources. Other than these, most evidence pointing to nectar feeding has been based on biochemical methods, the most common being cold anthrone test which provides evidence of recent nectar feeding (van Handel 1972). These include studies by Riensen *et al.*, (1986) who showed that 68% and 75% of males and females, respectively, from field collected *Culex tarsalis* were positive for fructose, a plant-specific sugar that is not normally synthesized by insects. Similarly, *Aedes albopictus* collected in coastal Israel tested positive for this important plant sugar (Müller et al. 2011), with up to a third of wild caught *Ae. aegypti* showing evidence of recent sugar feeding (Edman et al. 1992). Beier (1996) also demonstrated that 6.3% of the indoor-resting and 14.4% of host-seeking *Anopheles gambiae* and *Anopheles funestus* collected in western Kenya were positive for fructose. Evidence of plant tissue feeding has also been documented for *Culex pipiens molestus* and *Anopheles sergentii* (Schlein and Muller 1995). However, no direct observation or biochemical evidence of nectar feeding has been documented for flood water *Ae. mcintoshi* and *Ae. ochraceus*.

Other methods which have been used in the analysis of nectar feeding in mosquitoes include profiling of nectar sugars using gas chromatography (Nayar 1978, Burkett et al. 1999, Manda et

al. 2007a) and plant tissue analysis in the mid gut using cellulose staining method (Schlein and Muller 1995). Laboratory, semi-field and field assays using locally predominant plant species have also been employed to determine the acceptability of certain plant species as possible nectar sources for various mosquito species and their ability to sustain the survival and fecundity of such mosquitoes (Impoinvil et al. 2004, Manda et al. 2007a, Manda et al. 2007b, Müller et al. 2010b, Chen and Kearney 2015, Nyasembe et al. 2015). However, these plant sugar feeding detection methods are limited by the fact that they cannot specifically identify the preferred nectar sources under natural conditions. With the invention of new control measures targeting nectar feeding mosquitoes such as attractive toxic sugar baits (ATSB) (Müller and Schlein 2008, Müller et al. 2010a, Müller et al. 2010c) and the proposed nectar expressed Cry proteins from *Bacillus thurugensis* var *israensis* (Boisvert and Boisvert 2000, Chen and Kearney 2015), there is a need to accurately identify host plant species that are attractive to mosquitoes to improve on the specificity and efficacy of such tools. The use of molecular probes offers a promising tool for accurate determination of nectar sources, and has been employed in determining plant meals in a number of phytophagous insects among them mosquitoes (Miller et al. 2006, Matheson et al. 2008, Junnila et al. 2010, Staudacher et al. 2011). However, these molecular approaches have not been used in determining nectar sources of Afro-tropical mosquito disease vectors.

Identification of specific plant sources of important mosquito species will increase the potential of elucidating the specific semiochemicals which mediate their interactions with these plants. Up to now, efforts in developing odour-baits for mosquito disease vector management have centred mainly on human/animal-derived odours (Okumu et al. 2010, Mukabana et al. 2012, Tchouassi et al. 2013), which though effective, are limited in that they mainly target blood-seeking female mosquitoes (Foster 2008) and rely on the use of large amounts of CO_2 which present logistical

challenges for use in remote areas (Turner et al. 2011). An understanding of the chemical ecology of plant feeding offers a promising new target for vector control (Townson et al. 2005, Foster 2008, Ferguson et al. 2010, Nyasembe and Torto 2013). Besides the potential to trap mosquitoes of varying physiological states and sexes, plant-based lures have the potential to trap outdoor biting fractions of disease vectors and thus reduce human-vector contact (Foster 2008, Nyasembe and Torto 2013). Given that plants and fruits normally release low amounts of CO_2 at night as by-products of respiration (Richards 1952, Golding et al. 1998), mosquitoes are not expected to rely heavily on it for host-plant location. Hence, plant-based odours present the potential to minimize or even eliminate the reliance on CO_2 for trapping, if well formulated.

This study therefore sought to identify nectar sources for selected mosquito species which include; (1): *An. gambiae* s.l. for malaria; (2) *Ae. Aegypti* for dengue; and (3) *Ae. mcintoshi* and *Ae. Ochraceus* for Rift Valley fever, and their contribution to the survival and fertility of these vectors. The study also isolated attractive volatile chemical compounds from these plants and evaluated their potential to trap target mosquito species in the field.

Rationale of the study

The burden of mosquito-borne diseases is still enormous, especially in countries south of sub-Saharan Africa (WHO 2014). The available vector control tools, though effective to some extent, are still insufficient to curb these diseases. Besides, there is continuous evolution of viruses, parasites and vectors to new generations of vaccines, drugs and insecticides that are developed to control them. Exploiting the ecology of these disease vectors can potentially open new avenues for development of new surveillance and control tools. Nectar feeding forms an important pillar

in the biology of mosquitoes. While there have been efforts to understand the nectar feeding behaviour of *An. gambiae s.s.,* there is no information on the nectar feeding behaviour of its sibling species *An. arabiensis*, which is becoming increasingly important in sustained outdoor malaria transmission (due to its outdoor feeding habits) as it is less amenable to current indoor strategies such as insecticide treated nets and indoor residual sprays. Furthermore, there is little information on nectar feeding behaviour of the dengue vector, *Ae. aegypti*, and none on Rift Valley fever vectors, *Ae. mcintoshi* and *Ae. ochraceus*. Consequently, the chemical cues that mediate their nectar feeding behaviour and the odour receptors for these cues are not known. Therefore, exploiting the nectar feeding component of the ecology of these disease vectors offers a unique opportunity to develop new vector control interventions to complement currently available tools as a component of IVM.

Key research questions

The key research questions addressed in the present study are:

Chapter 2 Linalool oxide: generalist plant based lure for mosquito disease vectors

Q1: Is linalool oxide, a single component plant-based lure developed for malaria vectors effective in trapping dengue and Rift Valley fever mosquito vectors?

Chapter 3 DNA barcoding uncovers natural host plants of important Afrotropical mosquito disease vectors and evidence of their role on vector fitness

Q1: Do *Aedes mcintoshi* and *Aedes ochraceus* feed on plant sources in their natural habitats as has been reported for *Anopheles gambiae* s.s., *Aedes aegypti*?

Q2: Which the plant serve as food resources for the four mosquito species in their natural habitats?

Q3: What is the nutritional impact of plant sources on the fitness of these mosquito species?

Chapter 4: Patterns of host plant odour detection by Afrotropical mosquito disease vectors and their field evaluation

Q1: Are the natural host plants of the four mosquito species characterised by distinct or similar odour bouquet?

Q2: Do different mosquito species utilise different or similar chemical cues to locate a suitable host plant?

Q3: Are there distinct chemical cues utilised by different mosquito species that can be benchmarked as sugar feeding cues for novel vector control strategies?

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CHAPTER TWO

Linalool oxide: a generalist plant based lure for mosquito disease vectors

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Abstract

Background

Lack of effective vaccines and therapeutics for important arboviral diseases such as Rift Valley fever (RVF) and dengue, necessitates continuous monitoring of vector populations for infections in them. Plant-based lures as surveillance tools have the potential of targeting mosquitoes of both sexes and females of varied physiological states; yet such lures are lacking for vectors of these diseases. Here, we present evidence of the effectiveness of linalool oxide (LO), a single plant-based lure previously developed for malaria vectors in trapping RVF vectors, *Aedes mcintoshi* and *Aedes ochraceus*, and dengue vector, *Aedes aegypti*.

Methods

For RVF vectors, we used CDC traps to evaluate the performance of LO against three vertebrate-based lures: CO_2 (dry ice), BioGent (BG) lure, and HONAD (a blend of aldehydes) in 2 experiments with Completely Randomized design: 1) using unlit CDC traps baited separately with LO, HONAD and BG-lure, and unlit CDC trap + CO_2 and lit CDC trap as controls, 2) similar treatments but with inclusion of CO_2 to all the traps. For dengue vectors, LO was evaluated against BG lure using BG sentinel traps, in a 3 x 6 Latin Square design, first as single lures and then combined with CO_2 and traps baited with CO_2 included as controls. Trap captures were compared between the treatments using Chi square and GLM.

Results

Low captures of RVF vectors were recorded for all lures in the absence of CO_2 with no significant difference between them. When combined with CO_2, LO performance in

trapping these vectors was comparable to BG-lure and HONAD but it was less effective than the lit CDC trap. In the absence of CO_2, LO performed comparably with the BG-lure in trapping female *Ae. aegypti*, but with significantly higher males recorded in traps baited with the plant-based lure. When CO_2 was added, LO was significantly better than the BG-lure with a 2.8- and 1.3-fold increase in captures of male and female *Ae. aegypti*, respectively.

Conclusions

These results highlight the potential of LO as a generalist plant-based lure for mosquito disease vectors, pending further assessment of possible specificity in their response profile to the different stereoisomers of this compound.

Key words

Rift Valley fever, dengue, climate-change, stereoisomers, arboviral diseases, odor-bait, vector ecology, *Aedes mcintoshi*, *Aedes ochraceus*, *Aedes aegypti.*

Background

Vector-borne diseases exert a huge toll on global infectious disease burden. Rift Valley fever (RVF) and dengue represent two important mosquito-borne arboviral diseases, which continue to spread, evident from numerous disease outbreaks in various parts of the world [1, 2]. Rift Valley fever is an epizootic disease mainly occurring in Africa and the Arabian Peninsula, with outbreaks leading to devastating loss of millions of livestock and thousands of human deaths [3-7]. On the other hand, dengue, which mainly affects humans, has a worldwide distribution where outbreaks have been reported in over 110 countries [8]. Approximately 3 billion of the world population is at risk of dengue infection and over 100 million reported cases and up to 25000 fatalities annually [1, 9]. Both RVF and dengue have episodic outbreak patterns with low viral activities during the inter-epidemic periods [2, 10, 11]. Rift Valley fever outbreaks are associated with weather anomalies such as widespread elevated rainfall while that of dengue is closely linked to urbanization and transportation, which creates conducive breeding sites for the respective vector populations, and subsequent virus amplification and transmission [12-14]. In the recent past, there has been a growing concern of the possibility of further spread of both diseases to new areas, particularly to Asia and Europe, in the wake of current climate change [14, 15]. In Kenya, the key primary vectors implicated in the transmission of RVF are the flood water *Aedes mcintoshi* and *Aedes ochraceus* [16, 17] while that of dengue fever is *Aedes aegypti* [18]. Lack of safe and effective vaccines and therapeutics against both diseases [1,19], makes studies on the vectors geared towards developing efficient monitoring or control tools a priority [12, 20]. Effective monitoring of infection/viruses in vectors requires highly effective sampling tools.

The successful use of odor bait technology in population reduction of *Glossina morsitans morsitans* Westwood and *G. pallidipes* Austen in the Zambezi Valley of Zimbabwe [21], has heightened prospects for its similar application in the surveillance and control of blood

feeding insects [22]. For blood feeding mosquitoes, a number of odor baits targeting specific species have been developed with considerable success, but these baits are mainly based on vertebrate host odors [23-26]. These baits have been widely employed along with carbon dioxide, which is associated with vertebrate breath and is known to elicit long range activation of host seeking behavior in most mosquito species [27]. In addition, the synthetic carbon dioxide that is extensively employed together with these lures is expensive and presents logistical challenges for use in remote areas where these diseases are endemic [28]. These challenges can be circumvented by employing plant based lures as adult mosquitoes of all physiological states and both sexes utilize nectar for energy [29-30]. However, except for a few laboratory studies to identify plant odors attractive to *Ae. aegypti* [30], little effort has been made to develop plant based lures for the management of RVF and dengue vectors.

In an effort to develop more potent lures for these vector species, we investigated the potential of linalool oxide (LO), a single-component plant based lure initially developed for the malaria vectors [31, 32], in trapping the primary vectors for RVF, *Ae. mcintoshi* and *Ae. ochraceus*; and dengue fever, *Ae. aegypti*. This study presents the first evidence of effectiveness of a plant-based lure in trapping primary RVF and dengue vectors.

Methods

Study sites

The study was conducted at two sites in Kenya: Garissa County in North Eastern region where RVF is endemic [17] and Kilifi County in Coastal region where dengue fever is endemic [33]. The two sites were selected based on the relative abundance of the target

vectors of these diseases i.e. *Aedes* (*Neomelaniconion*) *mcintoshi* and *Aedes* (*Aedimorphus*) *ochraceus* for RVF, and *Aedes aegypti* for dengue [25, 34, 35].

Garissa County is largely semi-arid with two unreliable rainy seasons a year; short rains occurring between October and December and the long rains between March and May. Typical average rainfall ranges from 300 mm to 500 mm annually. Annual temperatures range from a minimum of 14 °C and a maximum of 34 °C. The region also experiences periodic ElNiño/Southern Oscillation (ENSO) phenomena which predispose it to epidemic RVF outbreaks [12]. The altitude of the study area varies between 18 m and 75 m above sea level with the coordinates of 1.5988°S and 40.5135°E. The area is inhabited mainly by pastoralists who engage in keeping livestock such as sheep, goats, cattle, camels, and donkeys, and migrate throughout the year in search of pastures and water. Vegetation in the area is predominantly shrubs and acacia bushes. Traps were set up in Sangailu, Ijara, Masalani, Korisa and Kotile communities of Ijara sub-County which have a lot of visible dambos in the landscape (shallow depressions that hold water during flooding and serve as breeding sites for flood water *Aedes*).

Kilifi County is relatively wet with two rainfall seasons; the short rains between October and December, and long monsoon rains between April and July, with an average annual rainfall of 300 mm to 2200 mm. The annual temperatures range from a minimum of 21 °C and a maximum of 32 °C. The area lies 3.6333°S and 39.8500°E with an altitude of between 9 and 50 m. In this area, trapping was conducted in an urban setting with largely modified topography and vegetation providing numerous breeding sites for *Ae. aegypti*.

Ethical statement

In Garissa, field trappings were conducted away from homesteads and on community land as authorized by area chiefs and community elders after explaining the purpose of the study to them. In Kilifi, informed consent was obtained from the persons in charge of public sites or heads of the homesteads where the studies were conducted as well as the area chiefs.

Chemicals used

The lures tested in this study included commercial synthetic linalool oxide, commercial BioGent (BG) lure (a 3-component blend comprising ammonia, lactic acid and caproic acid developed for *Aedes aegypti*), and HONAD (a 4-component blend comprising heptanal, octanal, nonanal and decanal, an animal-based lure developed at the International Centre of Insect Physiology and Ecology (*icipe*) in Nairobi for RVF vectors). The composition of HONAD is 2 mg/ml heptanal, 0.5 mg/ml octanal, 0.1 mg/ml nonanal and 0.1 mg/ml decanal [24].

The synthetic standards of the following compounds were used: linalool oxide (Aldrich, mixture of stereoisomers with furanoid form, 99.5% and 0.5% pyranoid form), heptanal (Sigma-Aldrich, 98%), octanal (Sigma-Aldrich, 98%), nonanal (Sigma-Aldrich, 98%), and decanal (Sigma-Aldrich, 98%). Both LO and HONAD were released from a rubber septa.

Optimization of field attractive doses of linalool oxide and determination of the release rate

Linalool oxide was tested at the concentration reported in our previous work (2 ng/µl) [32] including two ten-fold higher concentrations (20 ng/µl and 200 ng/µl) to find out if the threshold of odor response differs among the mosquito species. This was carried out at both Garissa and Kilifi field sites.

In Garissa, initial assessment of the dose responses was carried out in Sangailu involving three unlit CDC traps each baited with one of the three LO doses and CO_2 and randomly placed in the vegetation around dambos and away from homesteads at 40 m inter-trap distance. This was replicated three times with each replicate set at a new location daily. The traps were activated at 18:00 hr and retrieved 06:00 hr the following morning. The trapped mosquitoes were knocked down using dry ice, sorted, counted and then placed in eppendorf tubes and preserved in liquid nitrogen for transport to the laboratory at *icipe* in Nairobi. Once at *icipe* the samples were stored at -80^{o}C until identification using morphological keys [36, 37].

Similarly, BG sentinel traps baited with each of the LO doses and CO_2 was used to optimize for the most attractive dose for dengue vectors in Kilifi. Traps were placed at a distance of 40 m apart around three different locations (two breeding sites comprising abandoned tires and fish ponds which had *Ae. aegypti* larvae and one next to homestead with no obvious breeding sites) for two alternate days and one night. The daytime trapping was carried out from 06:00 – 18:00 hr while the night trapping was done from 18:00 – 06:00 hr. The trap captures were emptied and counted at the end of each trapping.

Release rate studies were carried out at the *icipe* Duduville campus in Nairobi (1.22°S, 36.88°E; ≈ 1,600 m above sea level) with temperature variations between 12 -28^{o}C and

humidity of 60 – 70 %. The release rate of LO at the optimal dose (20ng/µl) over 12 hr period was determined by applying 100 µl of the LO solution on a rubber septa, allowing the solvent to evaporate completely in a fume chamber before exposing the rubber septa outside. Volatiles were collected from the rubber septa in a 40 ml quickfit chamber (ARS, Gainesville, FL, USA®) and passing air over it at a flow rate of 260 ml/min into an adsorbent Super-Q trap for 1 hr. Volatiles were collected after every three hour-interval over a 12 hr period as follows: 1 hr, 3hr, 6hr, 9hr and 12hr; with the rubber septa re-exposed outside after every collection. The Super-Q trap was eluted with dichloromethane and analyzed on coupled gas chromatography-mass spectrometry (GC/MS). The GC/MS analysis was carried out in the splitless injection mode using an Agilent Technologies 7890 gas chromatograph coupled to a 5975C inert XL EI/CI mass spectrometer (EI, 70 eV, Agilent, Palo Alto, California, USA) equipped with an HP-5 column (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent, Palo Alto, California, USA). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was held at 35°C for 3 min, then programmed to increase at 10 °C/min to 280 °C and maintained at this temperature for 5 min. Three replicates were carried out at each time interval and the average peak areas for the two stereoisomers of LO ((*Z*)- and (*E*)-linalool oxide (furanoid form)) used to quantify release rates against an external calibration with synthetic LO. The release rate for BG lure is reported in Owino et al. [25].

Study design

Effectiveness of LO in trapping Rift Valley fever vectors

Selection of a suitable trap type: In a preliminary study, the performance of CDC light trap and BG sentinel trap were compared in terms of captures to determine the best trap type for

RVF vectors. Three unlit CDC traps and three BG sentinel traps separately baited with LO + CO_2, HONAD + CO_2 and BG lure + CO_2 were randomly set around vegetation with water-containing dambos away from homesteads. With an inter-trap distance of 40 m, the experiment was replicated five times. Based on consistent higher captures compared to BG sentinel trap (Additional Fig. 1), CDC light trap was selected for subsequent evaluation of the effectiveness of the different lures in trapping RVF vectors.

Evaluation of lures: Two sets of experiments were carried out. In the first experiment, 5 treatments comprising unlit CDC traps each baited singly with CO_2, LO, HONAD, BG lure and lit CDC trap without CO_2 were compared. A total of five replications were carried out in a Completely Randomized experimental design with each replicate set in a new location in either Sangailu or Kotile sites. In the second experimental setup, similar 5 sets of treatments comprising unlit CDC traps each baited with CO_2, LO + CO_2, HONAD + CO_2, BG lure + CO_2 and a lit CDC trap + CO_2. In both experiments, lit CDC traps were included as positive controls since they are widely used for mosquito surveillance. Completely randomized study design was conducted over five different sites (Sangailu, Ijara, Masalani, Korisa, and Kotile) located approximately 30 - 100 km apart. At each site, traps were set up following a Completely Randomized experimental design as described above. The study was carried out over 12 nights with each night treated as a replicate. Each site was sampled at least twice and all the treatments rotated through all the sites. In both experiments, traps were activated shortly after sunset (18:00 hrs) and removed in the morning (06:00 hrs). The synthetic carbon dioxide in the form of dry ice was released from an Igloos thermos container (2 L; John W Hock, Gainesville, FL, USA). Trapped mosquitoes were knocked down, preserved and transported to *icipe* as described above. Once at *icipe* the samples were

stored at -80^{o}C until identification using morphological keys [36, 37] and the number of target species counted.

Effectiveness of LO in trapping dengue vectors

Mosquito trapping was done within the urban centre in Kilifi. Two sets of experiments were carried out. In the first experiment, three treatments comprising BG sentinel traps each baited with CO_2, LO and BG lure were compared at six sites; two near productive breeding sites comprising abandoned tires or fish ponds (sites with *Ae. agypti* larvae), two in vegetation, and two around homesteads. A 3 x 6 Latin Square study design was used comprising six days and six nights. The traps were rotated through all the sites with each treatment replicated twice in each of the six sites day and night, giving a total of 12 replicates. In the second experiment, three treatments comprising BG sentinel traps each baited with CO_2, LO + CO_2 and BG lure + CO_2 were compared in a 3 x 6 Latin Square experimental design as described above with a total of 12 replicates. Day time mosquito trapping was done between 06:00 hrs and 18:00 hrs while night time trapping was done between 18:00 hrs and 06:00 hrs the following day. The carbon dioxide was dispensed as described earlier. Trap collections were removed at 06:00hr and 18:00hrs every day, knocked down, counted and similarly preserved and transported as described earlier. Once at *icipe* the samples were stored at -80^{o}C until identification using morphological keys [36, 37].

Statistical analysis

The difference in the release rates of (*Z*)- and (*E*)- linalool oxide (furanoid) over time was detected by one-way ANOVA after reciprocal transformation of release rates. The proportions of total number of mosquitoes trapped by each dose of LO were subjected to Chi square. Similarly, trap capture between CDC and BG sentinel traps to determine the best trap type for RVF vectors was compared using Chi square. To determine the effectiveness of the different lures in trapping disease vectors, the numbers of mosquitoes per treatment were first fitted with general linear model (GLM) with Poisson distribution and then negative-binomial error structure and log link in case of over dispersion as described by White and Bennetts [38] using R 2.15.1 software [39]. For RVF vectors, trap treatment (lure) was modelled as factors, with BG lure and BG lure + CO_2 serving as reference for traps without and with CO_2, respectively. Trap captures in the presence and absence of CO_2 was compared using Chi square. In the case of dengue vectors, trap treatment and time of the day, were modelled as factors, with BG lure or BG lure + CO_2 serving as reference for traps without and with CO_2, respectively. Similarly, trap captures in the presence and absence of CO_2 was compared using Chi square. The incidence rate ratios (IRR), a measure of the likelihood that mosquito species chose treatments other than the reference treatment (traps baited with BG lure or BG lure + CO_2) and their *P*-values were estimated. The IRR for the reference is 1 (unity) and values above this indicates better performance and values below under performance of the treatments relative to the control. Given the high number of male mosquitoes caught in the traps for *Ae. aegypti*, captures were compared for both sexes. Only female trap captures were considered for *Ae. ochraceus* and *Ae. mcintohi* since no males were caught in any of the treatments. In addition, day and night captures of *Ae. aegypti* were compared using Chi square goodness-of-fit test. All statistical analyses were done at 95 % confidence interval.

Results

Optimal field attractive dose of LO and its release rates

Dose 20 ng/µl, had the highest capture for *Ae. mcintoshi*, *Ae. ochraceus* and *Ae. aegypti*, but was only significantly different from the other doses in capturing the latter mosquito species ($P < 0.001$) (Table 1). The average release rate at this dose was 1.98 ng/hr and 1.92 ng/hr for (*Z*)- and (*E*)-linalool oxide (furanoid), respectively, with no difference in the release rates of both isomers over the 12 hr period ($F_{(4,10)} = 0.746$, $P = 0.582$ and $F_{(4,10)} = 0.965$, $P = 0.468$, respectively) (Figure 1A). GC/MS analysis further revealed that the commercial synthetic standard of LO used also contained trace amounts of the pyranoid form of (*Z*)- and (*E*)-linalool oxide (Figure 1B).

Table 1 Trap captures of Rift Valley fever and dengue vectors to different doses of Linalool oxide

Mosquito species	2 ng/µl	20 ng/µl	200 ng/µl	*P*-value
Aedes mcintoshi	6.33±0.88	9.33±1.45	8±0.58	0.513
Aedes ochraceus	3.66±0.67	7±0.58	5±0.58	0.977
Aedes aegypti	99±10.26	130.33±8.84	115.33±5.36	0.719

The values represent the total number of mosquitoes caught. Unlit CDC traps were used in trapping *Ae. mcintoshi* and *Ae. ochraceus*, while BG sentinel trap was used in trapping *Ae. aegypti*.

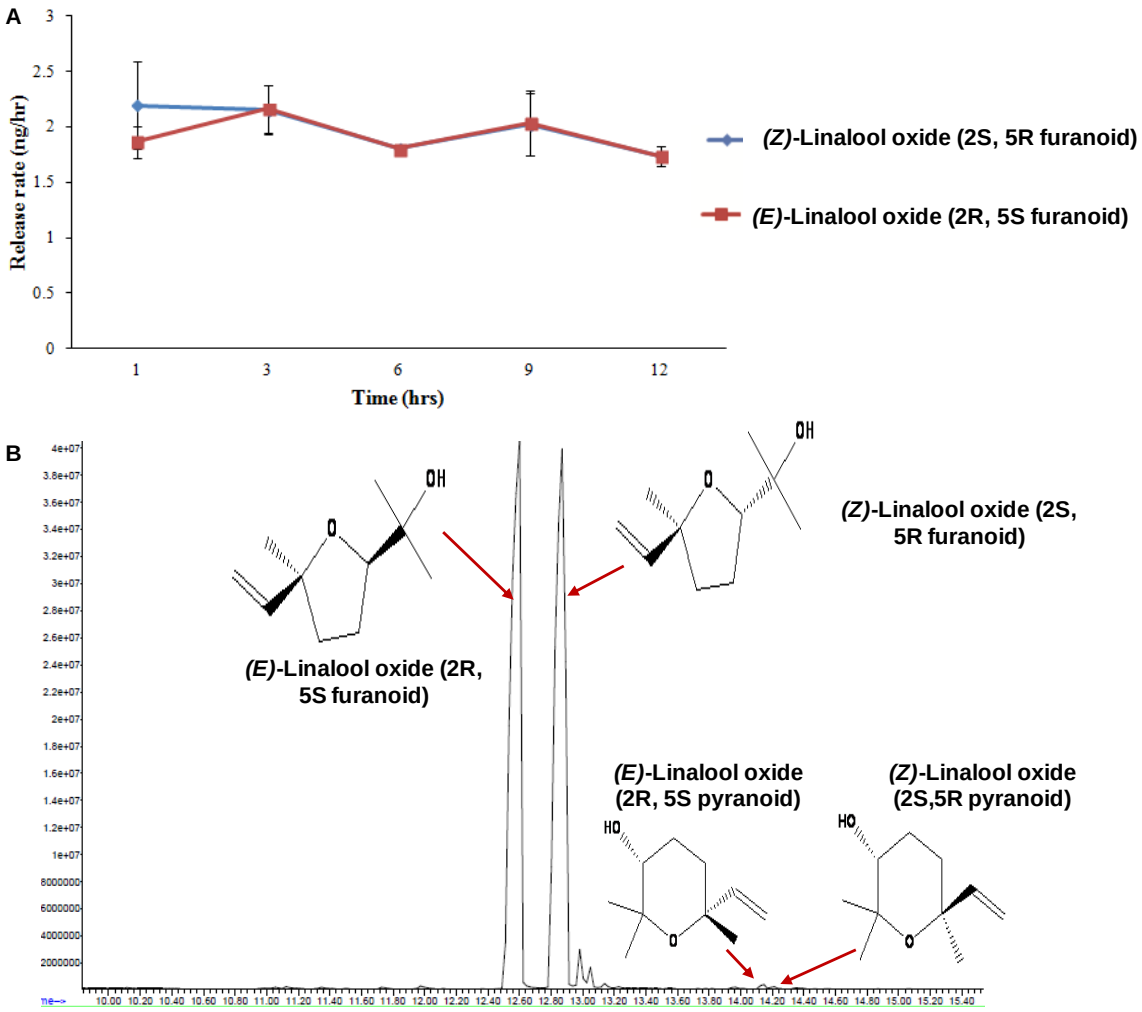

Figure 1 Release rates of the optimal dose of LO and its structural isomers: A) release rate of LO at dose of 20 ng/µl collected over 12 hr period, B) GC/MS chromatogram showing their retention time and structures of the four isomers of LO. N = 3. Differences in LO release rate over time was detected using ANOVA.

Linalool oxide effective in trapping Rift Valley fever vectors

In the first experiment, low numbers of *Ae. mcintoshi* and *Ae. ochraceus* were caught in the absence of CO_2 (*Ae. mcintoshi*: LO = 4, HONAD = 1, BG lure = 4, lit CDC trap =5; *Ae. ochraceus*: LO = 5, HONAD = 7, BG lure = 2, lit CDC trap =6) with no significant difference in the mosquito captures between the traps baited with these lures (P = 0.33). In the second experiment, a total of 267 *Ae. mcintoshi* and 141 *Ae. ochraceus* were caught in

12 replicate trials. The lit CDC traps + CO_2 caught two-fold more *Ae. mcintoshi* than the unlit CDC traps baited with BG lure + CO_2, while the unlit CDC trap + CO_2 caught six-fold less compared to the later. HONAD + CO_2 and LO + CO_2 were as good as unlit CDC lure + CO_2 (Table 2). Similar patterns were observed for *Ae. aegypti* (Table 2).

Table 2 Trap captures of Rift Valley fever vectors captured by CDC trap baited with different lures.

Mosquito species	Lure	Total caught	Mean ± SEM	*P*-value
Aedes mcintoshi	BG lure + CO_2	47	3.92 ± 0.93	
	CO_2	7	0.58 ± 0.19	< 0.001
	LO + CO_2	59	4.92 ± 1.56	0.24
	HONAD + CO_2	53	4.42 ± 1.22	0.55
	Lit CDC + CO_2	101	8.42 ± 1.05	< 0.001
Aedes ochraceus	BG lure + CO_2	24	2.00 ± 0.63	
	CO_2	10	0.83 ± 0.27	< 0.05
	LO + CO_2	26	2.17 ± 0.91	0.78
	HONAD + CO_2	19	1.58 ± 0.63	0.44
	Lit CDC + CO_2	62	5.17 ± 1.14	< 0.001

Lit CDC trap was not baited with any lure except CO_2. Total number of replicates (N) = 12. SEM = standard error of mean, LO = linalool oxide, BG = Biogent. BG lure + CO_2 was used as reference and *P*-values for each treatment relative to it calculated.

Linalool oxide effective in trapping dengue vectors *Aedes aegypti*

In the first experiment, 628 females and 804 males of *Ae. aegypti* were caught. There was no significant difference between LO and BG lure in trapping female *Ae. aegypti* (IRR = 1, P = 0.89, Table 3), but LO trapped 1.4 fold more males than the BG lure (P< 0.01, Table 3).. When the lures were each combined with CO_2, a total of 2087 female and 2415 male *Ae. aegypti* were caught. An additive effect on trap capture was observed (P < 0.001), with LO trapping significantly more females three-fold more males (IRR = 2.8, P < 0.001, Table 3).

Table 3 Trap captures of dengue vectors (*Aedes aegypti*) captured by BG trap baited with different lures.

Sex	Lure	Total caught	Mean ± SE	*P*-value
Females	**BG lure**	257	32.13 ± 9.48	
	LO	216	27.00 ± 6.43	0.059
	CO_2	155	19.38 ± 4.49	< 0.001
	BG lure + CO_2	998	124.75 ± 53.54	
	LO + CO_2	935	116.88 ± 66.03	0.152
	CO_2	163	20.38 ± 3.94	< 0.001
Males	**BG lure**	204	25.50 ± 9.87	
	LO	416	52.00 ± 17.08	< 0.01
	CO_2	174	21.75 ± 4.75	0.123
	BG lure + CO_2	710	88.75 ± 38.67	
	LO + CO_2	1521	190.13 ± 72.41	< 0.001
	CO_2	177	22.13 ± 3.49	< 0.001

Total number of replicates (N) = 12. SEM = standard error of mean, LO = linalool oxide, BG = Biogent. BG lure or BG lure + CO_2 was used as reference and *P*-values for the other non-CO2 and CO_2 baited lures, respectively, calculated.

Discussion

In our previous study, we had demonstrated the high efficacy of LO alone and in combination with carbon dioxide in trapping the malaria vectors *An. gambiae* s.l. [32]. Our results showed that despite targeting different mosquito disease vectors, the three lures LO, BG and HONAD alone and in combination with carbon dioxide varied in their effectiveness in trapping RVF vectors *Ae. mcintochi* and *Ae. ochraceus* and the dengue vector *Ae. aegypti*, as previously found for the malaria vectors *An. gambiae* s.l. [32]. Notably, whereas LO alone was effective in trapping both sexes of *Ae. aegypti*, LO, BG lure and HONAD were

only effective in trapping the two RVF vectors in the presence of CO_2. The failure of the three lures to trap RVF vectors in the absence of CO_2 perhaps explains the critical role played by this compound in host location and orientation by these vectors, which are highly zoophilic as opposed to the anthropophilic nature of *Ae. aegypti* and *An. gambiae* in agreement with previous studies [40].

Also, notably, besides a few laboratory-based bioassays to test the attractiveness of plant volatile extracts to *Ae. aegypti* [41, 42], this is the first field evidence of the potential of a plant-based compound in field trapping of RVF and dengue vectors. Linalool oxide compared favorably to the two vertebrate lures in trapping RVF and dengue vectors. This is particularly interesting given that LO is a single component lure capable of attracting a number of important mosquito species. Use of common chemical cues in host location has been demonstrated in several insect species. For instance, 1-octen-3-ol has been shown to play a key role in host location by several blood feeding insects including tsetse flies, stable flies, culicoides and mosquitoes [43-45]. These findings highlight the potential use of a single- or multi-component plant-based lure for mosquito vector surveillance and control.

The fact that CO_2-baited lit CDC traps performed better than CO_2-baited unlit CDC traps baited with each of the three lures for RVF vectors, suggests the importance of visual cues in the behavior of these mosquitoes. As such, the interaction of visual and chemical cues could be exploited further for effective monitoring of these vectors. Therefore, with further development and formulations, linalool oxide and perhaps other yet to be identified plant odors may hold promise as possible plant-based alternatives to CO_2 depending on the target mosquito species.

Furthermore, the presence of male *Ae. aegypti* in traps baited with LO is interesting. With the development of sterile insect technique as a population reduction tool for mosquito

control, there has been a need to improve field based lures to target the male segment of the mosquito population for the purpose of evaluating competency and survival of sterile males as compared to wild males [46]. Plant-based lures have been suggested to hold potential in targeting this segment of the population [28, 30]. Therefore, our finding here suggests the potential of a phytochemical in trapping both male and female mosquito disease vectors.

LO is a chiral compound and the commercial synthetic standard used in our study as shown by our GC/MS analysis consisted of tentatively identified racemic mixture of four enantiomers, (2R,5S)-(*E*)-furanoid, (2S,5R)-(*Z*)-furanoid, (2R, 5S)-(*E*)-pyranoid and (2S, 5R)-(*Z*)-pyranoid. Therefore, it is likely that the differential efficacy of LO in trapping the different mosquito disease vectors and sexes can be attributed to the different enantiomeric forms of this compound, which needs further investigation. Interestingly, differential responses of insects to stereoisomers of semiochemicals has been reported. For example, the (1S,2′S) form of 1-[3-cyclohexen-1-ylcarbonyl]-2-methylpiperidine was found to be two-fold more repellent against *Ae. aegypti* than the (1R,2′S) form [47]. Similarly, *Manduca sexta* was shown to have a higher preference for plants producing (+)-linalool compared to those producing (-)-linalool [48]. Hence the present study justifies further investigation into the potential of phytochemicals, especially those with chiral heterogeneity to address specificity in responses of different mosquito disease vectors to stereoisomers of these compounds.

Conclusion

We document the performance of LO in trapping the RVF vectors, *Ae. mcintoshi* and *Ae. ochraceus*, which compared favorably with the BG lure and HONAD, but better than the

BG lure for female *Ae. aegypti*, the major vector of dengue. In the absence of carbon dioxide, this compound performed dismally in trapping RVF vectors but was comparable to the BG lure for trapping dengue vectors. However, linalool oxide was superior to the BG lure in trapping male *Ae. aegypti* in the presence or absence of carbon dioxide. These results highlight the potential of LO as a generalist plant-based lure for mosquito disease vectors, with room for further development to obtain a potent phytochemical attractant for the management of these vectors.

Competing interest

The authors declare that they have no competing interest and that the donors had no role in the drafting of this manuscript.

Author contributions

Conceived and designed the experiments: VON DPT CLS CP BT. Performed the experiments: VON DPT CMM BT. Analyzed the data: VON DPT BT. Wrote the paper: VON DPT CMM CLS CP BT. All authors approved the final version for submission.

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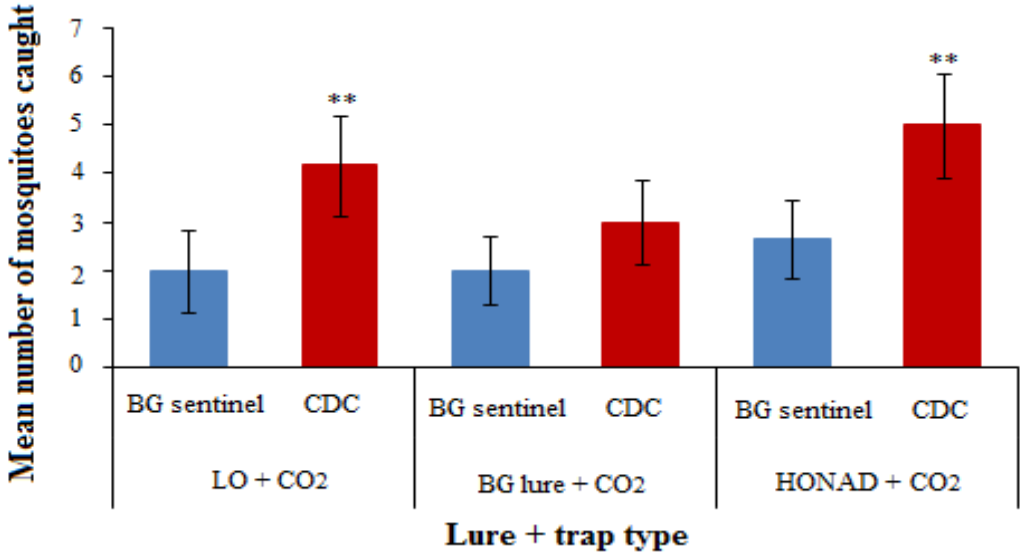
Additional information



Additional figure 1 Comparison of unlit CDC trap and BG sentinel trap in trapping Rift Valley fever vectors. N = 5, chi square was used to analyze count data. Bars capped with ** are significantly different at 0.01.

CHAPTER THREE

DNA barcoding uncovers natural host plants of important Afro-tropical mosquito disease vectors and evidence of their role on vector fitness

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Abstract

Plant feeding is important in the ecology of mosquito disease vectors, yet natural host plants for these vectors are scarcely known. Besides, current knowledge on mosquito plant feeding is attributed to the need for sugars as an energy source but little is known about the role of plants in modulating reproductive fitness of these disease vectors. We applied a combination of plant gene targets namely ITS2, trnH-psbA and matK to identify the host plants fed upon by four wild caught Afro-tropical mosquito species sampled from different habitats in Kenya. We report six plant species fed on by Afrotropical mosquito species as *Pithecellobium dulce* (Fabaceae) for *Aedes aegypti*, *Leonotis nepetifolia* (Lamiaceae), *Senna alata* (Fabaceae) and *Ricinus communis* (Euphorbiaceae) for *An. gambiae, Opuntia ficus-indica* (Cactaceae) for *Aedes mcintoshi*, and *O. ficus-indica* and *Nymphaea cyanea* (Nympheaceae) for *Aedes ochraceus*. Using *Ae. aegypti* as a model insect in subsequent host plant feeding and survival experiments, the study further shows that irrespective of sex and blood meal status, its survival on the natural host plant *P. dulce* was as good as survival on blood meals alone and *L. nepetifolia* but 3-fold greater than on the non-host plants *S. alata* and *O. ficus-indica*. However, life-time fecundity on *P. dulce* was about 2-, 3- and 5-fold less than that on blood meal alone, *L. nepetifolia* and *S. alata*, respectively. The study further shows that the hatching rate of eggs laid by adult *Ae. aegypti* fed on *P. dulce* was 2-fold less than those from blood meal diet but 6-fold higher than those from *S. alata* diet. Chemical analysis showed that plant sugar and amino acid contents directly correlated to *Ae. aegypti* survival and fertility on *P. dulce, L. nepetifolia* and *O. ficus-indica* but not on *S. alata* and *R. communis*. These results confirm the potential of DNA barcoding as a tool to unravel the preferred host plants of not only mosquitoes but other disease vectoring arthropods, and the role of plant sources in modulating vector fitness.

Key words: Plant feeding, Afrotropical disease vectors, natural host plant, plant DNA barcoding, amino acids, sugars, fecundity, survival

Significance of the study

Mosquito-borne infectious diseases pose a great health hazard to more than half of the world's population, with an increasing risk from re-emergence of arboviral diseases such as zika virus and dengue fever. As such, there is a need for the development of novel control tools to complement or substitute existing ones for these vectors. New vector control tools targeting plant feeding behaviour have been developed, but limited knowledge on plant species utilised by these vectors in their natural habitats hinder implementation of such strategies. We employed DNA barcoding to identify host plants for key vectors of dengue, malaria and Rift Valley fever in Kenya. The study further provides insights into the nutritional value of the identified host plants and their impact on vector survival and fecundity.

Introduction

Plant nutrients are known to be the sole source of nourishment for male mosquitoes (1-4), but the degree of its utilisation among females of most species has been the subject of debate until recently. Notably, previous studies have suggested that anthropogenic mosquito species such as *Anopheles gambiae sensu stricto* and *Aedes aegypti*, rarely sugar-feed, and that they utilise the nutrients obtained exclusively from blood for all their energy requirements as well as reproduction (5-8). However, a number of studies have reported a higher frequency of plant feeding among these vectors, with indication that plant feeding may be related to the availability of sugar sources in the environment (9, 10) and/or physiological states whereby females only seek blood when in need to develop eggs and resort to plant sugars for other metabolic needs (11). Recently, further evidence supporting frequent sugar feeding before or between blood meals among these species has been found (12-15). It is therefore evident that female mosquitoes invariably exploit plant resources for both their survival and reproductive fitness. However, this has been poorly investigated for most mosquito species (14-17), especially the identity of suitable plants that mosquitoes utilise in nature.

The recognition of the contribution of plants to the ecology of disease vectors and on transmission dynamics of diseases makes plants an attractive and important target for innovative ways to control diseases. New intervention measures are particularly required in the face of emergent and re-emergent mosquito-borne diseases such as dengue, yellow fever, zika, and chikungunya, whose control relies heavily on vector-based strategies. Furthermore, the current call by WHO to eliminate infectious diseases such as malaria (18) and the host of challenges facing current control tools for these diseases, underscore the need for more concerted control efforts. Specific plant-based targets for disease vector control include the attractive toxic sugar bait (ATSB) (19, 20) and the proposed nectar expressed cry proteins from *Bacillus thurugensis* var *israensis* (21, 22). In addition, secondary metabolites ingested

in the course of plant feeding by disease vectors can be exploited as potential sources for novel therapeutics against pathogens. For instance, a recent study highlighted the therapeutic potential of a key toxin, parthenin, in blocking transmission of the sexual stages of the malaria parasite, *Plasmodium falciparum* in a manner similar to arteminisin (23). This followed its isolation from *Parthenium hysterophorus* an invasive plant known to be a preferred host for feeding by the malaria vector *An. gambiae* established through experimental studies (24). Effectiveness of these measures will greatly benefit from knowledge on the identity of host plants in nature that are attractive to mosquitoes and the nature of their interactions.

Plant species composition and abundance vary greatly between time and space; as such, identification of definite plant feeding sources among disease vectors have proved challenging. Many studies on plant feeding among various mosquito species have relied on biochemical tests such as cold anthrone (25) and Seliwanoff (26) to detect evidence of recent nectar feeding. Chromatographic techniques that allow for profiling of nectar sugars in the mosquito mid-gut (27-29) and plant tissue analysis using cellulose staining (30) have also been applied to detect plant feeding in various mosquito species. In addition, laboratory, semi-field and field assays using locally predominant plant species have been employed to determine the acceptability of certain plant species for feeding by various mosquito species and their ability to sustain the survival and fecundity of such mosquitoes (12, 22, 24, 29, 31, 32). However, these plant sugar feeding detection methods are limited by the fact that they cannot specifically identify the preferred nectar sources under natural conditions. Recently, more sensitive and specific approaches targeting plant DNA in the gut of insects have been applied to increase our understanding of plant–insect interactions (33-36). A key aspect in these studies is the authentication of the feeding source, whereby the strongest evidence

linking herbivores to the plant source is provided through analysis of ingested host tissue (37, 38).

In this study, I employed molecular tools to identify the natural plant sources of four Afrotropical mosquito disease vector species: *Anopheles gambiae* s.l. (malaria vector hereafter as *An. gambiae*), *Aedes aegypti* (dengue vector) and *Aedes mcintoshi* and *Ae. ochraceus* (Rift Valley fever vectors). Plant feeding among mosquito disease vectors has largely been linked to sugar intake as an energy source with blood providing amino acids required for reproductive fitness (39). The direct effect of amino acids which is second major component of plant nectar after sugars on fecundity has not been considered. With the observed seasonal prevalence in gonotrophic discordance among mosquito species including *An. gambiae*, *An. funestus* and *Aedes aegypti* (5, 40, 41), the possible link of plant resources in influencing this phenomenon would be intriguing, but is yet to be investigated. To evaluate the impact of the identified plant species on vector fitness, we monitored the survival, fecundity and egg hatching rate of *Ae. aegypti* on these plants. *Aedes aegypti* was chosen as a model species because of ease of laboratory colonisation. Furthermore, we identified and quantified the amount of sugars and amino acids ingested by the vector from these plants and screened for their presence in the host plants using various mass spectrometric techniques.

Results

Afro-tropical mosquito species indulge in plant feeding to varying degrees

Applying the cold anthrone test to detect fructose as evidence of recent plant feeding, I established the extent of plant feeding among the females of four mosquito species *Anopheles gambiae* (malaria vector), *Aedes aegypti* (dengue vector) and *Aedes mcintoshi* and *Ae.*

ochraceus (Rift Valley fever vectors) trapped from different ecologies in Kenya during the long rainy season (April-June, 2014). We found evidence of recent plant feeding in *Ae. mcintoshi* (56 %, n = 180), *Ae. ochraceus* (65 %, n = 155), *An. gambiae* (24 %, n = 420) and *Ae. aegypti* (17 %, n = 245).

DNA barcoding identifies natural host plants fed upon by Afro-tropical mosquito vectors

To identify the plant species fed upon by these mosquito species in their natural habitats, we subjected aliquots of samples that tested positive for the anthrone test to DNA extraction followed by amplification targeting three plant genes; ITS2, trnH-psbA and matK; and sequencing. We observed that the success rates in amplification of plant DNA from the mosquito mid-guts differed significantly between the three gene targets; ITS2 (27.7%), trnH-psbA (24.5%) and matK (8.8%) ($P < 0.05$; Table 1). Similar patterns were observed for sequenced samples with differing success rates; highest for ITS2 (17.6 %), followed by trnH-psbA (16.4%) and least for matK (1.9%, $P < 0.05$; Table 1). Of the total samples sequenced, 5.2% were successful for all the three targets, 13.8% for two targets (trnH-psbA and ITS2) and 81% for individual targets (either trnH-psbA or ITS2). The sequenced fragment sizes ranged from 186 – 918 bp for ITS2, 276 – 617 bp for trnH-psbA and 133 – 846 bp for matK genes.

Table 1 Variable success rates of three gene targets in amplifying and sequencing plant DNA in the mid-guts of different mosquito species

		Amplified (Sequenced)		
Mosquito species	**N**	**ITS2**	**trnH-psbA**	**matK**
Anopheles gambiae	35	16 (9)	18 (15)	3 (3)
Aedes aegypti	54	16 (13)	8 (6)	0 (0)
Aedes mcintoshi	38	4 (0)	2 (2)	2 (0)
Aedes ochraceus	32	8 (6)	11 (3)	9 (0)
Overall	**159**	**44 (28)**	**39 (26)**	**14 (3)**

N = number of mosquitoes which were extracted for plant DNA.

Blast searches of the sequences for each target in GenBank and further phylogeny showed strong support (bootstrap values 70% and above) and identified host plants as *Senna alata* (Fabaceae), *Ricinus communis* (Euphorbiaceae) and *Leonotis nepetifolia* (Lamiaceae) for *An. gambiae*; *Pithecellobium dulce* (Fabaceae) for *Ae. aegypti*; *Opuntia ficus-indica* (Cactaceae) for *Ae. mcintoshi*; and *O. ficus-indica* and *Nymphaea cyanea* (Nympheaceae) for *Ae. ochraceus*. The plant identities were further corroborated by on-site botanical identification to confirm their presence and inclusion of matched sequences of extracted DNA in the analyses. These and putative sequences from the mosquito mid-gut clustered together with strong bootstrap support in the phylogenetic analysis for malaria (Fig 1), dengue and RVF (2A and B, respectively) vectors. However, one plant tentatively identified from GenBank hit as *Hibiscus heterophyllus* (Malvaceae) from the mid-gut of both *An. gambiae* and *Ae. aegypti* was not identified at the respective field sites and were subsequently labelled unknown (Fig 1 and 2A). Therefore, plant species identified as fed upon by the four mosquito species during the sampled period were confirmed as *S. alata*, *L. nepetifolia* and *R. communis* for *An. gambiae*; *P. dulce* for *Ae. aegypti*; and *O. ficus-indica* for *Ae. mcintoshi* and *O. ficus-indica* and *N. cyanea* for *Ae. ochraceus* (Fig 3).

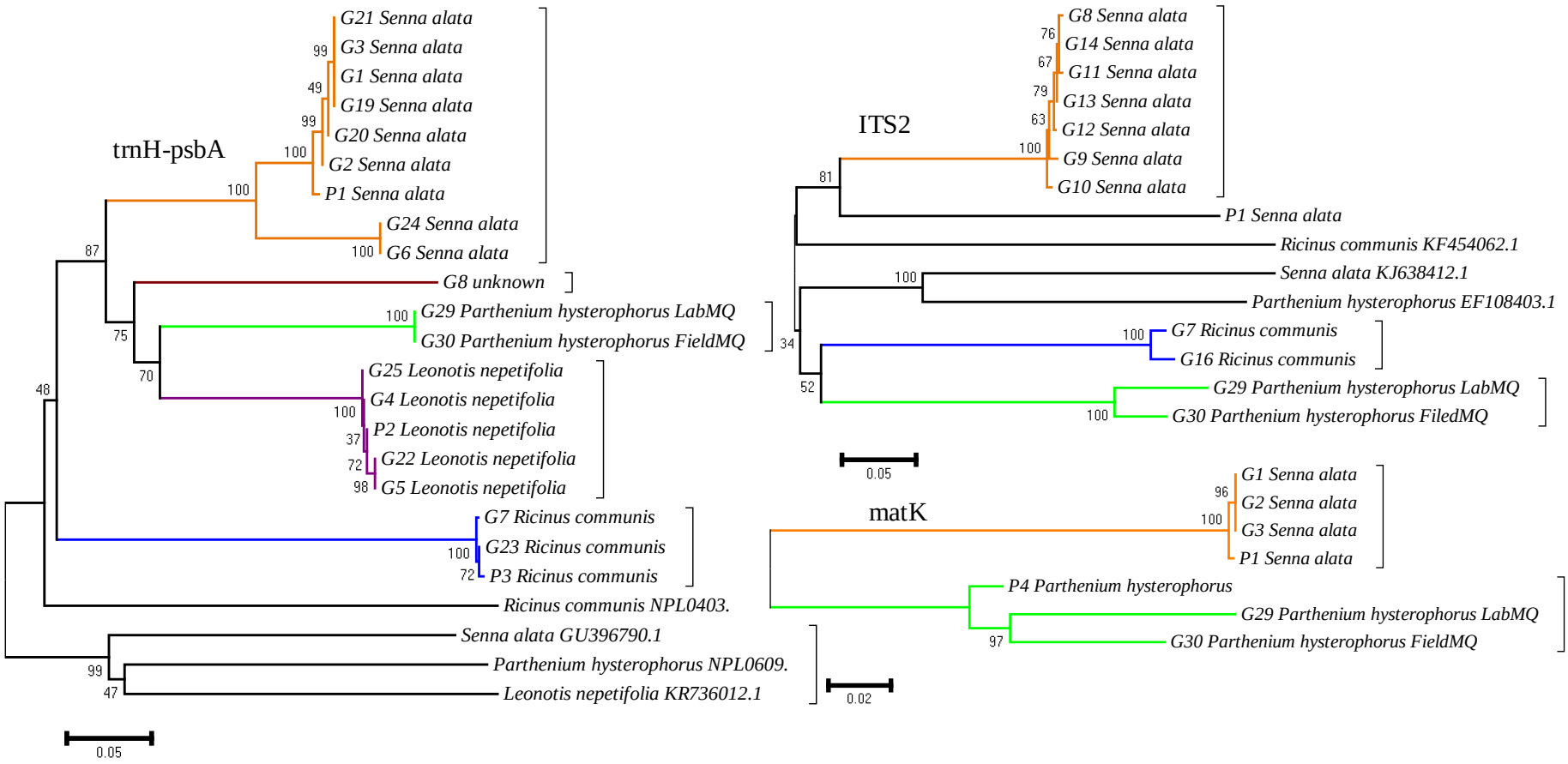

Figure 1 NJ phylogenetic trees from three gene targets showing plant species identified as natural host plants of *Anopheles gambiae* s.l. Plant species names with prefix G represent those that were identified from mosquito mid-guts with the numbers being sample ID. Plant species with prefix P1-4 represent the plant samples sequences to confirm the identity of the mosquito host plants while those with suffixes are outgroups from GenBank with extension being accession numbers. The phylogenetic trees were constructed in MEGA 6.

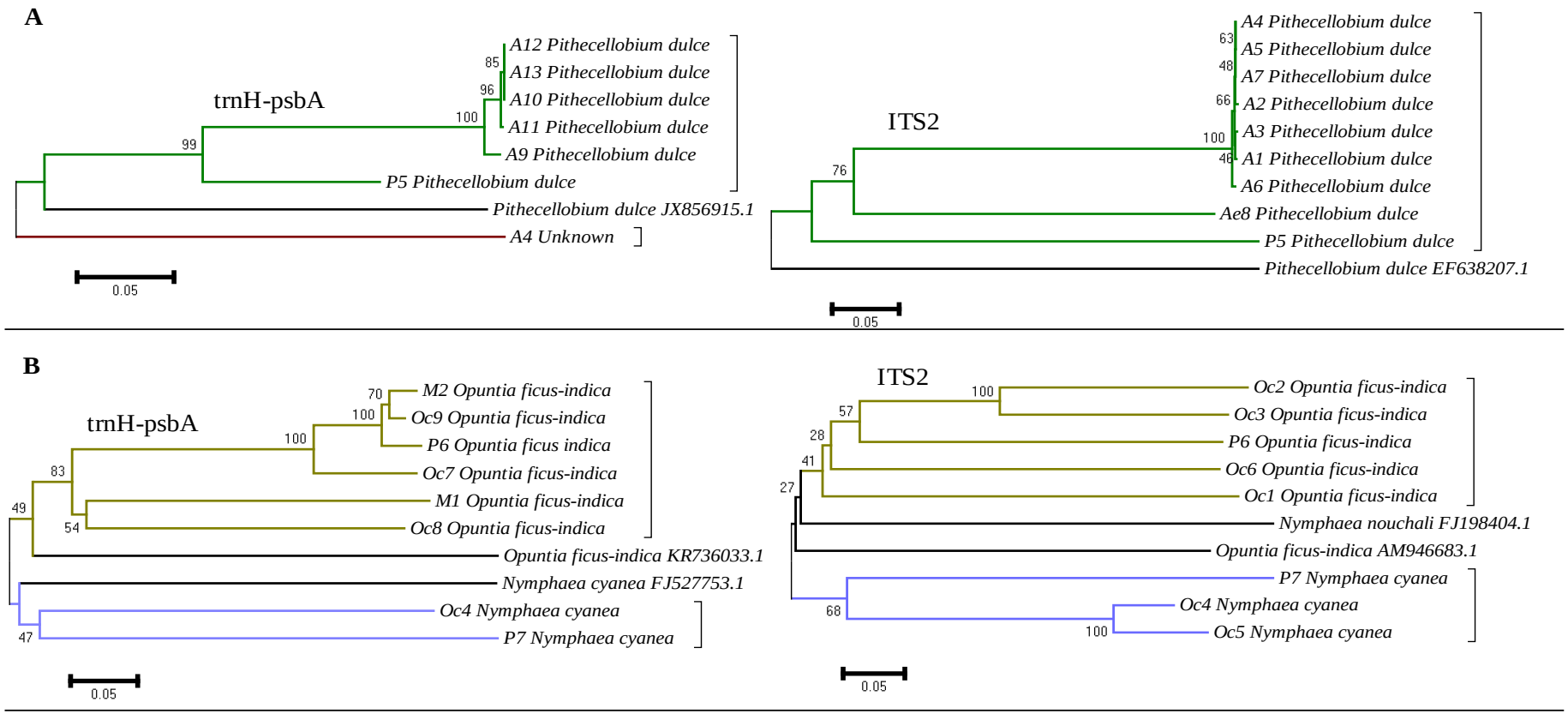

Figure 2 NJ phylogenetic trees from two gene targets showing plant species identified as natural host plants of dengue and Rift Valley fever vectors. A) Dengue vector *Aedes aegypti* host plants. B) RVF vectors *Aedes mcintoshi* and *Aedes ochraceus* host plants. Plant species names with prefixes A, M, and Oc represent those that were identified from the mid guts of *Ae. aegypti*, *Ae. mcintoshi* and *Ae. ochraceus*, respectively, with the numbers being sample ID. Plant species with prefix P5-7 represent the plant samples sequences to confirm the identity of the mosquito host plants while those with suffixes are outgroups from GenBank with extension being accession numbers. The phylogenetic trees were constructed in MEGA 6.

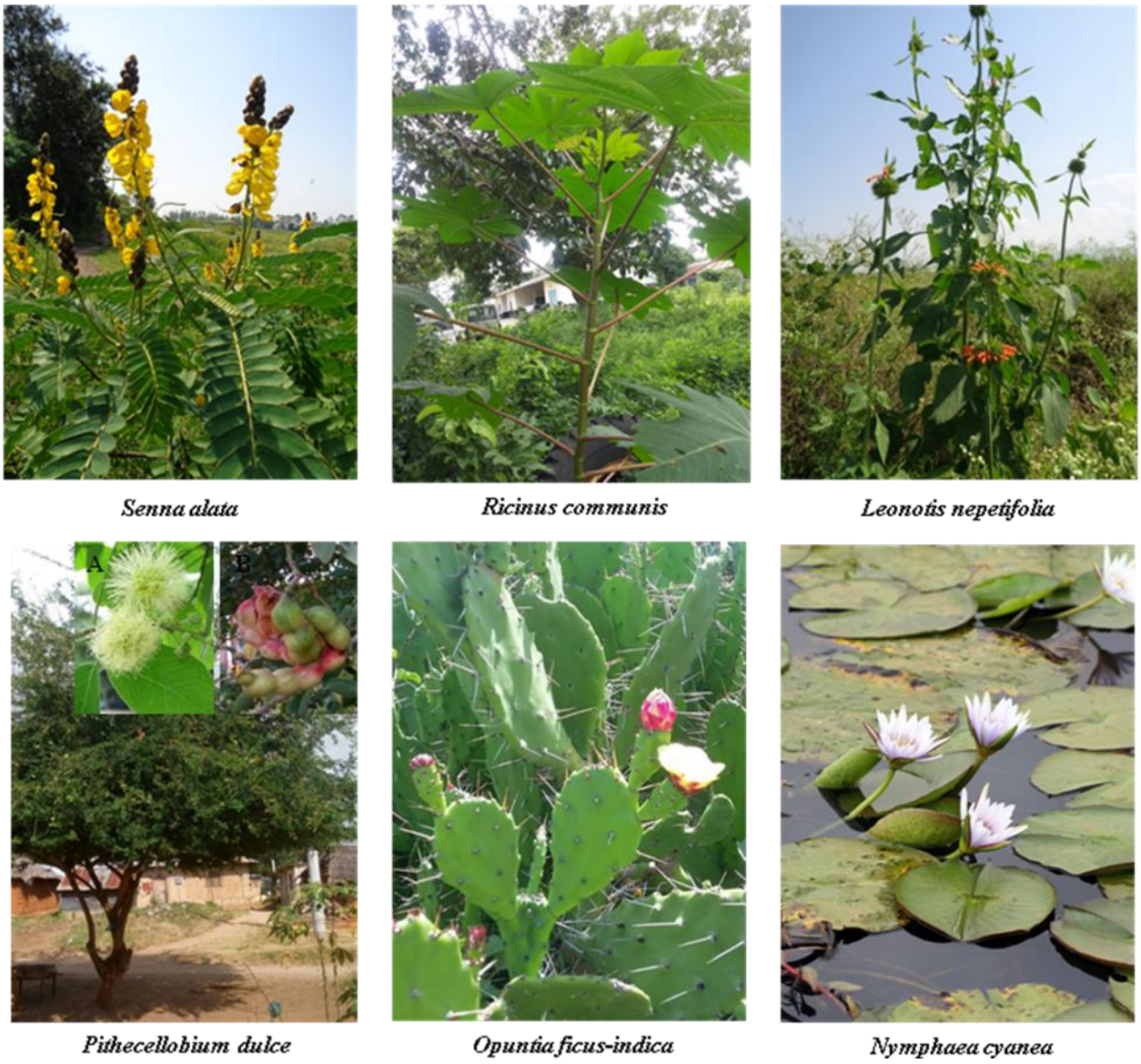

Figure 3 The identified natural host plants of *Anopheles gambiae*, *Aedes aegypti*, *Aedes mcintoshi* and *Aedes ochraceus*. Inset shows the flowers (A) and pods (B) of *P. dulce* (Photos by Vincent O. Nyasembe and Brian Mwashi – *icipe*).

Natural host plants impact on the survival, fecundity and fertility of *Aedes aegypti*

To assess how these plants influence vector fitness, we used an F1 generation of field collected *Ae. aegypti* as a model species to compare adult survival, fecundity and fertility on five of the identified host plants individually (although not all were identified as natural hosts

for this species) with and without a blood meal. We also included a diet of blood only and glucose as positive control and water as negative controls, respectively. Significant differences were detected in the survival time between different nutritional regimes among females either with no initial blood meal (Log Rank = 231.27, df = 6, $P < 0.001$, Fig. 4A) or with an initial three blood meals (Log Rank = 682.23, df = 7, $P < 0.001$, Fig. 4B), and males (Log Rank = 543.98, df = 6, $P < 0.001$, Fig. 4C). Overall, survival on *P. dulce*, the identified host plant for *Ae. aegypti*, was as good as survival on blood meal alone but 1.5-fold less than on glucose solution and 5.2-fold more than on water diet. Between the host plants, survival on *P. dulce* was as good as on *L. nepetifolia* but 1.6-fold more than on *R. communis*, 2.8-fold more than on *S. alata* and 3.2-fold more than on *O. ficus-indica*. Interestingly, initial provision of blood meal appeared to augment the survival of *Ae. aegypti* on *R. communis*, with females surviving almost twice as long as those fed on *R. communis* alone ($P < 0.001$) (Table 2). Both male and female *Ae. aegypti* were observed to probe on the flowers, leaf veins and stems of *L. nepetifolia* and *S. alata*, flowers, pods and leaf veins of *P. dulce*, flowers, fruits and succulent tissues of *O. ficus-indica*, and extrafloral nectaries, leaf veins and stem of *R. communis*.

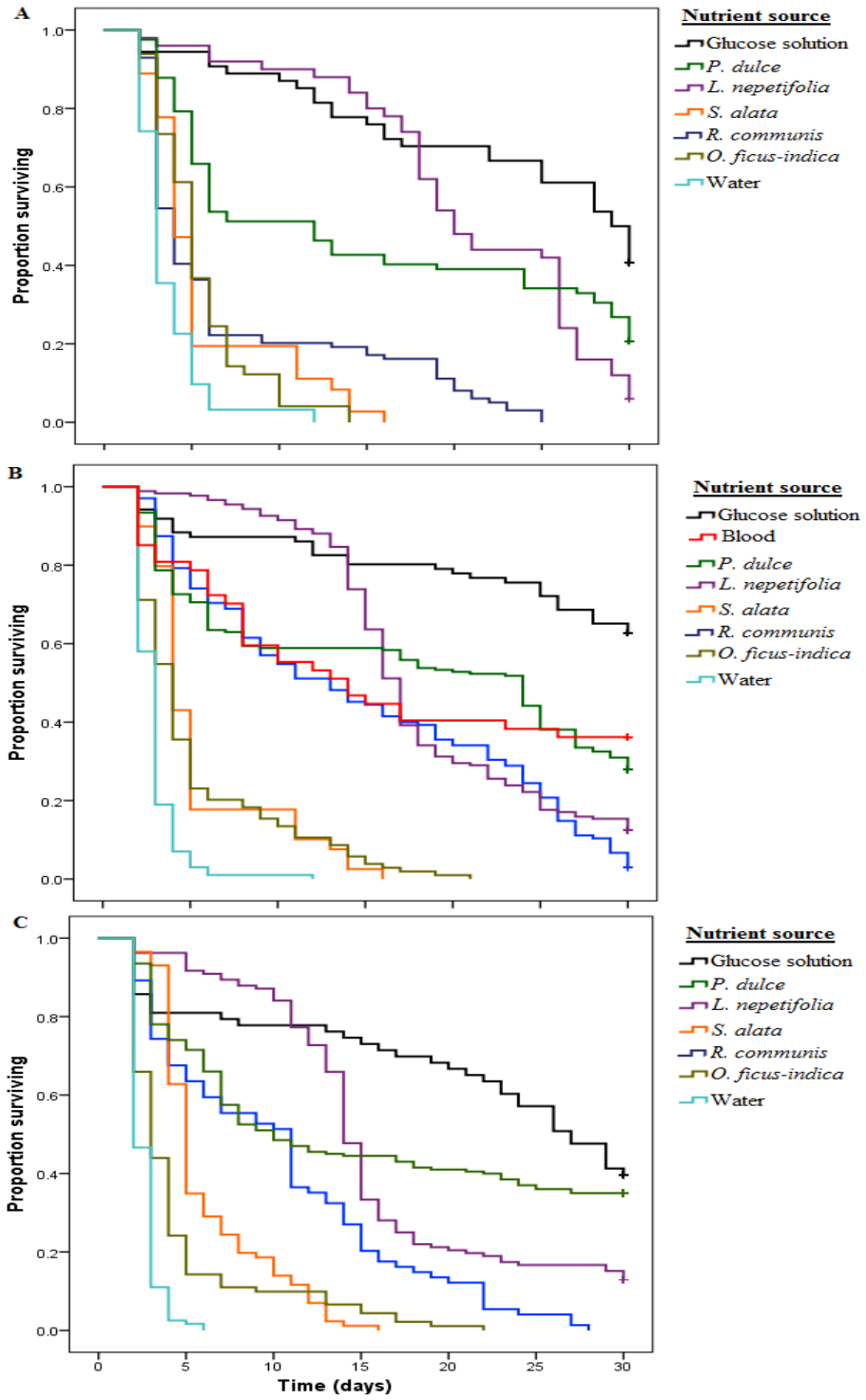

Figure 4 Impact of natural host plant on the survival of *Aedes aegypti*: Survival curves of A) females with no initial blood meal, B) females with three initial blood meals and C) males. Curves with + at the terminal end indicate censored mosquitoes which survived through the 30 days experimental period. N = 3. The difference in survival times of adult *Ae. aegypti* on different nutrient regimes was detected using Kaplan-Meier and Cox regression survival analyses.

Table 2 Mean survival times (days) ± standard errors of means

Nutrient source	Bloodfed females	Non-bloodfed females	Males
Glucose	24.51±1.04[a]	23.32±1.28[a]	21.38±1.34[a]
P. dulce	17.61±0.84[b]	15.23±1.28[b]	15.63±0.83[ab]
L. nepetifolia	18.30±0.51[b]	20.74±1.04[b]	15.88±65[b]
S. alata	5.52±0.41[d]	5.67±0.63[c]	6.16±0.34[d]
R. communis	14.62±0.83[c]	7.12±0.68[c]	10.31±0.84[c]
O. ficus-indica	5.20±0.42[d]	5.45±0.39[c]	4.54±0.44[e]
Water	2.93±0.13[e]	3.61±0.35[d]	2.62±0.08[f]
Blood	16.40±1.66[b]	-	-

Numbers marked with different letters in the table are significantly different down the column.

To determine the effect of these plant sources on mosquito fecundity, a similar experimental setup described above was used with oviposition cups provided in the cages. Eggs were collected daily over a 30-day period, counted and recorded against the treatment from which they were obtained. Irrespective of the nutrient regime, no eggs were laid in the absence of an initial blood meal. In addition, due to low survival rates, no females in the water treatment reached the egg laying stage which started between days 7-9 days post adult emergence. We found significant differences in the daily number of eggs laid per female fed on different host plants over the 30-day period when initial three blood meals were provided (Kruskal-Wallis chi-squared = 26.727, df = 6, $P < 0.001$, Fig. 5A and B). On average, *Ae. aegypti* fed on *S. alata* achieved the highest lifetime fecundity of 40 eggs/female over the 30-day period, followed by *L. nepetifolia* (26 eggs/female), blood (16 eggs/female), *O. ficus-indica* (13 eggs/female), *P. dulce* (12 eggs/female), glucose (10 eggs/female), and *R. communis* (8 eggs/female) (Fig. 5A). *Aedes aegypti* fed on *S. alata* had a high oviposition rate within a

four day window with no eggs laid thereafter, while those fed on glucose solution, *R. communis* and *O. ficus-indica* displayed an irregular oviposition pattern (Fig. 5B). A more consistent oviposition pattern over the 30-day period was observed in mosquitoes fed on *L. nepetifolia* and *P. dulce* (Fig. 5B).

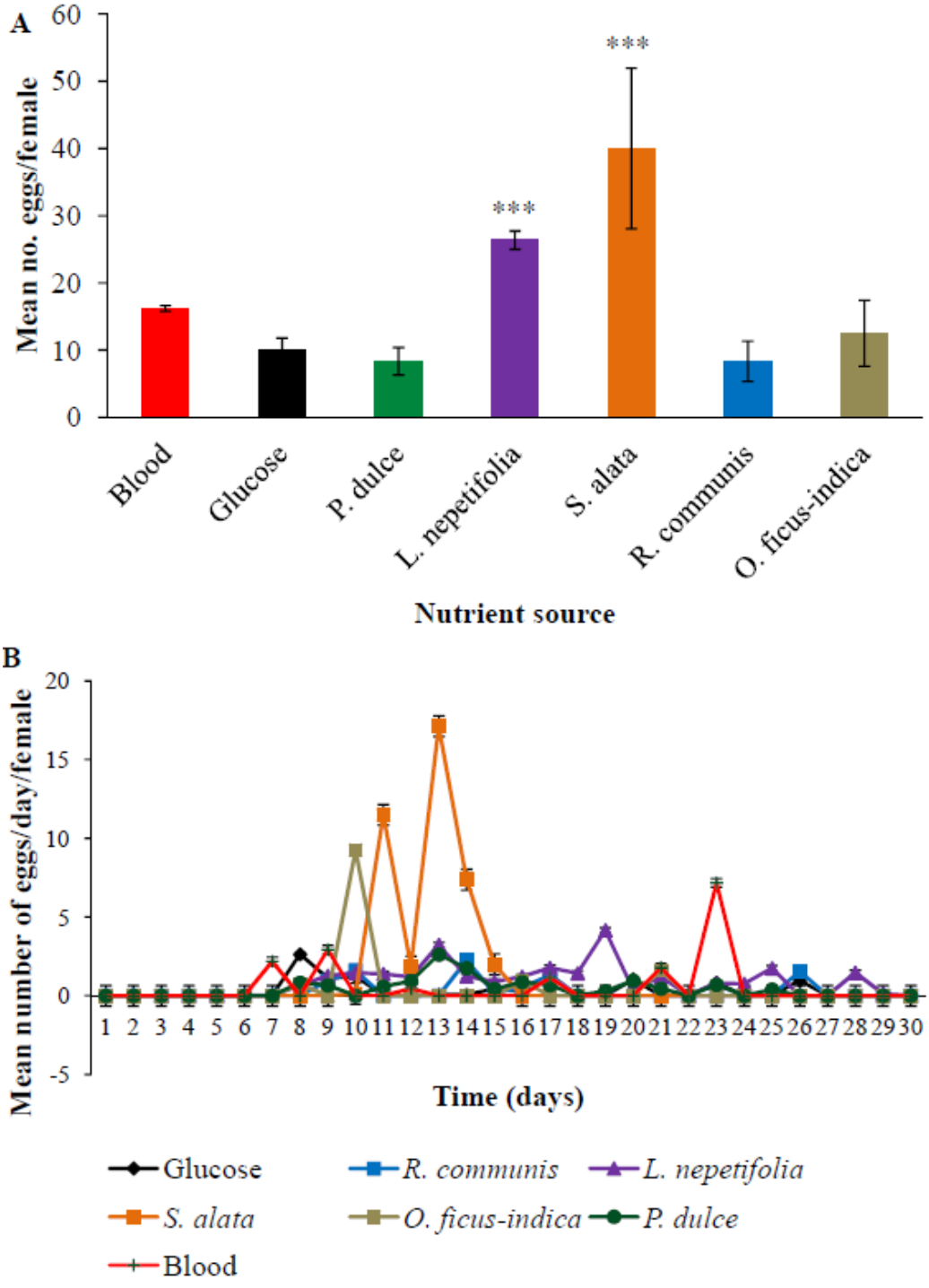

Figure 5 Impact of host plants on the fecundity of female *Aedes aegypti*: A) Average number of eggs laid per female over a 30-day observation period when fed on different host plants with an initial blood meal. B) Line graph showing average daily oviposition pattern of adults fed on different host plants. Error bars represent standard errors of mean. N = 3. The differences in daily fecundity between different nutrient sources were detected using Kruskal-Wallis test and the means separated with two-sample Wilcoxon test.

Next, we investigated the influence of host plants on the fertility of *Ae. aegypti*, which is an important factor in determining the sustenance of vector population within an ecosystem. We flooded the laid eggs from different regimes with double distilled water and counted the emergent larvae daily for 14 days, after which the eggs were considered non-viable. Significant differences in the proportion of eggs from each nutrient source hatching into larvae (χ^2 = 788.4, df = 6, $P < 0.001$). The proportion of hateched eggs was highest for eggs laid by females fed on blood meal alone followed by those fed on glucose solution, *P. dulce* , *R. communis, L. nepetifolia*, *O. ficus-indica* and *S. alata* (~10-fold less, $P < 0.001$) (Fig. 6). However, when the larvae were allowed to mature into adults, no difference was observed in the number of emerging males and females except for those from *R. communis* diet which had a bias towards females (Fig. S1)

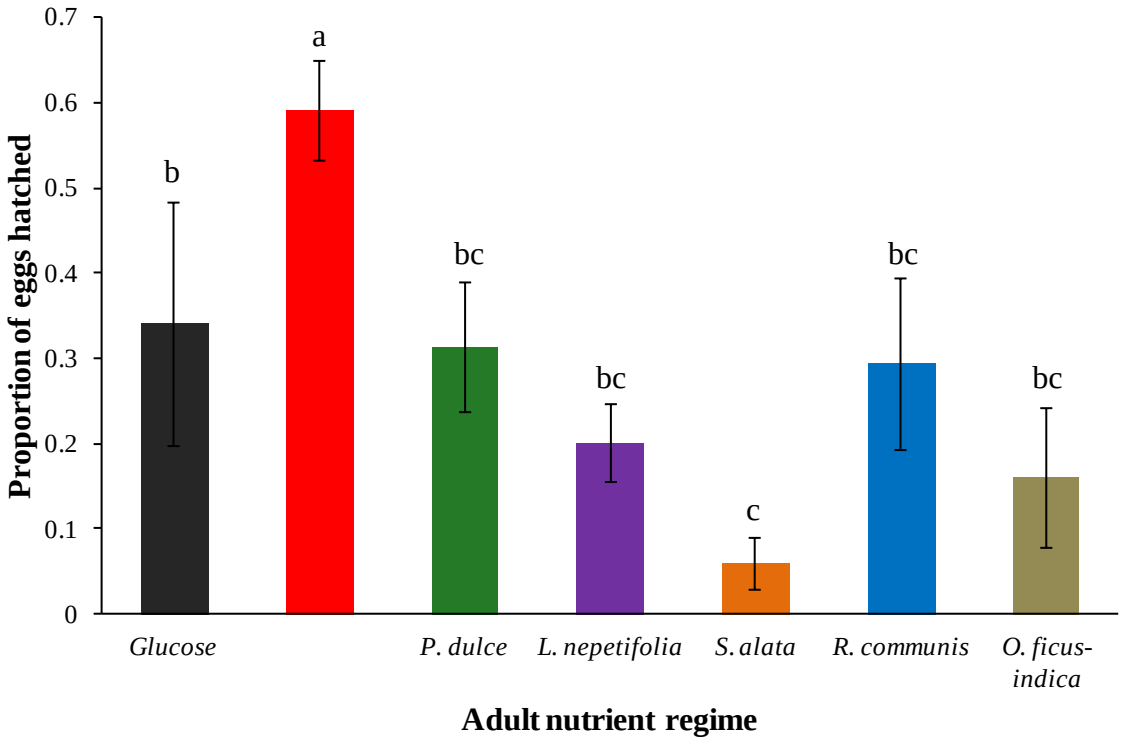

Figure 6 Mean proportion of eggs laid by adult females fed on different nutrient sources that hatched. Error bars represent standard errors of mean while bars capped with different letters are significantly different. N = 3. The difference in proportion of eggs hatching between different nutrient sources was detected using χ^2 test at $\alpha = 0.05$ level of significance**.**

Differential impact of host plants on disease vector survival is related to nutrient rewards

Although plants have been known to modulate important vectorial traits and survival of mosquitoes (39), how these parameters relate to the plant content and what nutrients they ingest has yet to be fully investigated. To address whether the differential fitness matrices observed is related to nutrient rewards, we hypothesised these could be related to the sugar and amino acid contents, the basis informed by nectar chemistry with these as major components (42). Since survival has previously been linked to sugar intake (39), we initially sought to determine if sugar intake from the different plant sources was commensurate with the survival patterns observed by quantifying the sugar content in the mid-gut of both sexes

of *Ae. aegypti* using coupled gas chromatography-mass spectrometry. With the exception of those held on *O. ficus-indica*, there was no significant difference in the total amount of sugar ingested from the plants by both males and females (Kruskal-Wallis chi-squared = 27.626, df = 5, $P < 0.001$ and Kruskal-Wallis chi-squared = 26.319, df = 5, $P < 0.001$, respectively, Fig. 7A). Further analysis of plant sugar content revealed that both *P. dulce* and *L. nepetifolia* had similar total sugar content (105.6 ± 21.1 µg/mg and 104.9 ± 21.4 µg/mg, respectively) with *R. communis* and *S. alata* having higher sugar content (3-fold and 4-fold more, respectively) while *O. ficus-indica* had the least total sugar content (2-fold less) (Kruskal-Wallis chi-squared = 30.1, df = 4, $P < 0.001$). In addition, GC-MS analysis showed variation in the type of sugar content with different plants having unique sugar profiles (Fig. 7B). To test if these qualitative differences in plant sugar content played a role in explaining the observed survival patterns, we subjected the data to Principal Component Analysis (PCA). PCA revealed that more than 97% of the variation in sugar content was explained by the first three principal components, suggesting that the benefits from plant feeding in relation to sugar content are derived primarily from the amounts of specific sugars. PC1 accounted for the highest variation (47%), which positively correlated with sugar constituents of *P. dulce*; with PC2 (32%) and PC3 (18%) positively correlated with the sugar constituents of *L. nepetifolia* and *S. alata*, respectively (Fig. 7C). A total of 27 sugars contributed to the observed variation, six of which were identified as glucose, fructose, mannose, ribose, rhamnose, galactose, sucrose and maltose. Together, these results suggest that both qualitative and quantitative value plant sugar content impact on mosquito survival.

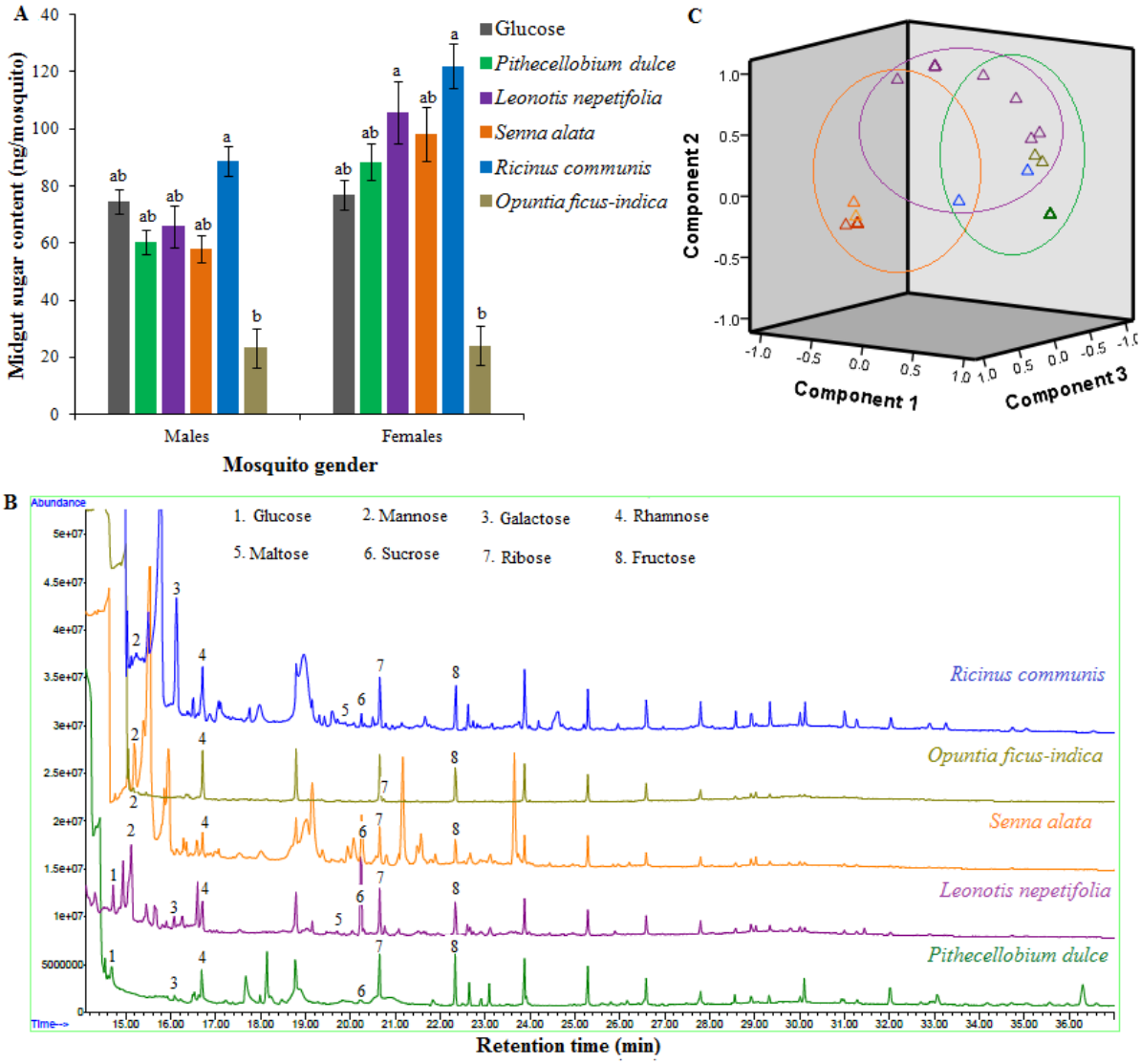

Figure 7 Plant and mosquito mid-gut sugar content. A) Total sugar content of the eight identified sugars in the mid-gut of male and non-blood fed female *Ae. aegypti*. The mid-guts were extracted for sugar analysis from mosquitoes which had been held on the respective plant for 15 consecutive days.B) Total ion chromatogram of N-Methyl-bis trifluoro acetamide sugar derivative of five host plant species. C) Three dimensional cluster plot based on the Principal Component Analysis of the eight most abundant sugar constituents of the five plant species. The cluster plot was generated using the first three principal components with PC1 explaining 47% of the variation, PC2 = 32.4% and PC3 = 18.1%. Quantitative differences in sugar content of mosquito mid-guts and host plants were detected using Kruskal-Wallis chi-squared test at 95 % confidence interval.

As the second major component of plant nutrients (42) amino acids are required for both the signalling of vitellogenesis and as building blocks for york protein synthesis (43). As such, we analysed for the amino acids imbibed by *Ae. aegypti* in the mid-gut after feeding on these different plant species and compared the profile to those available in the plants. Using coupled liquid chromatography-mass spectrometry, we found qualitative and quantitative differences in the amino acid content of blood fed and non-blood fed mosquitoes raised on the different host plants (Table 3). Key amino acids variably detected in the mid-gut of blood fed and non-blood fed *Ae. aegypti* included valine, serine, glutamic acid, proline, arginine, histidine, glycine, methionine, tyrosine, phenylalalnine, isoleucine, leucine and threonine. Overall, blood fed female *Ae. aegypti* had a higher total amino acid content (24.2 ± 3.3 nM/mosquito) than their non-blood fed counterparts (13.3 ± 4.6 nM/mosquito, V = 2135, $P < 0.001$). Among the blood fed *Ae. aegypti*, significant differences were detected between the nutrient regimes (Kruskal-Wallis $\chi^2 = 22.28$, df = 5, $P < 0.001$), with *O. ficus-indica* having the highest amino acid content (35.5 ± 1.2 nM), followed by *R. communis* (27.9 ± 0.5 nM), *S. alata* (25.8 ± 0.9 nM), *L. nepetifolia* (24.2 ± 0.3 nM), glucose solution (20.4 ± 0.4 nM), *P. dulce* (11.2 ± 0.1 nM). Significant differences were also detected among non-blood fed mosquitoes (Kruskal-Wallis $\chi^2 = 38.77$, df = 5, $P < 0.001$), with *S. alata* having the highest amino acid content (31.9 ± 0.5 nM) followed by *R. communis* (18.2 ± 0.6 nM), *L. nepetifolia* (15.5 ± 0.4 nM), *O. ficus* (8.4 ± 0.5 nM), *P. dulce* (5.5 ± 0.1 nM) and glucose solution (0.3 ± 0.5 0.3 nM) in decreasing order. With the exception of histidine which was detected only in mosquitoes fed on *S. alata* and *O. ficus-indica* irrespective of blood feeding status, all the other 13 amino acids detected in this study were present in all blood fed mosquitoes from all the host plants (Table 3). Qualitative differences in amino acid content were observed among the non-blood fed female *Ae. aegypti*, with those fed on *S. alata* having all the 13 amino acids

while *P. dulce* and *L. nepetifolia* each had 12, *R. communis* 10, *O. ficus-indica* 6 and glucose solution 1 (Table 3).

Significant differences were detected in the amino acid content of plant species and mice blood (Kruskal-Wallis χ^2 = 38.09, df = 5, P < 0.001). Mouse blood had the highest total amino acid content (2221.7 ± 9.7 nM/mg), followed by *P. dulce* (183.5 ± 4.4 nM/mg), *L. nepetifolia* (162.2 ± 18.0 nM/mg), *S. alata* (122.2 ± 12.7 nM/mg), *R. communis* (89.3 ± 3.4 nM/mg) and *O. ficus-indica* (77.9 ± 2.9 nM/mg). In addition, qualitative differences were observed in the number of amino acids with 15detected in mouse blood, 11 in *L. nepetifolia*, 7 in *S. alata* and 6 in *P. dulce*, *R. communis* and *O. ficus-indica* (Fig. 8).

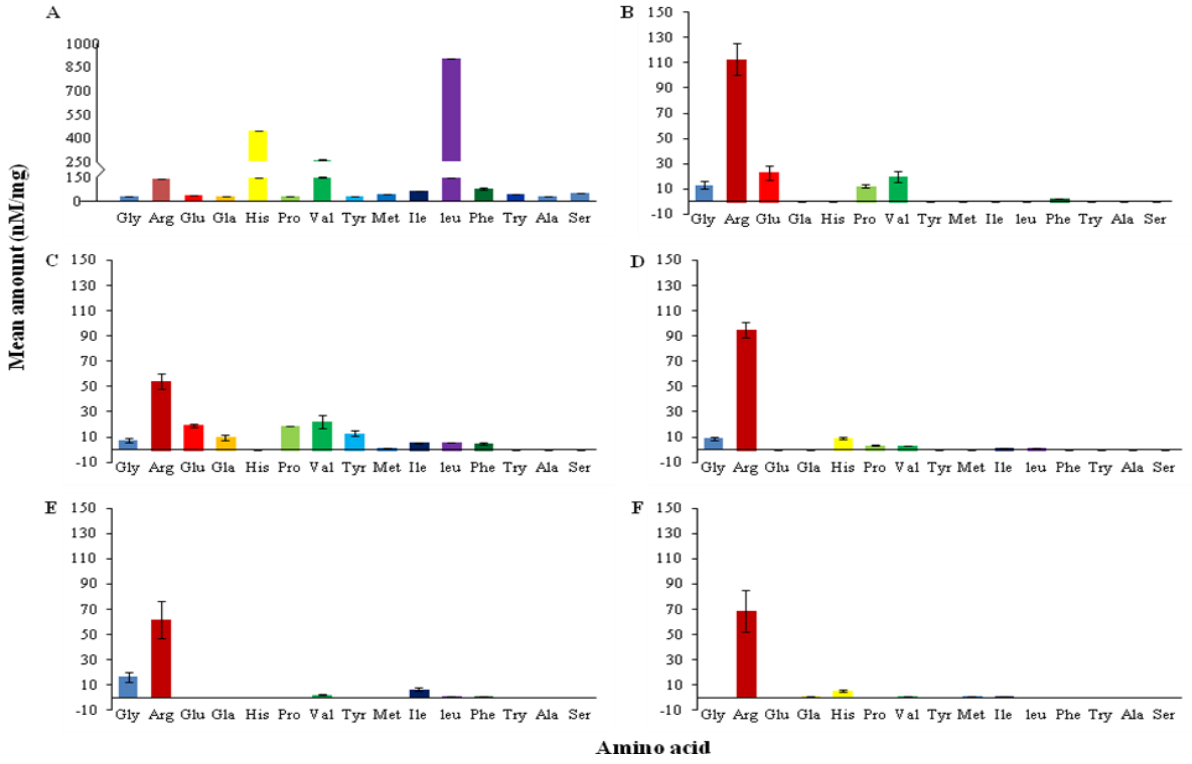

Figure 8 Free amino acid content/ingested (nM/mg) from mouse blood and natural host plants. A) Mouse blood amino acid content, B) *Pithecellobium dulce* amino acid content, C) *Leonotis nepetifolia* amino acid content, D) *Senna alata* amino acid content, E) *Ricinus communis* amino acid content, and F) *Opuntia ficus-indica* amino acid content. N = 3

Table 3 Mean ± SE mid-gut amino acid content (mM/mosquito) of *Aedes aegypti* fed on different host plants.

Nutrient regime		Val	Ser	Glu	Arg	His	Pro	Gly	Met	Tyr	Ile	Leu	Phe	Thr
Glucose	BM	10.5±1.9	6.0±2.1	0.5±0.2	0.3±0.0	0	0.3±0.1	0.9±0.5	0.4±0.1	0.3±0.0	0.4±0.1	0.3±0.0	0.3±0.1	0.3±0.0
	NBM	2.7±0.3	0	0	0	0	0	0	0	0	0	0	0	0
P. dulce	BM	1.2±0.5	0.4±0.1	4.0±0.2	2.2±0.2	0	0.3±0.0	0.6±0.1	0.3±0.0	0.3±0.1	0.5±0.1	0.4±0.1	0.5±0.1	0.6±0.2
	NBM	0.4±0.1	0.5±0.1	0.3±0.1	0.6±0.1	0	0.4±0.1	0.5±0.1	0.4±0.1	0.6±0.1	0.7±0.1	0.4±0.1	0.5±0.1	0.3±0.1
L. nepetifolia	BM	3.9±0.4	1.5±0.3	1.6±0.4	3.1±0.3	0	1.4±0.1	1.2±0.1	2.5±0.4	2.4±0.7	1.8±0.3	1.4±0.4	2.6±0.1	1.0±0.1
	NBM	3.0±0.3	0.6±0.1	0.7±0.1	2.2±0.4	0	1.3±0.2	1.1±0.2	0.3±0.0	1.7±0.7	1.2±0.3	1.3±0.7	1.3±0.9	0.8±0.3
S. alata	BM	9.8±3.7	0.6±0.0	4.3±1.8	2.9±1.6	1.1±0.7	1.5±0.7	0.5±0.2	0.9±0.6	1.0±0.4	0.5±0.2	1.3±0.9	0.9±0.5	0.5±0.1
	NBM	3.2±0.4	2.0±0.3	2.5±0.3	4.9±0.2	2.5±0.5	2.0±0.1	0.8±0.1	3.5±0.3	0.3±0.0	2.2±0.8	3.3±2.4	4.0±0.5	0.9±0.3
R. communis	BM	4.0±0.7	1.0±0.1	1.2±0.1	4.9±1.6	0	1.3±0.2	1.3±0.1	2.4±0.5	2.3±0.7	1.6±0.3	4.2±0.5	2.7±1.2	1.1±0.1
	NBM	1.6±0.6	1.0±0.6	0	4.3±0.3	0	2.0±0.3	2.4±0.8	1.6±0.6	0	0.8±0.1	2.5±0.7	1.4±0.4	0.5±0.0
O. ficus-indica	BM	9.2±3.0	1.9±1.1	4.2±1.0	5.9±0.9	1.0±0.7	1.4±0.5	2.0±1.1	1.6±1.1	0.6±0.3	3.5±2.6	1.8±1.4	1.7±1.4	1.0±0.4
	NBM	0	0.8±0.1	0	1.0±0.0	3.3±0.5	0.9±0.1	0	0	1.1±0.4	1.3±0.7	0	0	0
Blood meal only		0.8±0.2	3.1±1.1	2.6±0.6	2.6±0.1	0.3±0.0	2.7±0.4	1.8±0.4	0.8±0.4	0.5±0.1	0.8±0.1	0	3.6±1.2	1.1±0.1

The mid-gut was extracted on day 15 from the commencement of assays. BM = with initial blood meal, NBM = without initial blood meal. N = 15

Discussion

We employed a combination of biochemical and molecular approaches to document natural host plants of four Afro-tropical mosquito species which are known to transmit medically important human and animal diseases. For the malaria vector, one of the plants identified in this study *viz R. communis* has previously been implicated as a potential host plant due to its abundance in the peridomestic areas in malaria-endemic regions in western Kenya, rich sugar content and ability to support survival of *An. gambiae* s.s. (12, 24, 31) while *L. nepetifolia* and *S. alata* have not been reported before as host plants for the malaria vectors. To the best of our knowledge, this is the first attempt to identify plant species utilised by dengue and RVF vectors in tropical Africa. Vegetation cover is an important variable which impacts on the abundance of RVF vectors and has been widely incorporated into models to forecast outbreaks of RVF in East Africa (44). The preferred plant feeding observed for these vectors, suggests differential vegetation may be more important. Our results suggest that plant feeding constitutes an important aspect of mosquito biology and that the mosquitoes forage on suitable host plants within their ecological range to obtain nutrients essential for their survival, which corroborates previous studies (12, 14, 31). In addition, this study confirms the potential of DNA barcoding as a tool to unravel the preferred host plants of not only mosquitoes but other disease vectoring arthropods (34-36).

The potential of DNA barcoding to determine natural plant sources of phytophagous insects was first demonstrated by Matheson *et al.* (34), and later corroborated by Junnila, Müller and Schlein (36) who used this technique to identify the plant species that *Anopheles surgentii* collected in the Jordan Valley had fed on. Both studies utilised ribulose bisphosphate carboxylase large subunit (rbcL) gene target to identify the nectar sources. Unlike animal DNA where a universal gene target (cytochrome oxidase 1) has been established for wide application, no single universal primer exists for plant speciation due to lack of adequate

discriminatory powers among closely related plant taxa in a number of gene targets which have been proposed (45). This has led to a recommendation for use of multiple primer targets with varied specificity for different groups of plants (46, 47). In this study, we used three gene targets namely ITS2, trnH-psbA and matK to identify the host plants, informed by the fact that the four mosquito species were collected from habitats whose plant diversity are not well known. It is interesting to note the different success rates of the three gene targets at identifying the plant species from the three localities, with matK amplifying just a few samples of mosquitoes from Ahero and Garissa while trnH-psbA and ITS2 amplified samples from across the three sites with considerable success. The discrepancy between the number of mosquitoes which were positive in the cold anthrone test, indicating recent sugar feeding and those that were successfully sequenced for the presence of plant DNA is also intriguing. More striking is the low number of successful plant DNA sequencing and identification for *Ae. mcintoshi* and *Ae. ochraceus*, despite the fact that the two species showed high levels of plant sugar feeding. This can be attributed to a number of factors; lack of universality for any of the three targets in identifying all plant species (45), low plant DNA concentration in mosquito mid-gut possibly due to degradation, and lack or low concentration of DNA in nectars and honey dew (36, 48). Perhaps alternative primers not used here may improve success rates and would be worth exploring.

Our findings further provide evidence of differential nutritional benefits associated with five of the identified plant species. Besides glucose solution, *Ae. aegypti* survived well on *P. dulce* (the host plant identified from its mid-gut) and *L. nepetifolia* (detected from the mid-gut of *An. gambiae*), with survival on both plant species being slightly superior to that of a blood meal diet alone, irrespective of whether the mosquitoes had an initial blood meal or not. In contrast, *Ae. aegypti* did not survive well on *S. alata* and *R. communis* (both detected in *An. gambiae* mid-gut) and *O. ficus-indica* (detected in the mid-gut of *Ae. mcintoshi* and *Ae.*

ochraceus). This variable performance on different host plants points to either differences in nutrient rewards from such sources or specificity of *Ae. aegypti* to suitable host plants.

In depth analysis revealed discrepancy in vector fitness and the nutritional value of the host plants. While the higher survival of *Ae. aegypti* on *L. nepetifolia* and *P. dulce* and the low survival on *O. ficus-indica* were commensurate with the high to low sugar content of these plants and the amounts ingested by the mosquitoes, the high sugar content of *S. alata* and *R. communis* did not translate into prolonged vector survival as expected. Indeed, PCA separated the sugars into three distinct clusters with *L. nepetifolia* and *P. dulce* sharing a large number of sugars while the sugar profiles of *S. alata* were largely distinct. These results are suggestive of the qualitative rather than the quantitative sugar content as the key determinant of the suitability of a plant as source of nourishment for mosquitoes. Another possible reason for the low survival on *S. alata* and *R. communis* could be due to the presence of secondary metabolites that render this plant species unpalatable or toxic to *Ae. aegypti*, as was previously confirmed for *An. gambiae* s.s. fed on *R. communis* (24), or the inverse relationship of insect survival and fecundity in which they tend to dump rather than lay viable eggs when endangered. However, we note here that the nutrient analysis was based on dried plant tissues obtained from cut plant materials due to unsuccessful effort to directly extract plant sap and nectar. This might not reflect the actual nutrient gain by the mosquitoes whose mouthparts are adapted for sucking. In addition, *P. dulce* was used as cuttings as opposed to the intact potted plant. While this was necessitated by the perennial nature of this plant, both survival and nutrient benefits observed might not be reflective of the natural situation for intact plant given that cutting the plant might interfere with vascular activity and alter nutrients available for the mosquitoes.

Our results further show high fecundity displayed by blood fed female *Ae. aegypti* provided with *S. alata* and *L. nepetifolia* which was commensurate with gut amino acid content of mosquitoes fed on these plants. Plant amino acid analysis showed that plants only contain trace amounts of amino acids compared to mouse blood with quantitative and qualitative differences between the plant species. The insufficient amino acid reserves of all the plant species corroborate their inability to induce oviposition when no initial blood meal was offered. Nonetheless, the consistent and extended oviposition pattern observed in *L. nepetifolia* and high oviposition rate in *S. alata* are noteworthy. A number of reasons can be advanced to explain this phenomenon. Firstly, it is possible that mosquitoes obtain sufficient sugar reserves to meet their metabolic needs therefore allowing the amino acids obtained from blood to be fully dedicated to egg development as has been suggested by previous studies (17, 29, 30). Secondly, though in trace amounts, *L. nepetifolia* had up to 11 amino acids, key among them tyrosine and phenylalanine which are required for egg melanisation (49, 50), which may have provided continuous stimulation of vitellogenesis and sustained oviposition long after the initial blood meal. Thirdly, *S. alata*, just like *O. ficus-indica*, contains trace amounts of histidine which could be responsible for the high oviposition rate within a short time soon after blood meal, although this amino acid was shown not to be significantly important in the stimulation of vitellogenesis on its own (51). Finally, the arrival of blood in the mid-gut of *Ae. aegypti* has been shown to suppress the expression of the Reactive Oxygen Species (ROS) immune pathway resulting in proliferation of gut microbe populations (52), thereby increasing competition for nutrients. Since both plants possess antimicrobial properties (53, 54) it is possible the secondary metabolites from these plants suppress this microbial proliferation when ingested soon after a blood meal, thus allowing maximum assimilation of amino acids by the mosquitoes. Similar explanation could apply to

P. dulce, *R. communis* and *O. ficus-indica* which also posses antimicrobial properties (55-57), with other limiting factors coming into play to exert the difference in oviposition patterns.

Furthermore, the fact that *Ae. aegypti* kept on *L. nepetifolia* and *P. dulce* were able to lay subsequent batches of eggs without further blood meal points to possible role of natural plant sources in modulating traits such as gonotrophic discordance observed in a number of mosquito species including *An. gambiae*, *An. funestus* and *Ae. aegypti* (5, 40). However, this is subject to further studies to confirm the involvement of natural host plants in influencing these features. Taken together, these findings underpin the significant role played by plants in modulating the fitness among mosquito species that transmit pathogens to humans. It also highlights the potential of host plants in modulating nutrient assimilation by mosquitoes, which could play a role in vector-pathogen interactions and their potential as disease vectors.

Some of the limitations of this study were; mosquito sampling was not done across different seasons to determine possible seasonal variation of plant preference, the laboratory assays were done using *Ae. aegypti* only which might not fully reflect the actual impact of the other mosquito species, and *Ae. aegypti* were not fed on synthetic mixtures of the sugars and amino acids identified from the host plants to provide definitive proof of the impact of host plant nutrient rewards on mosquito vector fitness. Additional survival and fecundity studies using a single female and male mosquito to control for actual blood and plant feeding is also necessary to validate the impact of these plants on mosquito fitness. Further investigation to address these would be of immense interest. Nonetheless, these findings emphasise the significant role of plant feeding in mosquito behaviour and fitness. This study therefore lays a critical foundation for further exploration and possible adoption for control strategies to target the plant feeding behaviour of Afro-tropical mosquito species that are of public health importance.

Conclusion

Plant feeding is an integral component of the population dynamics of mosquito disease vectors, and DNA barcoding holds promise for accurate identification of natural plant sources of various mosquito species. In addition, different plant species utilised by mosquitoes have variable impacts on their fitness, which might not directly relate to the nutritional value of such plants. Accurate identification of specific natural nectar sources of these disease vectors and the exact nature of their interactions with such plants is therefore important in efforts to develop new environmentally viable vector control strategies.

Methods

Mosquito sample collection and preparation

Samples were obtained from three sites in Kenya: *An. gambiae* s.l. from Ahero (0°10'S, 34°55'E) which is a malaria endemic area in western Kenya (58), *Ae. aegypti* from Kilifi (3.6333° S and 39.8500° E) in coastal region with high dengue endemicity (59) and *Ae. mcintoshi* and *Ae. ochraceus* from Ijara (1.5988° S and 40.5135° E) in north eastern Kenya which a Rift Valley fever endemic region (60) (Fig. S2). *Aedes egypti, Ae. mcintoshi* and *Ae. ochraceus* used in this study were from our previous study (61), while *An. gambiae* were collected from Ahero using the same set of lures described in Nyasembe *et al*. (61). Using these trapping methods and lures, only a few males or no males at all were caught for *An. gambiae, Ae. mcintoshi* and *Ae. ochraceus*. Consequently, only females of the four species were considered for subsequent assays.

To prepare the samples for biochemical and molecular analyses, individual mosquitoes were submerged in a solution of 0.5% hypochlorite, agitated gently for 1 min with forceps, and

then rinsed in double distilled water (ddH_20) for 1 min. This was to remove any plant debris that may have been on the outside of the insect, which could otherwise contaminate the sample. They were then dissected on a microscope slide in distilled water and the mid-guts transferred individually on a 1.5 ml sterilised Eppendorf tube and macerated using round-tipped glass rods sterilised through the flame of a Bunsen burner. One hundred microlitres of absolute ethanol was added and the solution homogenised. Two sets of controls were used as follows: a) laboratory-reared *An. gambiae* s.s. fed on *Parthenium hysterophorus* (Asteraceae) overnight, and b) *An. gambiae* aspirated directly from *P. hysterophorus* field in Ahero using a backpack aspirator (3" IN-LINE BLOWER, John W. Hock Company, Gainesville, FL, USA). All the controls were cleaned as described above.

Determination of evidence of recent plant feeding in field collected mosquitoes

This was done using the cold anthrone test as described by van Handel, Haeger and Hansen (25) as a quick initial test to detect fructose. Aliquots (50 µl) of the prepared mosquito mid-gut homogenate were individually placed in the wells of a flat bottomed 96-well microtiter plate followed by 300 µl of the reaction solution comprising 0.15% anthrone (Sigma) (wt/vol) in 71.7% sulphuric acid. This was incubated at 25^{o}C for 60 min before being examined for colour changes. In the presence of fructose, the reaction mixture changed its colour from yellow to blue (Appendix 1). A fraction of fructose-positive samples were subjected to plant DNA extraction as described below.

Identification of natural host plant species of RVF, dengue and malaria vectors

DNA barcoding was used to detect and identify plant DNA in the mid-gut of the four mosquito species. Plant DNA was extracted from the homogenised samples of fructose-positive mosquitoes only using manufacturer's protocol described by DNeasy® Plant Minikit-(QIAGEN, USA) with a minor modification. The incubation period with lysis buffer AP1 and RNase was extended by 30 min while that with the elution Buffer AE was extended for 3 hrs. The extracted DNA was stored at -20^{o}C until use in PCR amplification.

PCR amplification was performed using published primers targeting the genes; Internal transcribed spacer region (ITS2), trnH-psbA intergenic spacer region (trnH-psbA) and maturase K (matK) (Table 1). The use of multiple gene targets was to maximise on the detection possibility as individual genes selectively amplify certain plant families (45, 62). Each PCR reaction (carried out in volume of 20 µl) consisted of 7 µl template DNA, 10 µl 2x HotStarTaq Master Mix (HotStaTaq® Plus Master Mix Kit, Quagen), 0.5 µM of each primer, and 2 µl of RNase free water. A PCR negative control (RNase-free water) was routinely used. Samples were amplified using Veriti™ 96-well Thermal Cycler (Singapore). For ITS2, the cycling parameters were 94^{o}C for 1 min, followed by 45 cycles of 94^{o}C for 1 min, 53^{o}C for 40 sec and 72^{o}C for 1 min, and final extension at 72^{o}C for 10 min. Similar cycling conditions were used for trnH-psbA and matK amplification with the annealing temperature set at 55^{o}C and 48^{o}C, respectively.

Table 4 Forward and reverse primer sequences for three gene targets used to identify natural host plants of dengue, malaria and Rift Valley mosquito disease vectors.

Primer	Direction	Sequence (5'-3')	Reference
ITS2	Forward	ATGCGATACTTGGTGTGAAT	(63)
	Reverse	GACGCTTCTCCAGACTACAAT	(63)
trnH-psbA	trnH	CGCGCATGGTGGATTCACAATCC	(64)
	psbA	GTTATGCATGAACGTAATGCT	(64)
matK	2.1 forward	CCTATCCATCTGGAAATCTTAG	(45)
	5 reverse	GTTCTAGCACAAGAAAGTCG	(45)

All successful amplifications were confirmed using 1% agarose gel electrophoresis (Appendices 2, 3 and 4). They were purified using the Exo/SAP-IT for PCR Product Kit (Affymetrix Inc., USA) according to the manufacturer's instructions and outsourced for bidirectional sequencing to Inqaba Biotechnological Industries (Pty) Ltd (South Africa).

The obtained plant DNA sequences for each gene were cleaned, edited and compared to reference sequences in the GenBank (65) database. In GenBank, the 'megablast' search option of nucleotide Basic Local Alignment Search Tool (BLASTn) (66) algorithm was used with the default search parameters. The hits with sequence identity above 96% were retrieved and added to the original sample query sequences. The sequences were aligned using ClustalW in MEGA 6 (67). Aligned matrices were used to construct p-distance phylogenetic tree based on Neighbour Joining method for individual genes with 1000 bootstraps. Nodal support was evaluated by bootstrapping with values of 70% or more considered significant according to Hillis and Bull (68). Further steps to confirm the plant identity included on-site identification and presence within the specific ecologies from where the mosquitoes were

sampled by a plant taxonomist (Simon Mathenge) and comparison to established botanical database by the National Museum of Kenya (http://www.museums.or.ke).

To confirm the plant sources, leaf and flower (where applicable) samples of the tentatively identified plants were directly obtained from the respective field sites. The samples were cleaned using double distilled water before obtaining approximately 100 mg wet weight of the sample to be placed in sterile 1.5 ml Eppendorf tubes. The plant samples were homogenised and DNA extracted using the DNeasy® Plant Mini Kit as described above. The obtained plant DNA was similarly amplified for ITS2, trnH-psbA and matK genes, processed and sent for sequencing as described earlier. The sequences were then aligned with those from the mosquitoes and phylogenetic tree obtained. Nodal support was evaluated by bootstrapping with values of 70% or more considered significant.

Survival, fecundity and egg hatchability of *Aedes aegypti* on the identified mosquito host plants

To determine the potential of the identified host plants to support these important life parameters of mosquito vectors, I used F1 generation of *Ae. aegypti* collected as eggs from Kilifi field site. *Aedes aegypti* were selected for survival assays due to their ease of adaptability hence the required F1 generation could easily be obtained from field collected eggs. Besides, attempts to obtain F1 generation from field collected *An. gambiae* were unsuccessful while efforts to rear *Ae. mcintoshi* and *Ae. ochraceus* in the laboratory was not made due to insecurity in north eastern Kenya where these mosquitoes could be obtained. The hatching larvae were reared in plastic trays (25 cm long × 20 cm wide × 14 cm high) to adults with a daily ration of Tetramin fish food (Tetramin1, Melle, Germany) of 0.3 g /100 larvae/day. The rearing room was maintained at a temperature of 31^{o}C and relative humidity

of 80 %. The emerging adults, 1-3 days old with no prior exposure to any other nutrient source, were used in survival and fecundity assays. Five of the plant species identified as natural host plants of the four mosquito species were used in these assays although specific for the vectors from their respective ecologies. They included *P. dulce* (*Ae. aegypti* host plant), *L. nepetifolia*, *S. alata* and *R. communis* (*An. gambiae* s.l. host plants), and *O. ficus-indica* (*Ae. mcintoshi* and *Ae. ochraceus* host plants). The sixth plant species, *N. cyanea* (*Ae. ochraceus* host plant), was not considered for fitness assays due to technicality in obtaining it at the time of the assays due to insecurity issues in Sangailu in north eastern Kenya where the plant could be obtained. *Leonotis nepetifolia*, *S. alata*, *R. communis* and *O. ficus-indica* were obtained as potted plants and transported to our laboratories at *icipe*, Nairobi where the assays were conducted with flowering stages (during the first blossoming stages) of the plants. *Pithecellobium dulce* which is a perennial tree could not be potted hence was evaluated as fresh cuttings consisting of leaves, flowers and pods at the field site and replaced twice a day. Although effort was not made to confirm actual plant feeding, the observed longer survival of non-blood fed mosquitoes provided host plants compared to those fed on water only was attributed to actual plant feeding. This data was further supported by mid-gut nutrient analysis. Plants under aphid attack were excluded from further experiment.

Two sets of experiments were conducted. In the first experiment, 100 male and 100 female *Ae. aegypti* were introduced into a 30 x 30 x 100 cm cage containing individual potted plant. In addition to the plant which was continuously available, they were provided with initial three mice blood meals at day 3, 5 and 7 from the onset of the assay. The blood meal was provided by placing a live caged mouse on top of the mosquito cages. Oviposition cups were provided in all the cages. They were monitored for survival and fecundity on a daily basis for 30 days. Mortality and the daily number of eggs laid were recorded. Control experiments comprised 100 female and 100 male *Ae. aegypti* with access to a) distilled water and three

blood meals (negative control), b) 6% glucose solution and three blood meals (positive control 1) and c) blood meals only on alternating days for thirty days with a total of 15 blood meals (positive control 2). The second experimental set up was similar to experiment one above except with the exclusion of the initial blood meals. A total of three replicates for all treatments were carried out. Ten live male and live female mosquitoes were randomly selected from each replicate of all the treatments on day 15 for nutrient analyses as described below.

To measure the hatchability of the laid eggs, the eggs were put in 10 x 20 cm trays and covered with double distilled water. The eggs were hatched according to the date laid and nutrient source. The numbers of larvae were counted for two weeks after which the non-hatched eggs were considered not viable. The hatched larvae were reared until adult emergence and the sex ratio based on diet of parent generation determined.

Nutrient value of plant sources and the ingested nutrient by *Aedes aegypti*

To understand the differences in the performance of *Ae. aegypti* on different nutrient sources, we quantified the amount of sugar and amino acids in the five plant species and the corresponding amounts ingested by the mosquitoes. Initially, I attempted to extract plant sap from vascular tissues and nectars from the flowers using tapered capillary tubes and immediately diluting the extract in water as described in Chen and Kearney (13) as well as fresh plant tissues (stem, leaves, flowers and pods). However, no sugar and amino acids were detected in the subsequent nutrional analysis. Consequently, plant samples were dried slowly at room temperature under shade. The mosquito samples were prepared by dissecting the mid-gut of mosquitoes preserved from survival assays and pooling the mid-gut of three mosquitoes together, with a total of five replicates.

For sugar analysis, 3 mg of dry leaves and flowers (*L. nepetifolia* and *S. alata*), leaves, flowers and pods (*P. dulce*), leaves and extra-floral nectaries (*R. communis*), and tissues and flowers (*O. ficus-indica*) were separately weighed into sterile 1.5 ml Eppendorf tubes and ground to fine powder. The samples were then transferred to a 5 ml reaction vial, 100 µl of pyridine added and allowed to macerate for three days. The samples were then derivatised by adding 100 µl N-Methyl-bis trifluoro acetamide (MBTFA) (Sigma, 98%) and incubating them at 60°C for 1 hr. The products were sieved through glass wool and subjected to GC/MS analysis. GC/MS analysis was carried out in the splitless injection mode using an Agilent Technologies 7890 gas chromatograph coupled to a 5975C inert XL EI/CI mass spectrometer (EI, 70 eV, Agilent, Palo Alto, CA) equipped with an HP-5 column (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent, Palo Alto, CA). Helium was used as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was held at 35°C for 3 min, then programmed to increase at 10°C/min to 280°C and maintained at this temperature for 10 min. For comparison of total sugar content of the five plant species, the ions 69 and/or 319 were used to identify plant sugars. Plant sugars were then identified by comparison of spectra of their trimethylsilyl derivatives with library data (69), and with those of authentic standards derivatised similarly. This was repeated three times with samples obtained from different plant for each host plant species. Similarly, the extracted mid-guts were macerated in pyridine for three days, derivatised and analysed as described above. The presence and amount of sugars was determined as described above. This was replicated five times for each nutrient regime. For quantification, a calibration curve was prepared by derivatisation and analysis of 0.01, 0.1, 1, 10 and 100 µg of synthetic glucose, fructose, rhamnose, mannose, sucrose and maltose and analysing them on GC/MS as described above. The absolute peak areas were used to generate external calibration curves.

To detect plant amino acid content, 3 mg of each dry plant part described above was weighed, ground to fine powder and transferred to reaction vials. The samples were then hydrolysed with 6 M HCl for three hours as described by Moran-Palacio *et al* (70) with a slight modification. The product was re-suspended in 200 µl mmol 1-1 EDTA solution, pH 7.5. The samples were incubated for 90 min in the dark in a sealed chamber equilibrated at 25°C with a dish of saturated KH_2PO_4 to maintain high humidity. The EDTA samples were subsequently diluted in water - acetonitrile solvent mixture in a ratio of 80:20 and analysed on a liquid chromatography attached to 6120 quadrupole mass spectrometer Agilent Technologies. The mass spectrometer was operated in positive ion mode, with a capillary voltage of 3 KV, con voltage 50-180 eV, mass range 50-300 *m/z*. The source temperature was 130^{o}C, desolvation temperature 350^{o}C, desolvation gas flow 100 ml/min (nitrogen) and con gas flow 0.7 ml/min (nitrogen). Samples were injected via Agilent Technology 1260 Infinity series sample manager, injecting 10 µl on to Agilent SB-C18 3.5 µm 4.6 x 250 mm column. The run time was 22 min at a flow rate of 0.7 mL/min. The solvent system consisted of A (water + 1% formic acid) and B (acetonitrile + 1% formic acid). The mobile phase used a gradient program, initially 95:5 (A: B), to 70:30 at 3 min, 20:80 at 7.5 min, 0:100 at 13 min, 95:5 at 20 min. Three replicate samples from different plants of each host plant species were analysed. Similarly, the extracted mid-gut samples were dissolved in 200 µl mmol l–1 EDTA solution, pH 7.5 and incubated as described above. The resultant product was then diluted in 100 µl of the 80:20 water: acetonitrile solvent mixture and centrifuged at 1300 r.p.m. The supernatant was then analysed on LC-MS as described above. Five replicates each comprising three mosquitoes pooled were obtained from each nutrient regime. The amino acids were identified by comparing their mass spectra with those described by Özcan and Şenyuva (71).

Data analysis

The difference in survival times of adult *Ae. aegypti* on different nutrient regimes was detected using Kaplan-Meier and Cox regression survival analyses. Mosquitoes sampled for nutrient analyses and those surviving after the 30-days observation period were treated as censored. The differences in hatching rate and sex ratios from different nutrient sources were compared using Chi-square Goodness of Fit test. To compare the sugar content of different plants, eight most abundant sugars in each plant species was then extracted and retrieved from the rest of plant species, yielding a total of 55 different sugars. The absolute areas of these sugars were then measured and converted into a percentage of the total sugar content of each plant. These percentages were then subjected to Principal Component Analysis (PCA) to determine which ones are important in explaining the variation in sugar content of five plant species. The difference in amount of sugar ingested from different nutrient sources was detected using Kruskal-Wallis test and two sample Wilcoxon test. The mid-gut amino acid content was quantified for the different nutrient sources and the differences detected using one-way analysis of variance. All statistical analyses were done in R 2.15.1 software (72).

Author's contribution

Conceived and designed the experiments: VON DPT BT. Performed the experiments: VON DPT. Analysed the data: VON DPT CLS CWWP BT. Supervised the work: DPT CMM CLS CWWP BT. Wrote the paper: VON DPT CMM CLS CWWP BT. All authors approved the final version for submission.

Conflict interest

The authors declare no conflict of interest.

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Supplementary information

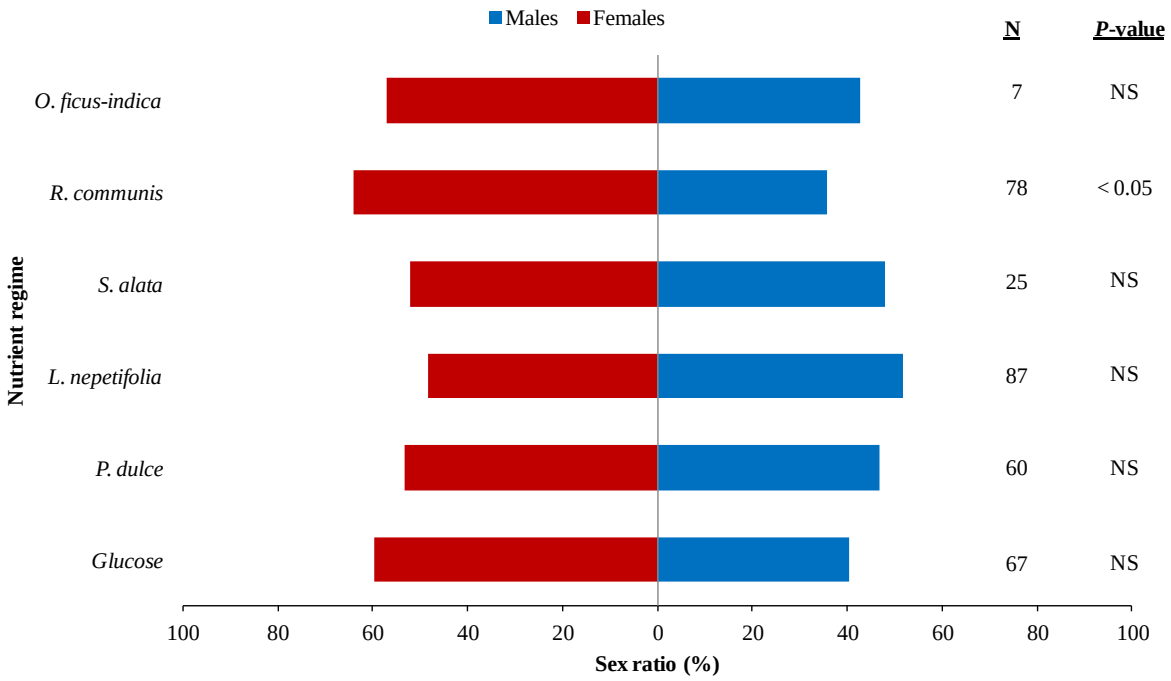

Figure S1 Influence of adult *Aedes aegypti* nutrition on the sex ratio of the offspring. No statistical difference was detected between different nutrient regimes. N = 3. Differences in proportion of males and females were detected using $\chi 2$ goodness of fit at 95 % confidence interval.

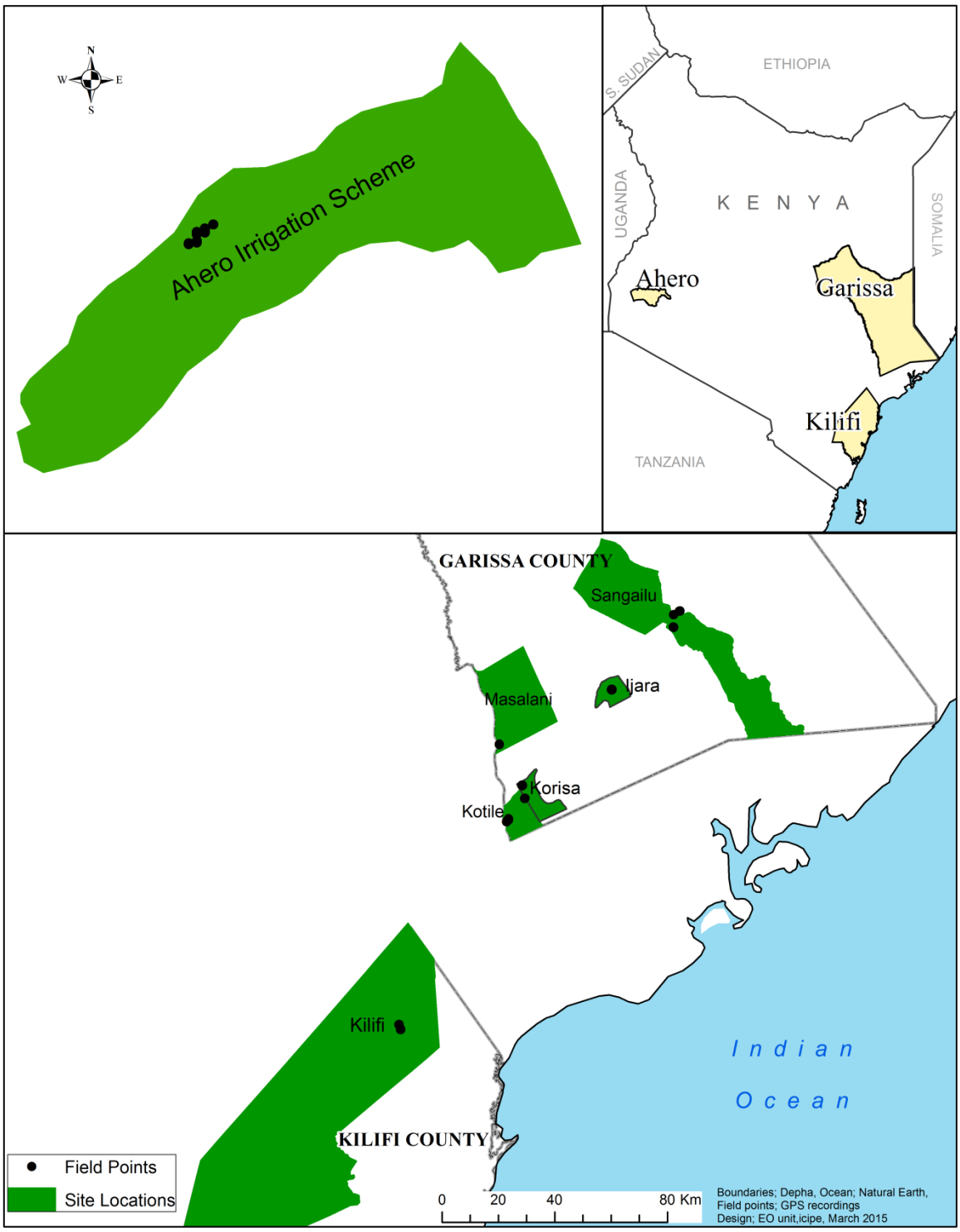

Figure S2 Map of Kenya showing study sites where samples were collected. The map was created by Jackson Kimani (*icipe*) using ArcGIS for Desktop 10.2. (http://arcview-gis.software.informer.com/10.2/).

CHAPTER FOUR

Patterns of host plant odour detection by Afrotropical mosquito disease vectors and their field evaluation

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Abstract

Chemical cues are known to play an important role in the location and acceptance of plants as sources of nourishment by a number of insect species. However, the chemical cues that mediate the interactions of mosquito species with their natural host plants have scarcely been studied. This study sought to develop a potent plant-based lure by exploiting chemical cues utilised by different Afrotropical mosquito species to locate their preferred natural host plants. The headspace volatile organic compounds (VOCs) of five plant species previously identified as host plants of *Aedes aegypti* (*Pithecellobium dulce*), *Anopheles gambiae* (*Leonotis nepetifolia, Senna alata* and *Ricinus communis*) and *Aedes ochraceus* and *Aedes mcintoshi* (*Opuntia ficus-indica*) were analysed using gas chromatography/mass spectrometry. Biologically-active VOCs for *An. gambiae* and *Ae. aegypti* from their respective host plants were detected by gas chromatography/electroantenography (GC/EAD). Variation in volatile constituents among the plants was detected using Principal Component Analysis (PCA). The results show significant variation in the VOC profiles of three of the host plant species with *P. dulce*, *L. nepetifolia* and *R. communis* having distinct classes of volatiles. Linalool oxide, previously shown to be attractive to the four mosquito species, was dominant in the headspace volatiles of *P. dulce* but only present in trace amounts in the volatiles of the other plant species. In terms of odour perception, both *An. gambiae* and *Ae. aegypti* detected variable VOCs from their respective host plants. Chemical convergence in detection of hexanal, ocimene, linalool oxide decanal and hexenol isomers were observed for both species of mosquitoes. EAG puffs using *Ae. aegypti* confirmed dose response to (*E*)-2-hexenol, (*Z*)-3-hexenol, ocimene, linalool oxide and decanal with variable antennal depolarisation. Field evaluation with the five EAG-active compounds revealed dose-dependent attraction and potential masking effect between some of the compounds. A three-component plant-based lure was developed and found to be highly attractive to both male and

female of *Ae. aegypti* in field evaluations. The study highlights the qualitative and quantitative basis of chemical interaction of Afrotropical mosquito species and their natural host plants. These findings reflect the complex pattern of host plant odour recognition by disease vectors, indicating that careful understanding of these interactions and their behavioural effect can propel the development of potent plant-based lure against these vectors.

Key words: *Anopheles gambiae*, *Aedes aegypti*, *Aedes mcintoshi*, *Aedes ochraceus*, EAG puffs, plant volatiles, plant feeding, mosquito-plant interactions, plant-based lures.

Introduction

Chemical cues have been shown to play a major role in the location of vital resources by insects, more so among nocturnal species such as is the case for a number of mosquito species (Takken 1991, Foster 1995, Zwiebel and Takken 2004, Balkenius et al. 2006). The role of chemical cues in the location of vertebrate hosts for blood meals by disease vectoring mosquitoes has been extensively studied (Syed 2015). Some of the well studied olfactory systems in relation to blood feeding behaviour are those of *Anopheles gambiae* s.s. and *Aedes aegypti*, from peripheral perception of human skin and breath odours to the genetic coding of the olfactory receptors involved (Acree et al. 1968, Carey et al. 2010). Today, both narrowly- and broadly-tuned receptors for human odours in both of these highly anthropophilic species are well characterised (Carey et al. 2010), and several lures based on chemical compounds mediating human/animal-vector interactions have been formulated for their management (Bernier et al. 1999, Bernier et al. 2000, Syed and Leal 2009, Okumu et al. 2010, Mukabana et al. 2012, Tchouassi et al. 2013, Owino et al. 2014). In addition, effort has been made to identify chemical cues that mediate oviposition site selection in a number of *Culex* species as well as *An. gambiae* s.l. (Millar et al. 1992, Lindh et al. 2008, Lindh et al. 2015) and *Aedes aegypti* (Ponnusamy et al. 2008). One area which, until recently, has received little attention is the olfactory mediation of the nectar feeding behaviour of mosquito disease vectors. With the exception of a few studies on chemical cues mediating the interactions of the malaria vectors *An. gambiae* s.s. (Nyasembe et al. 2012, Nyasembe et al. 2014), *Ae. aegypti* (Jhumur et al. 2007, Oppen et al. 2015) and *Culex pipiens* (Jhumur et al. 2007, Otienoburu et al. 2012) with their potential host plants, scant information exists on the chemical cues mediating interactions of most disease transmitting mosquitoes with their natural host plants. With the promise of odour bait technology playing a critical role in the management of haematophagous insects (Kline 2007), a multifaceted approach taking into account the entire

chemical bouquet that inform mating, blood feeding, oviposition and plant feeding behaviours is necessary.

Previously, the plant species fed upon by malaria vectors *An. gambiae*, dengue vectors *Ae. aegypti* and Rift Valley fever vectors *Aedes mcintoshi* and *Aedes ochraceus* in their natural habitats were identified using DNA barcoding (Chapter three). This presented a significant milestone in harnessing this aspect of vector ecology for novel control strategies. It has been suggested that the choice of either plant or vertebrate host by female mosquitoes for feeding is dependent on the physiological need, time and the availability of either of the hosts (Foster 1995). In addition, as is the case with many insects, both female and male mosquitoes need to assess the quality of their nutrient sources which is vital for their own fitness and the viability of their progeny (Soberon 1986). In an ecosystem with thousands of plant species, this assessment becomes confounded by a number of factors; key among them the similarity of classes of compounds released by different plant species and a large array of volatile organic compounds released by plants even within a single species (Bruce et al. 2005). In this complex set up, it is expected that mosquitoes have evolved olfactory discriminatory ability to isolate suitable from non-suitable resources (Bruce et al. 2005, Nyasembe and Torto 2014). However, it is not known whether different mosquito disease vectors discriminate their host plants based on specific unique compounds or the amounts of certain common compounds present in their volatiles.

In previous studies (Nyasembe et al. 2012, Nyasembe et al. 2014), a plant based compound, (*E*)-Linalool oxide, from an *An. gambiae* s.s. potential host plant was identified and shown to be highly attractive to the females of this species. This compound was later confirmed to be attractive to both male and female *Ae. aegypti* as well as female *Ae. mcintoshi* and *Ae. ochraceus* (Nyasembe et al. 2015; Chapter two). However, this compound was not attractive

to male *Ae. ochraceus* and *Ae. mcintoshi*, and even both sexes of the later species in the absence of carbon dioxide. The current study sought to develop a more attractive lure by exploiting chemical cues utilised by different mosquito species to locate their preferred natural host plants. The study was guided by three questions; 1) Do natural host plants for different mosquito species produce unique volatile profiles? 2) Do different mosquito species use unique chemical cues to locate their preferred host plants? 3) Can these chemical cues be exploited to develop a potent plant-based lure for these mosquito disease vectors?

Materials and methods

Headspace volatile profiles of natural mosquito host plants

Five of the plant species identified in the previous chapter as natural host plants for *An. gambiae, Ae. aegypti*, *Ae. mcintoshi* and *Ae. ochraceus* were evaluated for unique volatile organic constituents. The five plants included *Pithecellobium dulce* (Fabaceae), *Leonotis nepetifolia* (Lamiaceae), *Senna alata* (Fabaceae), *Ricinus communis* (Euphorbiaceae) and *Opuntia ficus-indica* (Cactaceae). This was done by collecting the headspace volatiles from these plants *in situ* at their natural habitats using portable field pumps (Analytical Research System, Gainesville, Florida, USA). The aerial part of an intact plant was gently enclosed in an air-tight oven bag (Reynolds^{TM}, Richmond, VA, USA) and charcoal filtered air passed over the plant at a flow rate of 350 ml/min into adsorbent Super-Q traps (30 mg, Analytical Research System, Gainesville, Florida, USA) (Fig. 1). The aerial plant parts enclosed in the oven bags included leaves, flowers and pods of *P. dulce*, leaves and flowers of *L. nepetifolia* and *S. alata*, leaves of *R. communis* and leaves, flowers and fruits of *O. ficus-indica*, characteristic of each of the plant species. For all plant species, volatiles were collected for 12

hr during the day and 12 hr at night and replicated three times using different plants for each type. The Super-Q traps were eluted with 200 µl GC/GC-MS-grade dichloromethane (DCM) (Burdick and Jackson, Muskegon, Michigan, USA) and the eluent stored at -80 °C until analysis.

Figure 1 Headspace volatile collection set-up comprising portable pump, Teflon tubing channelling airflow in and out of the oven bag which encloses the aerial part of *Pithecellobium dulce*. The Super-Q trap was connected to the Teflon tubing leading air out of the bag into the vacuum pump (Photo by Vincent O. Nyasembe).

For quantification and identification of the constituent compounds of the plant volatiles, an aliquot (1 µl) of each sample was injected into gas chromatograph (Agilent technologies-7890) coupled to inert XL EI/CI mass spectrophotometer (5975C, EI, 70eV, Agilent, Palo Alto, Califonia, USA) (GC/MS) in a splitless injection mode. The GC was equipped with an

HP-5 column (30 m x 0.25 mm ID x 0.25 µm film thickness, Agilent, Palo Alto, California, USA), with helium as the carrier gas at a flow rate of 1.2 ml/min. The oven temperature was held at 35 °C for 5 min, then programmed to increase at 10 °C/min to 280 °C and maintained at this temperature for 10 min. The volatile organic constituents were identified by comparing their mass spectra with library data at > 95 similarity (Adams2.L, Chemecol.L and NIST05a.L) and with those of authentic standards where possible (see sources and purity under chemical section below). The absolute areas of each constituent as calculated by the NIST05a.L software was used to estimate their amounts using an external calibration equation generated from known amounts of authentic compounds.

Electrophysiologically-active volatile constituents of mosquito natural host plants

To identify electrophysiologically-active (EAG-active) compounds mediating the interactions between mosquitoes and their host plants, wild caught adult *An. gambiae* s.l. collected from Ahero irrigation scheme in western Kenya (malaria-endemic area) and *Ae. aegypti* collected from Kilifi in the coastal Kenya (dengue-endemic area) were transported to the International Centre of Insect Physiology and Ecology (*icipe*) laboratories in Nairobi under high containment level and used in electrophysiological assays. *Aedes mcintoshi* and *Ae. ochraceus* were not included in these assays due to unprecedented insecurity in north eastern Kenya which rendered the region inaccessible. In addition, *An. gambiae* s.s. antennal responses to *R. communis* headspace volatiles had been tested in our previous study (Nyasembe et al. 2012), hence antennal responses to the volatiles of this plant was not repeated in this study. Live mosquitoes were sampled using BG sentinel traps baited with carbon dioxide and either BG lure or linalool oxide as described in Nyasembe et al. (2015) and chapter two. Briefly, the mosquitoes were sampled from Kilifi and Ahero field sites using

linalool oxide and BioGent (BG) lures baited with carbon dioxide. BG sentinel traps were used in both sites to trap adult mosquitoes with CO_2 dispensed from igloos thermos container (2 L; John W Hock, Gainesville, FL, USA) as described in Nyasembe et al. (2015) and chapter two. *Aedes aegypti* were trapped both during the day and night while *An. gambiae* were trapped during the night. In both sites, traps were setup outdoors near oviposition sites and vegetation. Trapped mosquitoes were then aspirated into a 30 x 30 x 30 cm cages and provided with 10% glucose solution soaked on cotton wool during transportation. The tops of the cages were covered with a moist towel to maintain high humidity. Once at *icipe*, the mosquitoes were kept in a high containment animal rearing unit at a temperature of 27-31^{o}C and average humidity of 80%. Mosquitoes used for electrophysiological assays were starved for 2 hr before experimentation. Since no male *An. gambiae* were caught in the field using our trapping methods, only females were tested for antennal responses against *L. nepetifolia*, and *S. alata* volatiles. On the other hand, both male and female *Ae. agypti* were tested for antennal responses against *P. dulce* volatiles.

The coupled gas chromatography/electro-antennographic (GC/EAD) analyses were performed as described by Nyasembe et al. (2012) using 5 µl of the remaining volatile samples. EAG activity was confirmed by male and female *Ae. aegypti* (as a model organism) antennal responses to synthetic standards of the identified compounds. Volatile components that elicited consistent antennal response in at least three EAG traces were considered for identification of active compound.

The activity of five of the tentatively identified compounds were confirmed by EAG puffs at different doses of 1, 2 and 4 µg/µl of the synthetic standards using female *Ae. aegypti*. The synthetic standards were dispensed on a filter paper (ca. 0.8 cm^2). The solvent was allowed to evaporate and the filter paper placed inside a Pasteur pipette and delivered on the antennae

through a stream of air at a flow of 100 mm/second for one second puffed using GC-EAG pedal. For each replicate (= one antennae) three puff treatments comprising the three doses in increasing order were delivered 30 seconds apart with a total of nine replicates for each synthetic standard. A blank of solvent (hexane) was insuflated before and after every puff treatment. The net EAG depolarisation due to the synthetic standard was obtained by subtracting EAG responses for each dose from the average of before and after EAG depolarisation to the solvent for each replicate.

Formulation and field evaluation of plant-based attractive blends for *Aedes aegypti*

Five synthetic compounds with confirmed EAG-activity were further evaluated for their attractiveness to *Ae. aegypti* in a three-step field trial. These included linalool oxide, (*E*)-2-hexenol, (*Z*)-3-hexenol, decanal and ocimene mixture ((*Z*)-β-ocimene = 27%, (*E*)-β-ocimene = 67% and allo-ocimene = 6%, hereafter referred to as ocimene) (See the chemical section for the sources and purity). Field trials were carried out at the Kilifi field site described in details in Nyasembe et al. (2015) using BioGent sentinel traps. All test compounds were individually dispensed in rubber septa (7 mm, Aldrich, Milwaukee, WI, USA) which were replaced every two days.

Firstly, the attractiveness of the compounds was tested in a dose response experiment at three different concentrations of 20 ng/µl, 200 ng/µl and 2000 ng/µl. The compounds were tested in two groups comprising (*E*)-2-hexenol, (*Z*)-3-hexenol and decanal (Blend A), and linalool oxide and ocimene (Blend B) in two separate sites located 2 km apart within Kilifi town. The components of each blend were dispensed separately in a clean rubber septum. Each blend was tested in a 3 x 3 Latin Square study design with a total of six replicates (three days and three nights). The daytime trapping was carried out from 06:00 – 18:00 hr while the night

trapping was done from 18:00 – 06:00 hr. At each site, the traps were placed 40 m apart. The trap captures were emptied and counted at the end of each trapping.

In the second step, components of Blend A were then tested separately at the optimal dose determined above against the full blend. This was aimed at eliminating antagonistic effects. A 4 x 4 Latin Square study design was used with each treatment rotated through each of the pre-identified four points. eight replicates (four days and four nights) were collected.

Thirdly, linalool oxide, ocimene, and (*E*)-2-hexenol were evaluated at the optimal dose in additive assay to optimise blend performance. This resulted in three blends *viz* linalool oxide (LO), linalool oxide and ocimene (Blend B) and linalool oxide, ocimene and (*E*)-2-hexenol (Blend C). These were tested alongside commercial BioGent (BG) lure and solvent as controls. All lures were baited with carbon dioxide dispensed in igloos thermos container (2 L; John W Hock, Gainesville, FL, USA) as described in Nyasembe et al. (2015). Fourteen replicates (seven days and seven nights) were collected.

Chemicals used

The synthetic standards of the following EAG-active compounds were used: (*Z*)-3-hexenol (Aldrich, 98%), (*E*)-2-hexenol (Aldrich, 96%), ocimene (Chemika, (Z)-β-ocimene = 27%, (E)-β-ocimene = 67% and allo-ocimene = 6%), linalool oxide (Aldrich, mixture of stereoisomers with furanoid form, 99.5 % and 0.5 % pyranoid form), carbon dioxide (Carbacid Ltd, Kenya, as dry ice) and commercial BioGent (BG) lure (a 3-component blend comprising ammonia, lactic acid and caproic acid developed for *Aedes aegypti*).

Statistical analysis

To determine if there was any significant difference in the volatile profiles of these five plant species, ten most abundant volatile constituents in each plant species were selected. Attempts were then made to retrieve each of these compounds from the VOCs analysed for the rest of the plant species, yielding a total of 26 different compounds. The absolute areas of these compounds were then measured and converted into a percentage of the total. These percentages were then subjected to Principal Component Analysis (PCA) to determine which ones if any are important in explaining the variation in the odour profiles of the five plant species. Difference in antennal response to different doses of a given synthetic standard was detected using ANOVA and Tukey post hoc test. To determine the effectiveness of the different lures in trapping disease vectors, the numbers of mosquitoes per treatment were first fitted with a general linear model (GLM) with Poisson distribution and then negative-binomial error structure and log link in case of over dispersion as described by White and Bennetts (1996). All statistical analyses were carried out at 95% confidence interval using R 2.15.1 software (R Development Core Team 2010).

Results

Patterns of host plant odour profiles for different Afrotropical mosquito species

The odour profiles of the five species of plants that have been identified as natural host plants of malaria, dengue and RVF mosquito vectors were differentiated by unique chemical constituents of varying abundance (Fig. 2A). PCA resolved these chemical constituents into three clusters which accounted for more than 90% of the total variation (Fig. 2B). PC1 explained 38% of the variation; PC2 explained 32% while PC3 explained 22% of the

variation. PC1 was weighed positively by monoterpenoids and benzenoids, predominantly unique to *P. dulce*, while PC2 was positively contributed by sesquiterpenes which were characteristically abundant in *L. nepotifolia* (Fig. 2C). PC3 was positively characterised by monoterpenes which were the key constituents detected in the VOCs of *R. communis* (Fig. 2C). The headspace volatile constituents of *S. alata* and *O. ficus-indica* contained benzenoids (Fig. 2C). (*E*)-β-Ocimene was detected as a VOC in all the five plant species while (*Z*)-3-hexenol, α-pinene, n-nonanal, n-decanal and germacrene D were variably present in the volatiles of two or three plant species.

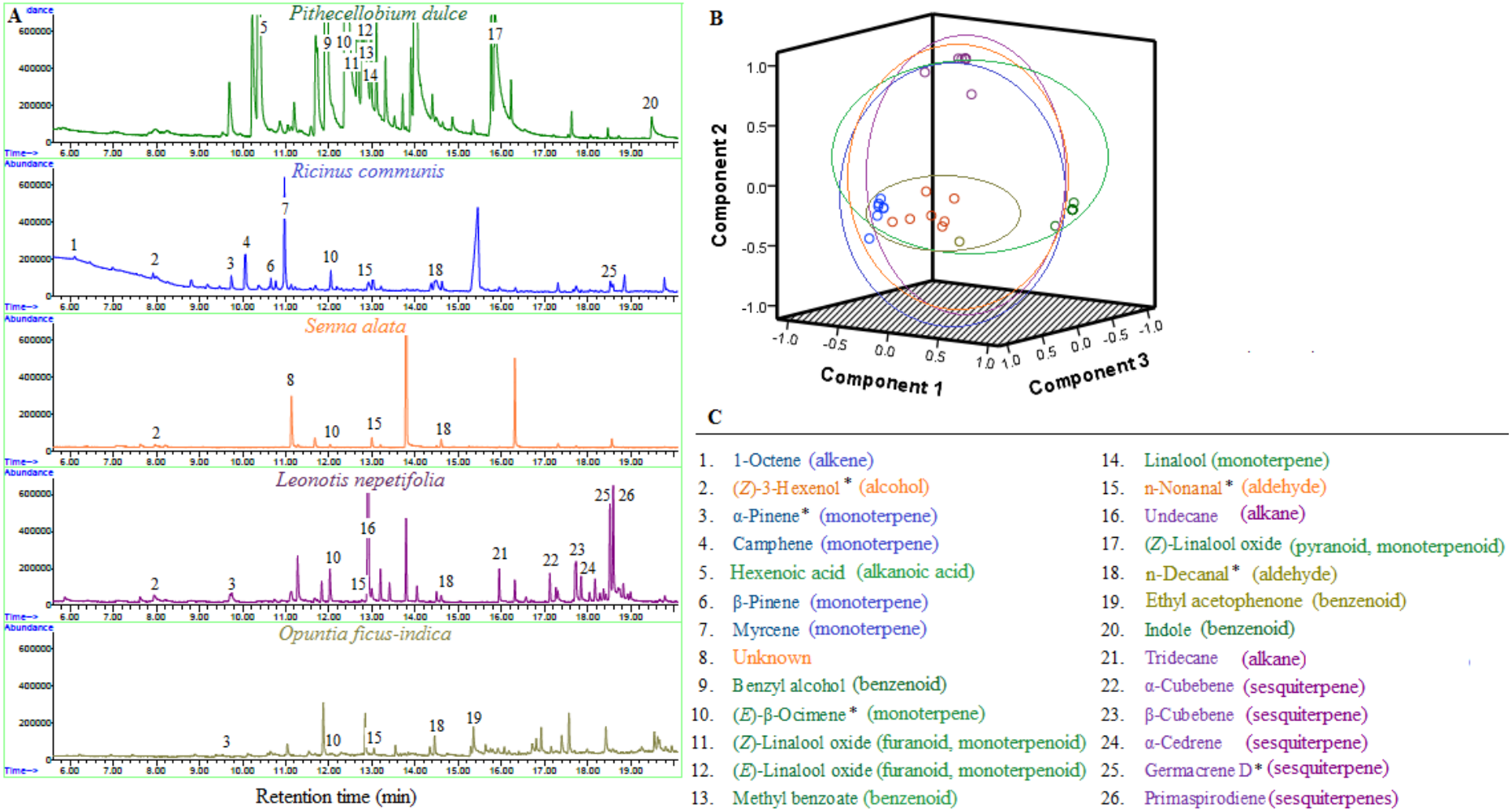

Figure 2 Variable chemical profiles of plant species used by different mosquito species as host plants. A) Representative profiles of headspace volatile chemical constituents of different plant species as measured by gas chromatography-mass spectrometry. B) Three

dimensional graphical representation of PCA which resolves the volatile profiles of the five plant species into three distinct clusters. PCA1 = 38%, PCA2 = 32% and PCA3 =22%. C) The chemical compounds contributing to significant variation in the headspace volatile profile of the five plant species. N = 6. The colour coding of the compound match the plant species in which the specific compound is uniquely abundant while those capped with asterisk are also present in the other plant species in varying amounts.

Afrotropical mosquito species detect variant volatile compounds in their host plants

The antennae of *Aedes aegypti* responded to 10 compounds in *P. dulce* headspace volatiles (Fig. 3) while *An. gambiae* s.l. detected 12 and 10 compounds in the volatiles of *L. nepetifolia* and *S. alata*, respectively (Fig. 4 and 5). Notable was the fact that both *Ae. aegypti* and *An. gambiae* detected hexanal, (*E*)-hexenol, (*E*)-β-ocimene, (*E*)-linalool oxide and decanal with *An. gambiae* also detecting allo-ocimene Both male and female *Ae. aegypti* detected (*E*)-β-ocimene.

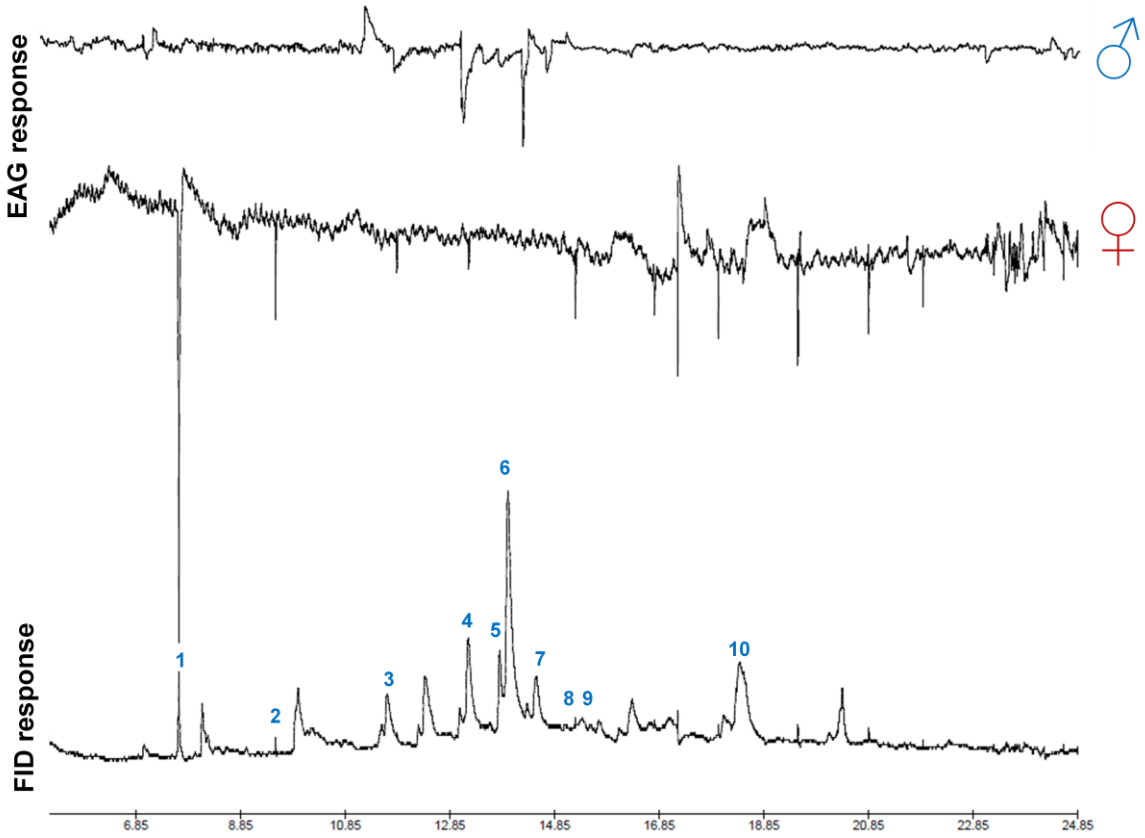

Figure 3 Specific antennal responses of male (♂) and female (♀) *Aedes aegypti* to *Pithecellobium dulce* volatile constituents. The antennaly-active compounds include Hexanal (1), (*Z*)-3-Hexenol (2), Benzaldehyde (3), , (*E*)-β-Ocimene (4), (*Z*)-Linalool oxide (furanoid) (5), (*E*)-Linalool oxide (furanoid) (6), (*E*)-Linalool oxide (Pyranoid) (7), unknown (8), Decanal (9), (*E*)-Caryophellene (10).

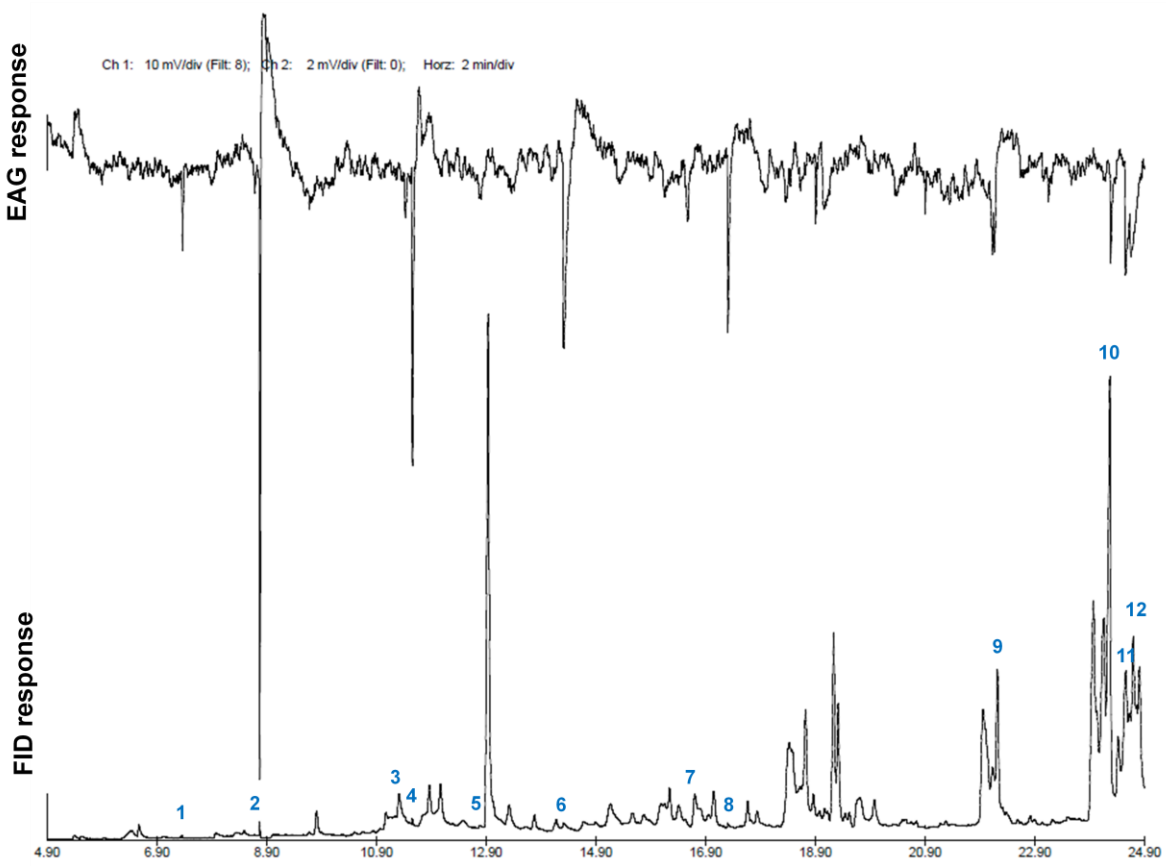

Figure 4 *Anopheles gambiae* s.l. antennal responses to headspace volatile constituents of *Leonotis nepetifolia*. The EAG active volatile constituents include Hexanal (1), (*E*)-2-Hexenol (2), (E)-3-Hexenyl acetate (3), unknown (4), (*E*)-β-Ocimene (5), (*E*)-Linalool oxide (6), allo-Ocimene (7), β-Cedrene (8), (*E*)-β-Caryophellene (9), α-Humulene (10), γ-Elemene (11) and Aromadendrene (12).

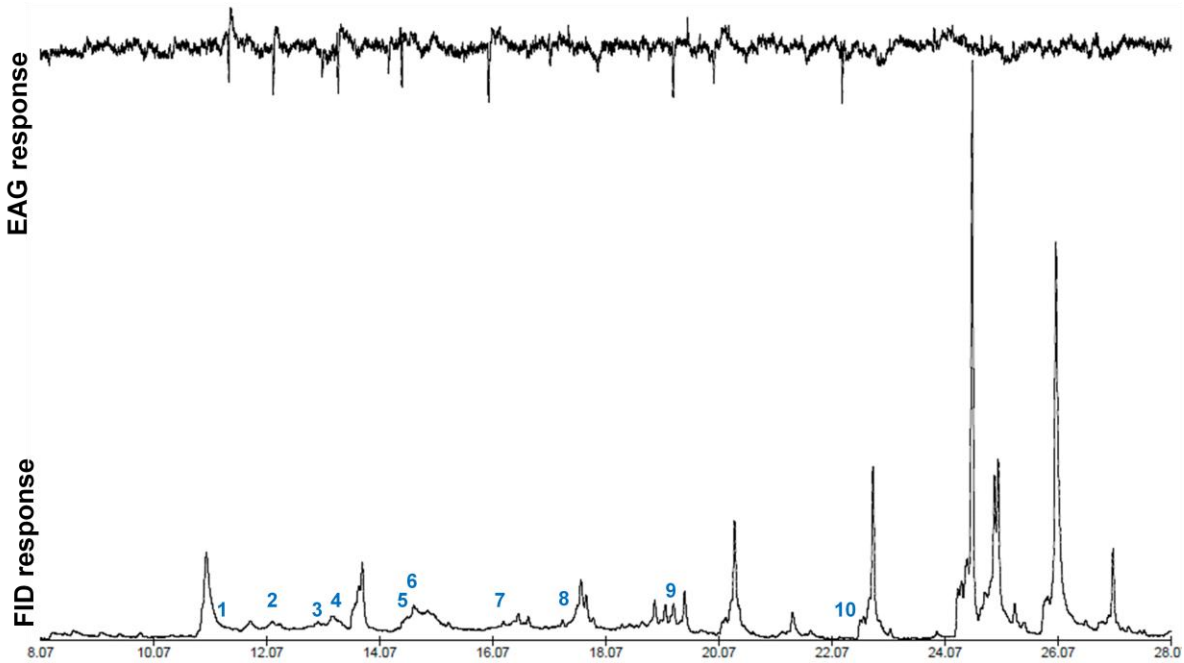

Figure 5 *Anopheles gambiae* s.l. antennal responses to headspace volatile constituents of *Senna alata*. The EAG active volatile constituents include unknown (1), (*E*)-β-ocimene (2), (*Z*)-Linalool oxide (3), (*E*)-Linalool oxide (4), unknown (5), n-Decanal (6), β-Cedrene (7), 1-Hexadecene (8) 1-Docosene (9) and unknown (10).

Variable dose response of female *Ae. aegypti* to different doses of five of the EAG-active compounds was recorded using EAG puffs. Significant dose response was detected for linalool oxide ($F_{(2, 24)} = 5.40$, $P < 0.05$) and (*E*)-β-ocimene ($F_{(2, 24)} = 4.25$, $P < 0.05$), but not for (*E*)-2-hexenol, (*Z*)-3-hexenol and decanal (Fig. 6).

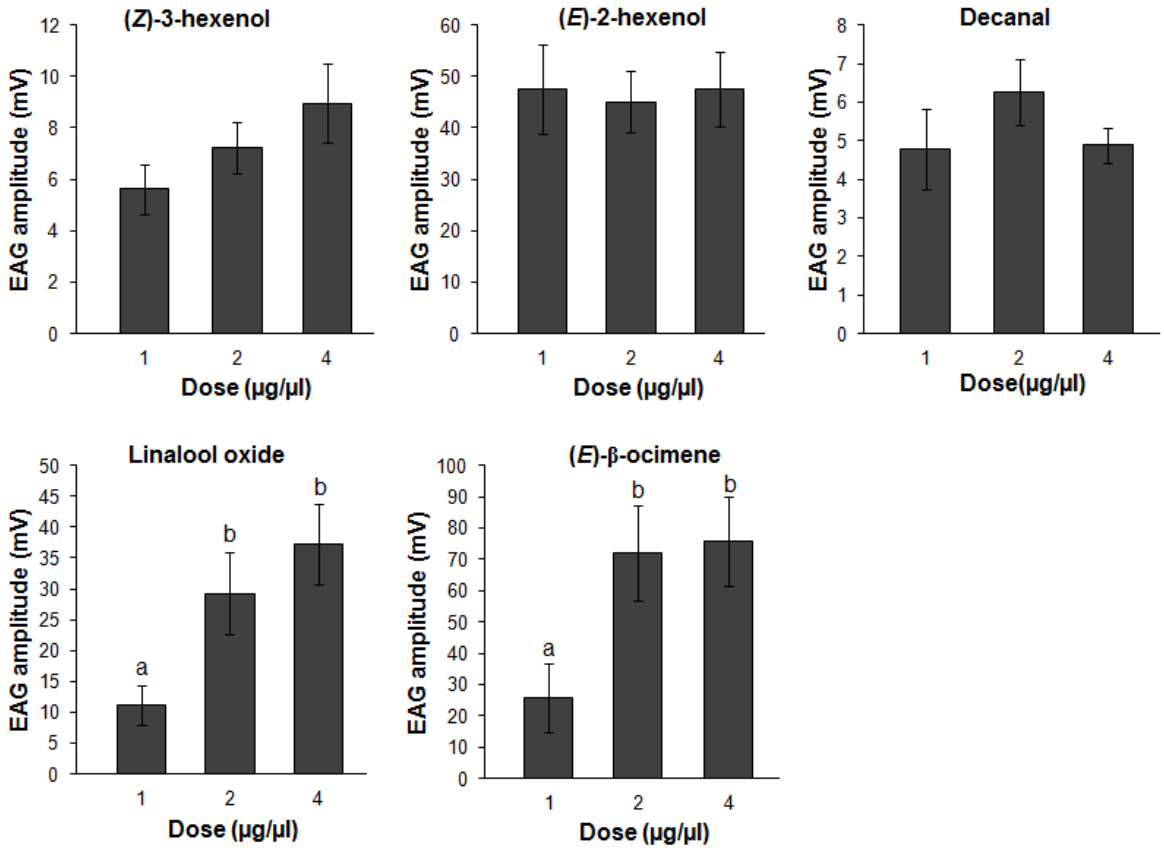

Figure 6 Average female *Aedes aegypti* electroantennograph responses to different doses of five synthetic standards. The EAG response for each replicate and dose was obtained by subtracting them from response to the solvent (hexane). A total of three puffs per antennae with at least 3 different female *Ae. aegypti*. Differences in EAG responses to different doses of each standard was detected by ANOVA and means separated by Tukey test at 95 % confidence.

Field evaluation of antennally-active host plant volatile compounds

Differential attraction of EAG-active compounds to both male and female *Ae. aegypti* was observed in field evaluations. In the first experiment, Blend A had a marginally significant dose response for female *Ae. aegypti* (χ^2 = 5.9, df = 2, 18, P = 0.05; Fig 6A) while Blend B had a significant dose response for males *Ae. aegypti* (χ^2 = 10.9, df = 2, 18, P < 0.01, Fig. 6B). In both blends, 20 ng/µl was the most attractive dose except Blend B against male *Ae. aegypti* (Fig. 7A and B).

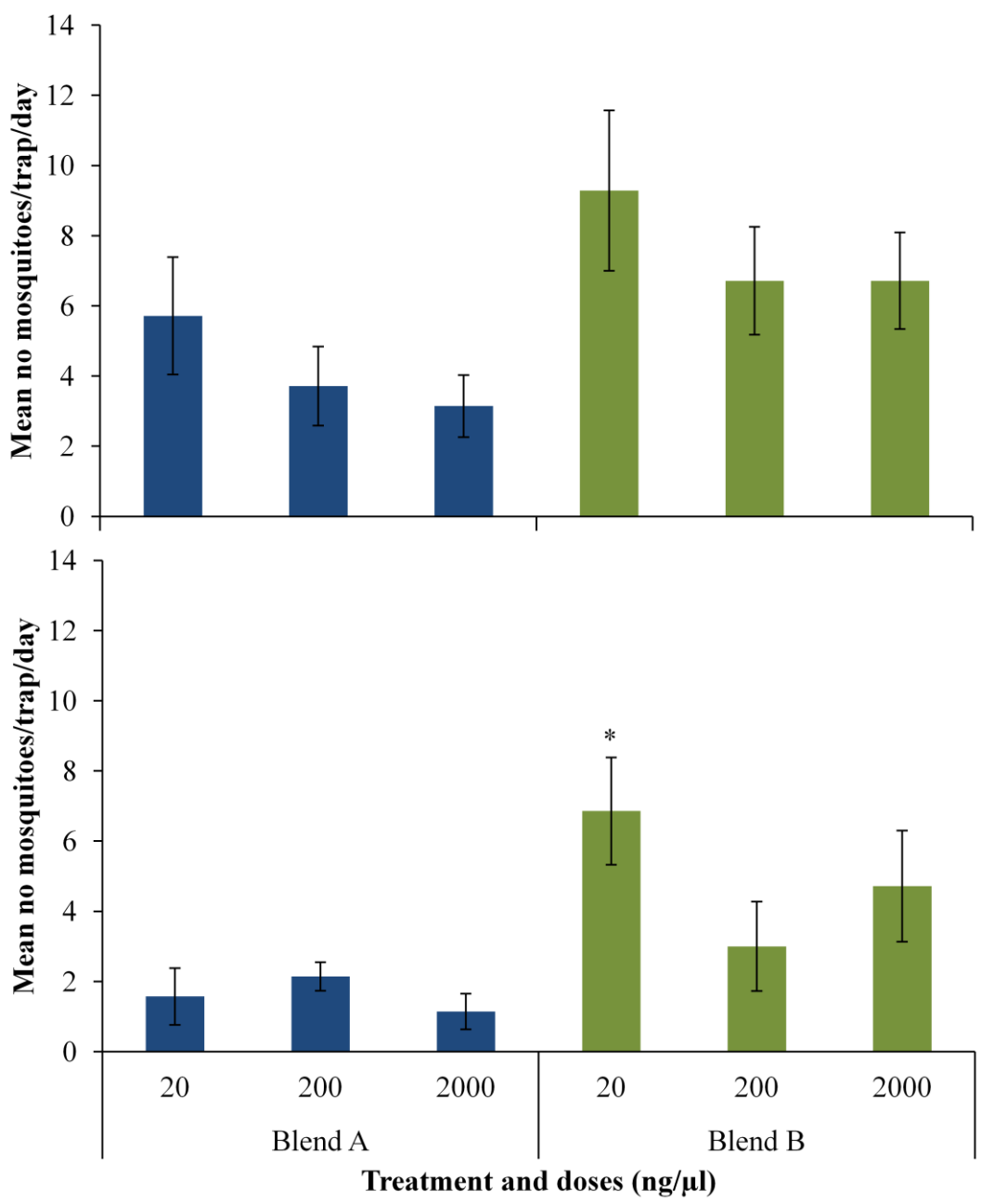

Figure 7 *Aedes aegypti* dose response to different classes of synthetic standards in field trials. A) Mean female capture ± SEM, B) Mean male capture ± SEM. Blend A = (*E*)-2-hexenol + (*Z*)-3-hexenol + decanal, and Blend B = linalool oxide + ocimene. N = 6. Bars capped with asterisk are significantly different from Blend A. · = $P < 0.1$, * = $P < 0.05$.

In the second experiment, (*E*)-2-hexenol was 2-fold more attractive than blend B for both females ($P < 0.05$; Fig 8A) and males ($P < 0.01$; Fig 8B) *Ae. aegypti*. All the other

individual components of Blend B were as good as the full blend in trapping both sexes of *Ae. aegypti* (Fig 8A and B).

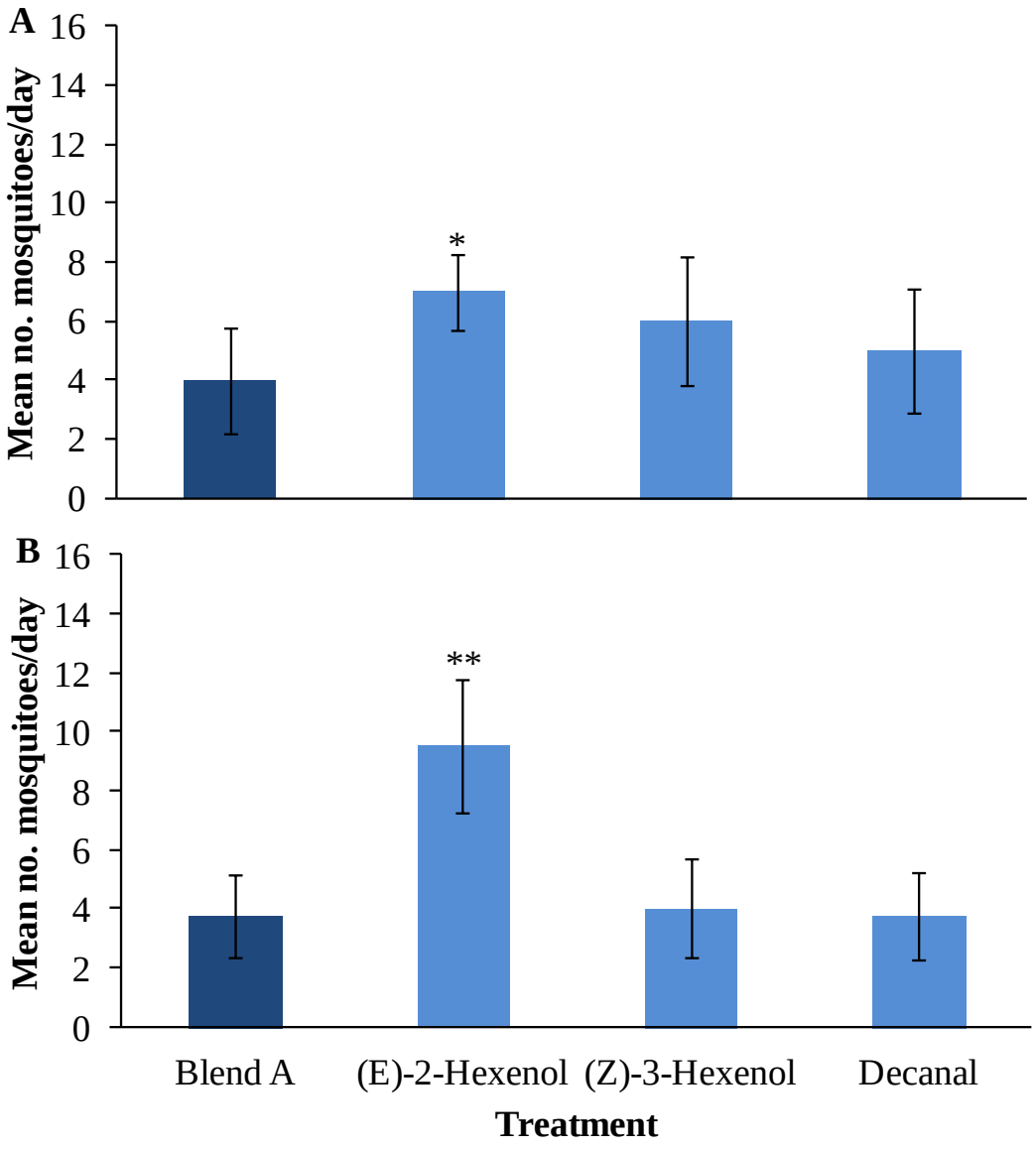

Figure 8 *Aedes aegypti* field responses to Blend A and its individual components. A) Mean female capture ± SEM and B) Mean male capture ± SEM. N = 8. Bars capped with asterisk are significantly different from Blend A. * = $P < 0.05$, ** = $P < 0.01$.

In the third experiment, the addition of ocimene to linalool oxide (Blend B) did not have any effect but the addition of a (*E*)-2-hexenol to make a three-component blend (Blend C) increased the trap capture of both male and female *Ae. aegypti* by about 2-fold (Fig 9A and

B). Overall, significant differences in trap captures of both female and male *Ae. aegypti* was detected (χ^2 = 65.9, df = 4, 60, $P < 0.001$ and χ^2 = 305.1, df = 4, 60, $P < 0.001$, respectively). Blend C had 1.7-fold higher female *Ae. aegypti* capture than the control ($P < 0.001$) while LO and Blend C had 1.3- and 3.3-fold higher male *Ae. aegypti* capture than the control ($P < 0.01$, $P < 0.001$), respectively.

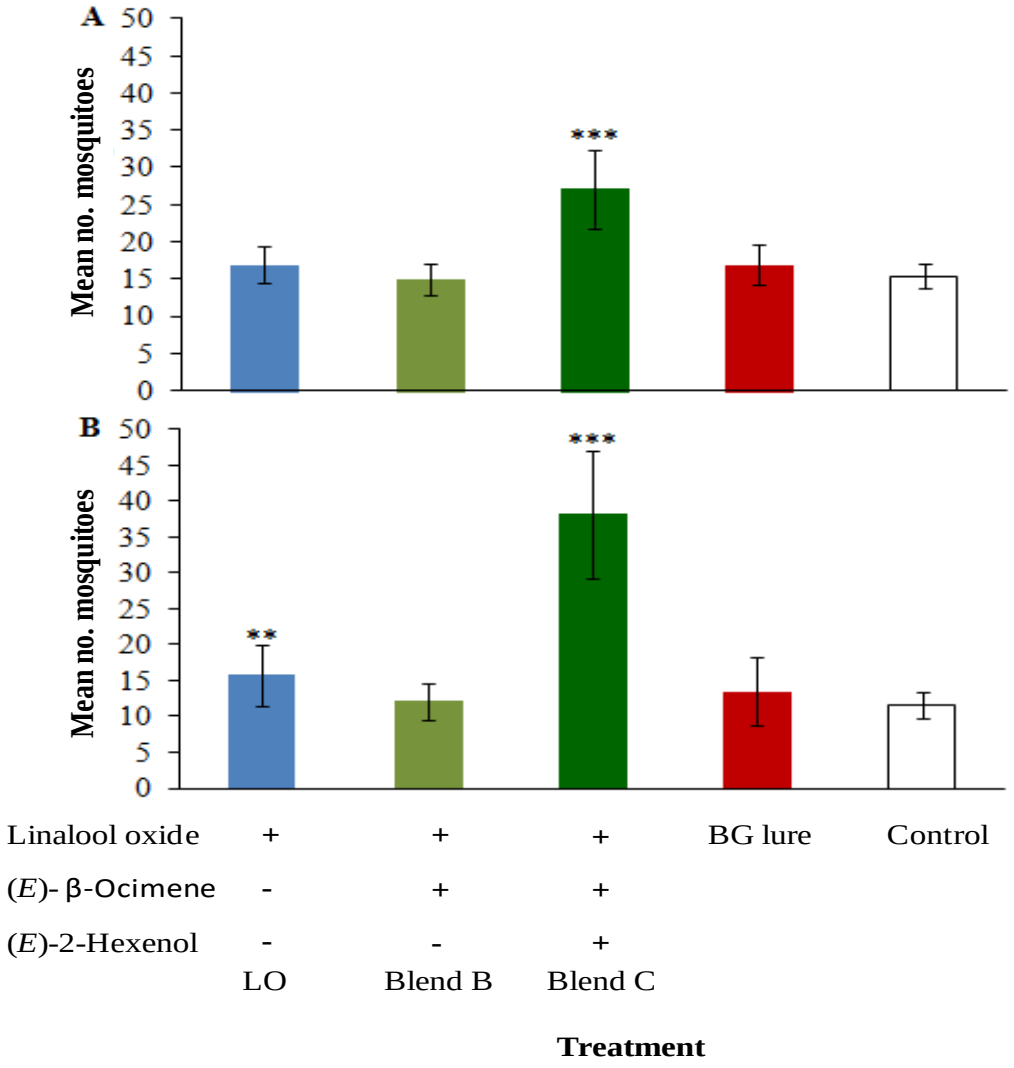

Figure 9 Additive effects of plant volatile compounds in field trapping of *Aedes aegypti*. A) Mean female capture ± SEM and B) Mean male capture ± SEM. All treatments were baited with synthetic CO_2 with control comprising solvent + CO_2. LO = linalool oxide, Blend B = linalool oxide + ocimene, and Blend C = linalool oxide, ocimene + (*E*)-2-hexenol. N = 14. Bars capped asterisk are significantly different from control. ** = $P < 0.01$, *** = $P < 0.001$.

Discussion

In addition to morphological traits, plants produce volatile organic compounds that attract insects including terpenoids, benzenoids/phenylpropanoids, fatty acid derivatives and amino acid derivatives (Pichersky et al. 2006, Dudareva et al. 2013). Some of these products are utilised by the plant for its own metabolic processes while others are used as chemo-attractants for beneficial insects or arsenals against harmful insects. The present study shows that the five plant species varied in their volatile profiles with *P. dulce* volatiles dominated by monoterpenoids and benzenoids, *L. nepetifolia* dominated by sequiterpenes while *R. communis* had a higher abundance of monoterpenes. *Senna alata* and *O. ficus-indica* volatiles were dominated by fatty acid derivatives as well and a few monoterpenes. All the plant species had considerable amount of (*E*)-β-ocimene as in their headspace volatiles. The qualitative variation in VOCs detected is not surprising since similar observations even within the same plant species from different geographical locations had been made before (Jhumur et al. 2008). Besides, it is probable that these plants utilise different metabolic pathways giving them the unique fragrance necessary for a competitive advantage in the event of scarcity of certain shared resources (Dudareva et al. 2013).

In such a complex environment permeated with many odour plumes from different plant species, plant feeding insects are expected to evolve mechanisms that allow them to discriminate biologically relevant chemical cues for their fitness (Visser 1986). This study reveals a range of specific compounds from different host plants which elicit antennal activity in two wild caught mosquito species. In *An. gambiae* s.l., n-decanal linalool oxide and two isomers of hexenol detected by the antennae were common across its two host plants while different isomers of hexanal, (*E*)-hexenol, (*E*)-β-ocimene, (*E*)-linalool oxide and decanal were detected by both *An. gambiae* and *Ae. aegypti* from their respective host

plants. In previous studies, hexanal, (*E*)-β-ocimene, (*E*)-linalool oxide and decanal were identified as some of the compounds eliciting antennal activity in laboratory reared *An. gambiae* s.s (Nyasembe et al. 2012). The fact that these compounds are present in the headspace volatiles of the three plant species used in this study, albeit in variable proportions and were detected by both mosquito species, suggests their significance as an odour signature in mosquito-plant interactions. (*Z*)-3-Hexenol and (*E*)-2-hexenol, were reported for the first time in this study as eliciting antennal response in mosquitoes. (*Z*)-3-Hexenol has been previously reported as a plant compound that was strongly detected by antennae of the codling moth *Cydia pomonella* (Ansebo et al. 2004). These findings are in tandem with co-evolutionary hypothesis (Thompson 1988), indicating that different mosquito species could have co-evolved with their preferred host plants by exploiting unique as well as common chemical signatures.

Whereas female *Ae. aegypti* detected up to eight different VOCs, males detected only two compounds, (*E*)-β-ocimene and (*E*)-linalool oxide. Male mosquitoes are known to feed entirely on plants while females feed on both plants and vertebrates. The specificity in odour perception by male *Ae. aegypti* could be an indication of adaptation to limited resources, with most receptors having atrophied over the evolutionary period as opposed to their female counterparts which are tuned to respond to a variety of stimuli. However, further characterisation of male *Ae. aegypti* odour receptors is necessary to find out if this is the case or if they use a different set of receptors to locate vital resources.

Interestingly, linalool oxide which was shown to be effective in trapping dengue and RVF vectors albeit with some limitations (Nyasembe et al. 2015), dominated the headspace volatiles of *P. dulce*. While this compound was only present in trace amounts in *Parthenium hysterophorus* and *R. communis* (Nyasembe et al. 2012), all the three isomers of this

compound were present in considerable amounts in *P. dulce* volatiles, the host plant for dengue vectors. However, this compound was absent in the identified host plants for RVF vectors, perhaps explaining why it performed dismally in the absence of CO_2 and against the males of these vectors in field evaluations (Nyasembe et al. 2015). These findings suggest possible adaptation by different mosquito species to fine-tune their olfactory reception to specific chemical compounds that dominate the headspace volatiles of their preferred host plant. However, since there was convergence in some of the chemical compounds detected, it is possible that there are certain key compounds that serve as odour signature for suitable mosquito host plants which merit additional investigation.

EAG puffs using female *Ae. aegypti* antennae revealed dose response (*Z*)-3-hexenol, linalool oxide and ocimene but not to (*E*)-2-hexenol and decanal. In addition, (*E*)-2-hexenol, (*E*)-linalool oxide and (*E*)-β-ocimene elicited strong antennal depolarisations. While dose response in antennal depolarisation to synthetic chemicals is reminiscent to those reported in a number of plant-seeking insects (Visser, 1979; Bruce et al., 2005; Bruce and Pickett, 2011), the difference in the level of depolarisation indicate that these five compounds are either perceived by different odour receptors and/or have different relevance in *Ae. aegyti* host plant seeking behaviour. These results further support the hypothesis of the use of specific chemical compounds by plant feeding insects as odour signature to detect host suitability.

This study further explored the potential of exploiting confirmed antennally-active compounds in developing more attractive plant-based lures for these disease vectors using wild *Ae. aegypti* under field conditions. The results of this study reveal two key insights into mosquito plant interactions. Firstly, the dose response shows that *Ae. aegypti* were optimally attracted to a lower concentration of 20 ng/µl compared to the higher doses for

both blends A and B, similar to the concentration detected in Nyasembe et al. (2015). Compared to human/animal-based lures (Mukabana et al., 2012, Owino et al. 2014), these are relatively low concentrations for field trials, indicating that mosquitoes could have adapted to detect very low concentrations of VOCs from their host plants. Evolutionarily, this could be a strategy to navigate confounding odours from non-host plants and to avoid being located by their natural enemies such as the spider *Evarcha culicivora* which also known to be responsive to plant volatiles (Cross and Jackson, 2009, Nelson and Jackson, 2013). Besides, a number of plant compounds including terpenes are known to be repellent to most mosquito species at high concentrations (Becerra, 1997, Tawatsin et al. 2001, Maia and Moore, 2011). Secondly, the finding that individual components of Blend A were more attractive than the full blend are indicative of possible antagonistic or masking effect of various plant compounds, similar to the findings in Nyasembe et al. (2014). Given that electrophysiological analysis shows that mosquitoes often detect specific VOCs which are often in trace amounts, these observations further lend support to the argument for qualitative and quantitative basis of evolutionary mosquito-plant interactions as is the case for a number of phytophagous insects (Visser, 1986).

Field trials also recorded an improved three component plant-based lure for *Ae. aegypti*. This lure was superior to the one component lure previously tested (Nyasembe et al. 2015) as well as commercial BG lure developed for this species. More interesting is the improved efficacy of this three component lure found against male *Ae. aegypti* in the field assays, a segment which has become increasingly important with the invention of male targeted control strategies (Harris et al. 2011). Together, these findings emphasise the potential of plant-based lures in the management of Afrotropical mosquito species. However, since only three of the identified EAG-active compounds were evaluated in the field, additional

behavioural and field studies are needed to confirm the role of the other compounds in host plant odours toward development of even more potent lures for mosquito disease vectors.

Conclusion

Overall, these findings confirm the significant role of chemical cues in mosquito-plant interactions and the need for more accurate identification of plants fed upon by various mosquito species in their natural habitats for insightful exploitation in the management of mosquito-borne diseases. The study indicates that both qualitative and quantitative value of plant VOCs are important with no clear specificity across different mosquito species. The study presents a significant step in the exploitation of plant feeding in the control of important Afrotropical mosquito species.

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Author contributions

Conceived and designed the experiments: VON DPT CLS CP BT. Performed the experiments: VON. Analysed the data: VON DPT BT. Wrote the paper: VON DPT CLS CP BT. All authors approved the final version for submission.

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CHAPTER FIVE

General conclusions and recommendation

Although the significance of plant-derived nutrients in the biology and fitness of disease vectoring mosquitoes has been known for long (Sandholm and Price 1962, Nayar and Sauerman 1971, Magnarelli 1977), interest in this aspect of vector ecology has only gained momentum in the last decade, with focus having been mainly on the blood feeding and oviposition aspects. The recent resurgence of vector-borne diseases such as dengue and chikungunya (Gubler 2002, Bhatt et al. 2013), the re-emergence of those that had hitherto been brought under control such as Zika virus (Musso and Gubler 2015) and the persistence of other endemic/epidemic ones such as malaria and Rift Valley fever (Sang et al. 2010, WHO 2014), which together puts more than half of the world population at risk of infection with more than a million deaths annually (WHO 2004, 2014), calls for new concerted effort to combat these diseases. Countries in sub-Saharan Africa bear the greatest burden of these diseases - driven by insecticide resistance, lack of vaccines and chemotherapy for some of these diseases, poor infrastructure and sanitation, lack of proper response strategies during outbreaks, urbanisation, climate change and political instability in some countries (Colwell et al. 2011, WHO 2014). The need for improved vector surveillance and control in tackling these diseases is of paramount importance. In attempting to comprehensively understand vector ecology this study provides unique opportunities for novel environmentally sound tools and control strategies. This study explored nectar feeding behaviour of important mosquito species that are implicated in the transmission of malaria (*An. gambiae* s.l.), dengue (*Ae. aegypti*) and Rift Valley fever (*Ae. mcintoshi* and *Ae. ochraceus*) in Kenya with the view of gaining insight into info-chemicals involved in their interactions with their preferred host plants.

In chapter two, the study sought to establish a baseline for plant-based lures in the surveillance of Rift Valley fever and dengue mosquito vectors. The effectiveness of (*E*)-Linalool oxide (LO), a single-component blend initially developed for the malaria vectors (Nyasembe et al. 2014a), in trapping *Ae. aegypti*, *Ae. mcintoshi* and *Ae. ochraceus* was evaluated. Human odour-based commercial BG lure served as positive control for *Ae. aegypti* while animal-based HONAD lure (Tchouassi et al. 2013), BG lure and lit CDC trap served as positive control for *Ae. mcintoshi* and *Ae. ochraceus*. The lures were tested in combination with or without CO_2 with traps baited with CO_2 alone serving as negative control in all trials. LO performed as well as the BG lure in trapping female *Ae. aegypti* but was superior to the commercial lure in trapping male *Ae. aegypti* whether baited with or without CO_2. On the other hand, it performed as well as BG lure and HONAD in trapping female *Ae. mcintoshi* and *Ae. ochraceus* but was outperformed by the lighted CDC trap in the presence of CO_2. LO along with the other lures tested performed dismally in trapping the RVF vectors in the absence of CO_2 and was not effective in trapping males, a portion of the mosquito population which is becoming increasingly important in evaluating male targeted control strategies. LO was equally effective in trapping females of the malaria vector *An. gambaie* and *An. funestus* in combination with or without CO_2 but ineffective in trapping males of these species (Nyasembe et al. 2014a). Plant-based lures have been touted to have the potential of not only targeting the blood seeking potion of a vector population but also an understudied portion of the population with varying physiological states and ages of both sexes (Foster 2008, Nyasembe and Torto 2014). This chapter highlights the potential of plant-based lures in the management of these vectors but also points to the need for further research to address the shortfalls.

To address the limitations of the single component blend, the hypothesis that identifying specific cues mediating interactions of disease vectoring mosquitoes with their preferred

natural host plants could improve the performance of plant-based lures was proposed. Chapter three sought to establish plant feeding among the RVF vectors for the first time and to identify the natural host plant species for malaria, dengue and RVF vectors. DNA barcoding employing a combination of three gene targets was used to identify the specific plants upon which these vectors feed. The study successfully identified seven plant species utilised by these vectors for nourishment. This approach of mosquito host plant identification is intriguing as it is the first time this approach is being used for Afrotropical mosquito species, with previous attempts having mainly relied on potential plants (Impoinvil et al. 2004, Manda et al. 2007b, Müller et al. 2010, Gu et al. 2011). The use of DNA barcoding to identify plant species upon which various phytophagous insect species feed was first demonstrated by Miller et al. (2006), and has gained interest in the recent past (Matheson et al. 2008, Junnila et al. 2010, Lima et al. 2016). One of the recent arsenals in tackling vector-borne diseases in the attractive toxic sugar bait technology (ATSB), which targets plant feeding and resting mosquitoes (Müller et al. 2008, Müller and Schlein 2008). However, the success of this approach would be greatly improved by identification of specific plants utilised by target vectors for maximum contact and to avoid killing non-target plant visiting insects (Qualls et al. 2014). While providing a benchmark for further exploitation of chemical cues involved for the development of more potent lures, these findings further present an opportunity for deployment of ATSB in the control of Afrotropical mosquito species.

Chapter three further presents evidence of variable contribution of the identified natural host plants to *Ae. aegypti* survival and fecundity, the findings which are invariably supported by the nutritional content of the host plants. While *S. alata* scored highly as natural host plant for the malaria vectors *An. gambiae* s.l. and had the highest total sugar content and benefit, it nonetheless was among the least supportive of vector survival and fertility but stimulated

the highest fecundity. On the other hand, *P. dulce* and *L. nepetifolia* which had moderate sugar and amino acid content supported vector survival, fecundity and fertility. *Opuntia ficus-indica* which was highly scored as natural host plant for RVF vectors had an inferior performance by comparison. While these findings could be a pointer for mosquitoes resorting to plant species readily available within their locality for feeding irrespective of their nutritional value, they also suggest possible multiple plant feeding with specific plant species providing specific benefits other than sugar. This is in line with previous suggestions that some mosquito species such as *Ae. aegypti* and *An. gambiae* only feed on plants when they are available but resort to blood for all their metabolic requirements when plants are scarce (Van Handel et al. 1994, Beier 1996, Martinez-Ibarra et al. 1997). In addition, these findings further augment suggestions of added benefits derived by mosquitoes from plants made by Schlein and Muller (1995), Manda et al. (2007a) and Nyasembe et al. (2014b).

Finally, this study presents evidence of involvement of chemical cues in mosquito-host plant interactions and the potential for exploiting these cues for development of potent plant-based lures for the management of Afrotropical mosquito species. The study documents variable volatile profiles among the five natural host plants with *L. nepetifolia* volatile profiles dominated by sesquiterpenes, *R. communis* dominated by monoterpenes, *P. dulce* by monoterpenoids and benzoids while *S. alata* and *O. ficus-indica* have benzenoids. Hexanal, (*E*)-β-ocimene, (*E*)-linalool oxide and decanal was present in the volatiles of all the five plants, albeit in variable amounts. Different isomers of hexenol (i.e. (*E*)-2- and (*Z*)-3-hexenol) were also detected in the headspace VOCs of the five plants. In terms of what the antenna detects, there was variation in the number of electrophysiologically active compounds detected by *Ae. aegypti* and *An. gambiae*. Both male and female *Ae. aegypti* detected (*E*)-β-ocimene in *P. dulce* volatiles while female *An. gambiae* detected different isomers of (*E*)-β-ocimene, decanal and β-cedrene in the volatiles of *L. nepetifolia* and *S.*

alata. Both species detected different isomers of hexanal, different isomers of hexenol, (*E*)-β-ocimene, (*E*)-linalool oxide, decanal and (*E*)-caryophellene. The differences in the classes of volatile profiles of different mosquito host plants is not surprising given that the plants are from different families hence are likely to utilised variable metabolic pathways (Pichersky et al. 2006, Jhumur et al. 2008). It is possible that the mosquitoes have adapted to respond to certain chemical cues associated to their locally available host plants. The development and field evaluation of a three-component plant-based lure that was more attractive than LO and commercial BG lure is an indication of the potential of phytochemicals in the management of disease vectors. The three-component lure was particularly effective for male *Ae. aegypti*. However, further evaluation of this lure across different ecologies and seasons is necessary. In addition, the confirmation of the role of the other compounds detected by the two mosquito species and their involvement in mosquito-plant interactions is necessary.

Overall, this study provides a detailed insight in the nectar feeding behaviour of key Afrotropical mosquito species and the potential of harnessing this aspect of vector ecology in developing environmentally safe and viable control strategies. A two-pronged vector control approach is concluded from this study; 1) the identified natural host plants for these vectors can be targeted in an area wide insecticide application targeting out-door biting fractions of mosquito populations or deployment of ATSB as a control strategy, 2) chemical signatures identified in this study as antennaly-active can be further investigated in addition to LO and Blend C in order to develop a more potent plant-based lure for vector surveillance or control when used in combination to a killing agent. Together, these approaches when integrated into the existing control strategies will provide additional weapons important in the war against the burdening vector-bone diseases.

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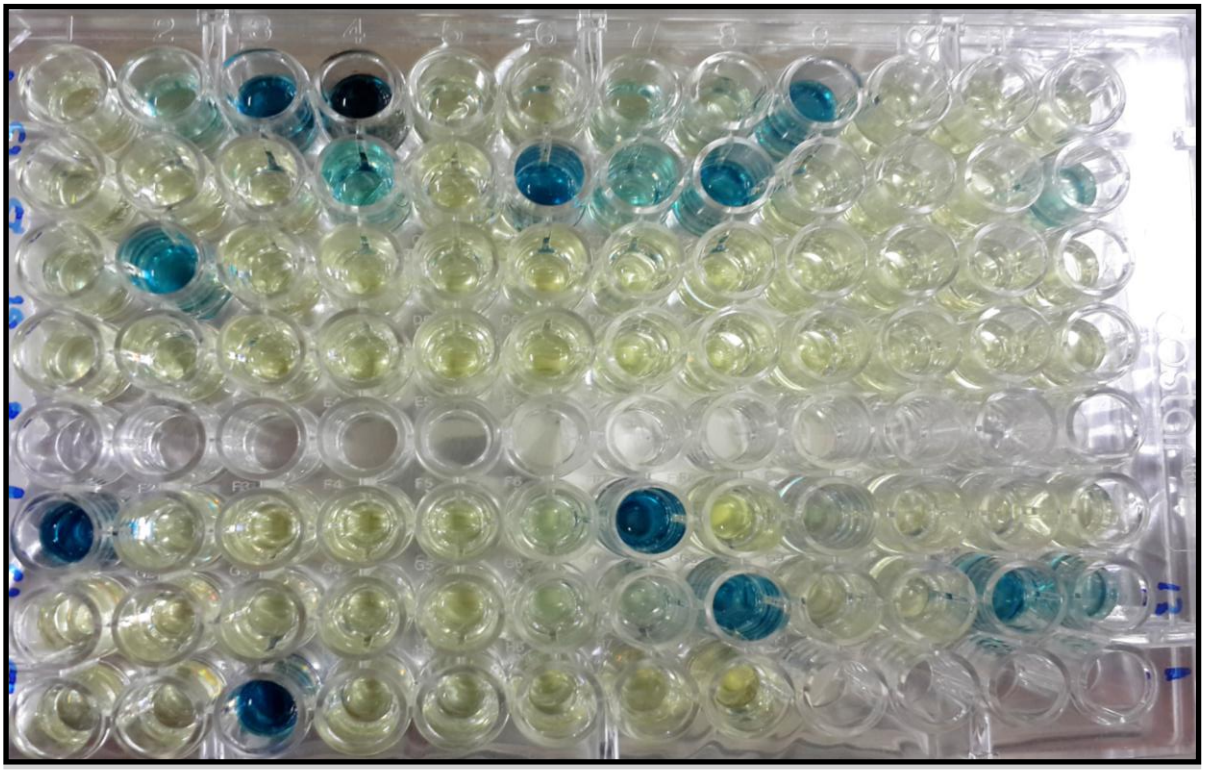
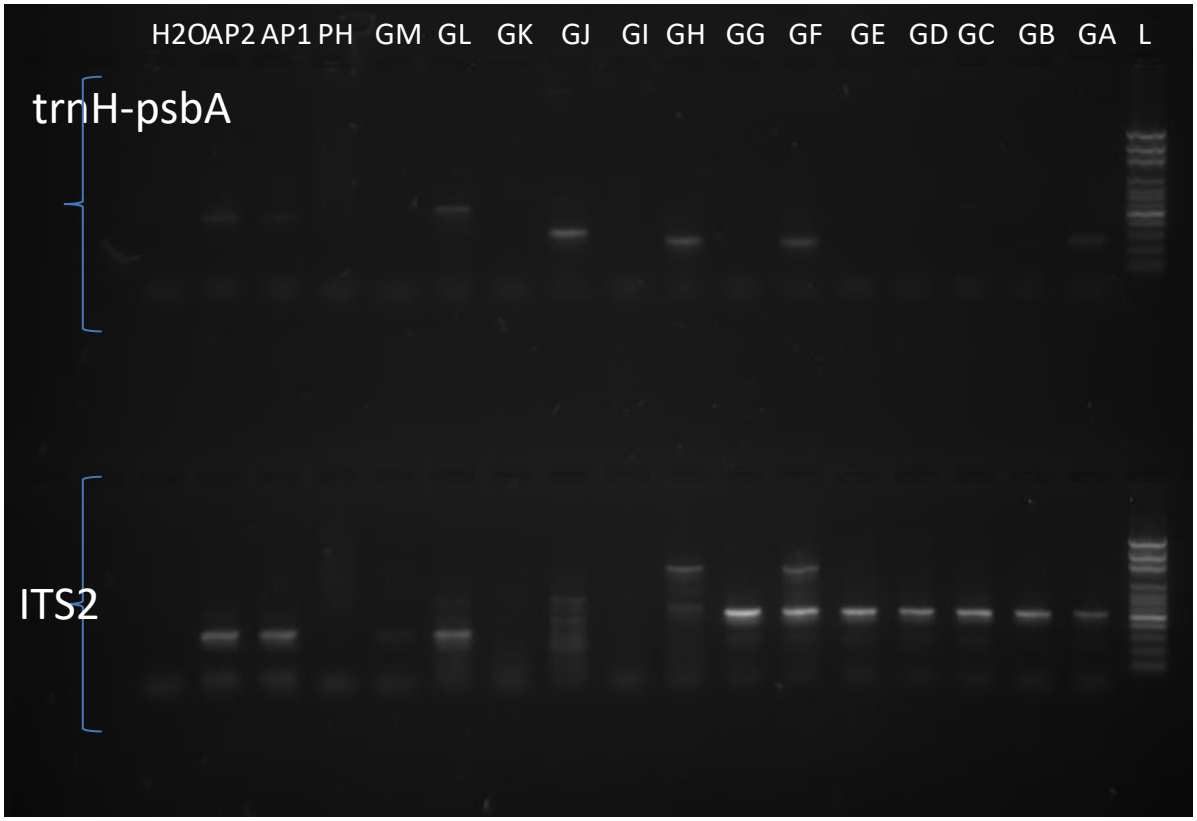
APPENDICES

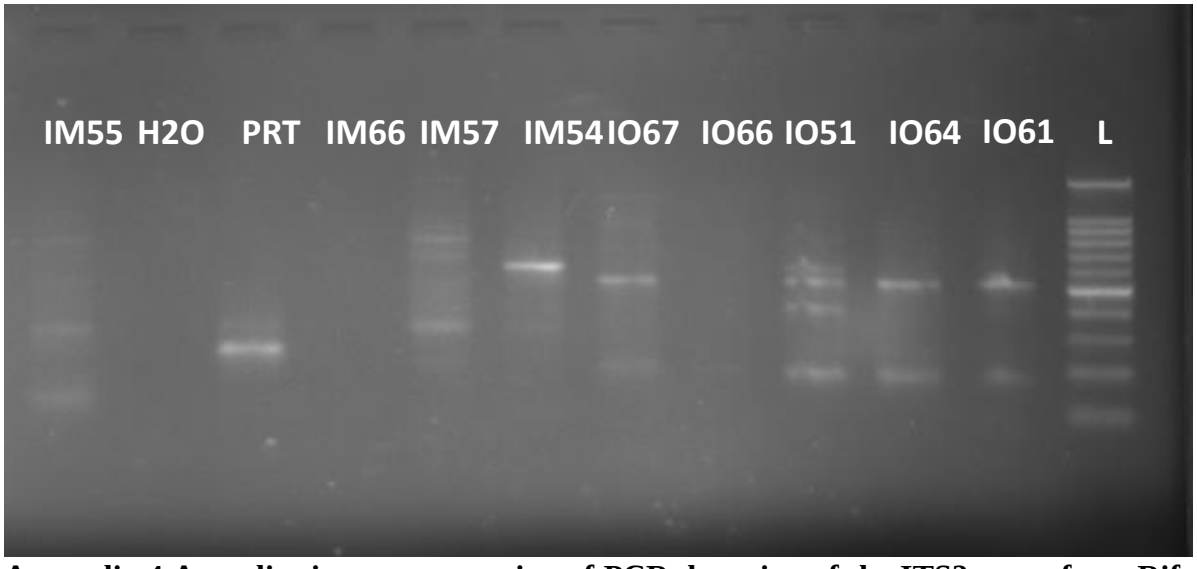


Appendix 1 Anthrone reaction product showing fructose positive samples (deep blue wells) and negative samples (light green wells).



Appendix 2 A qualitative representation of PCR detection of thetrnH-psbA and ITS2 genes from malaria vectors. Lanes GA-GM: *Anopheles gambiae* mid gut amplicons; lane PH: *Parthenium hysterophorus* amplicon serving as positive control; lane AP1 and AP2: Aphid gut amplicon serving as second positive control; H2O: PCR negative control; L: molecular marker.

Appendix 3 A qualitative representation of PCR detection of the ITS2, trnH-psbA and matK genes from dengue vectors. Lanes KAF 6 – KAF30 and KAM 13 – KAM 30: female and male *Aedes aegypti* mid gut amplicons, respectively; lane PRT: *Parthenium hysterophorus* amplicon serving as positive control; lane KB1: Leaf beetle gut amplicon serving as second positive control; H_2O: PCR negative control; L: molecular marker.



Appendix 4 A qualitative representation of PCR detection of the ITS2 genes from Rift Valley fever vectors. Lanes IO51, IO61, IO64, IO66 and IO67: *Aedes ochraceus* mid gut amlicons; lanes IM54, IM55, IM57 and IM66: *Aedes mcintoshi* mid gut amplicons; lane PRT: *Parthenium hysterophorus* amplicon serving as positive control; H_2O: PCR negative control, L: molecular marker.