Development of efficient tools for improved surveillance of vectors of dengue and chikungunya fever.

By

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Declaration

I, Eunice Anyango Owino hereby declare that this thesis which is submitted for the degree of Doctor of philosophy (Entomology) at the University of Pretoria, is my own work and has not been previously been submitted by me for a degree at this or any other tertiary institution.

Signed

30/06/2015

Date

Disclaimer

This thesis consists of a series of chapters that have been prepared as standalone papers already published or manuscripts for different scientific journals. Consequently, unavoidable overlaps and/or repetitions may occur and the references style and formats may differ between chapters.

Thesis summary

In the past 3 decades, arboviruses have become a major cause of re-emerging epidemic diseases in the world. Amongst the arboviruses, dengue and chikungunya fevers which are transmitted by *Aedes spp* have become more prevalent and have spread far beyond traditional areas of distribution - mainly in Africa and Asia - to cause severe morbidity, mortality, and economic harm. Currently control of these diseases solely depends on vector control as there is no treatment or vaccine. This calls for efficient vector surveillance tools. However, the currently available vector sampling tools are inadequate in sampling *Ae. aegypti.* The popularly used Centers for Disease Control (CDC) and prevention light trap, under represents *Ae. aegypti* abundance as they are not attracted to its incandescent light bulb. Man landing catches (MLC) technique on the other hand is ethically unacceptable as it exposes the catchers to infective bites while the BG lure baited BG sentinel trap is reported to be less effective in sampling *Ae. aegypti*. This lack of an adequate sampling tool has led to underestimation of the magnitude of the two diseases in endemic areas consequently leading to un predicted outbreaks that have caused high morbidities and mortalities. Therefore, developing an effective surveillance tool that would aid in timely control campaigns could be a contribution of the utmost importance. We conducted our studies in two dengue and chikungunya endemic regions of Kenya-Busia and Kilifi counties.

We tested the efficacy of various colored light emitting diode (LED) traps against the (CDC) light trap in sampling *Ae. aegypti* using replicated, randomized field experiments and observed that the violet trap caught significantly more *Ae. aegypti* in Busia than the control trap. Viral testing using *Flavivirus* and *Alphaviru*s universal primers showed that the *Ae. aegypti* in Busia were infected with insect specific flaviviruses (ISFs) and there was a preference for the violet LED trap by the ISF Infected *Ae. aegypti*. Replicated randomized field experiments were also used to test the efficacy of Biogents (BG) sentinel traps baited with human feet odors trapped in socks and human trunk odors trapped in T-shirts against a control trap baited with the Biogents (BG) commercial lure. We observed that the traps baited with human odors caught more *Ae. aegypti* than the

BG lure baited trap. We also observed that some individual’s odors attracted more *Ae. aegypti* than others. Gas chromatography coupled with mass spectrometry (GC-MS) analysis of the human volatiles and the BG lure revealed that the BG-lure mainly emitted hexanoic acid while human volatiles had several compounds mainly aldehydes, carboxylic acids, ketones and a couple of alcohols. A further GC coupled with electro-antenno- grams (GC-EAG) identified electrophysiologically active compounds from the human odors some of which were then formulated into attractant blends and tested against whole human odors and the BG lure in the field. It was observed that some single compounds like hexanoic acid were better attractants to *Ae. aegypti* than the BG commercial lure and some formulated blends and that some compounds when dispensed together produced antagonistic and inhibitory effects against *Ae. aegypti*. Additionally, we conducted population genetics studies on the *Ae. aegypti* samples from Busia and Kilifi using a 653-bp region of the mitochondrial DNA cytochrome oxidase I (*COI*) gene and observed that there was no genetic differentiation between *Ae. aegypti* from the two regions suggesting that their vector competency and susceptibility to insecticides might not be different.

We thus believe that our work gives greater insight into the efficient use of LED traps in sampling not only *Ae. aegypti* in the field but also other diurnal insects (Chapter 2). Our work also adds information on the development of attractant synthetic odor baits from host volatiles for effective sampling of *Ae. aegypti* in the field (Chapter 3 & 4). Additionally, our work provides vital information on the population genetic structure of mosquitoes in Busia and Kilifi which would be important in planning control measures and help immensely in understanding disease transmission risks (Chapter 5).

To improve our understanding on the effect of all the biologically active compounds in human skin volatiles, we recommend further investigations on the other active compounds that might were included in our odor blends but were identified to be biologically active. This might help improve odor baits for *Ae. aegypti*. We also recommend further investigations on preference of the violet colored LED traps by *Ae. aegypti* especially the *Flavivirus* infected. It would also be important to determine the

interaction between ISFs and arboviruses like dengue and chikungunya in a co-infected mosquito as this may potentially impact on vector competence and thus transmission.

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Table of Content

Table of content	
Declaration	iii
Disclaimer	iv
Thesis summary	v
Acknowledgements	viii
Table of content	ix
List of figures	x
List of tables	xiii
Chapter 1: General Introduction, Rationale and key questions	1
Chapter 2: Potential of the violet baited light emitting diode (LED) in improving the sampling of adult *Aedes aegypti* in dengue and chikungunya endemic zones in Kenya	16
Chapter 3: Field evaluation of natural human odors and the biogent-synthetic lure in trapping *Ae. aegypti*, vector of dengue and chikungunya viruses in Kenya	43
Chapter 4: An improved odor bait for monitoring populations of *Ae. aegypti*-vectors of dengue and chikungunya viruses in Kenya	69
Chapter 5: *Ae. aegypti* in Busia and Kilifi counties of Kenya: Population genetics.	101
Chapter 6: General conclusions and Recommendations	128

List of Figures

Chapter 2

Figure 1. Trapping sites in Busia and Kilifi counties in Kenya	21
Figure 2. A set up light emitting diode (LED) trap	25
Figure 3. The mean number of *Ae. aegypti* captured by the various light traps per night in Busia and Kilifi counties. Panel A – Busia, Panel B- Kilifi. Asterisks indicate that the mean catch of the trap is significantly different from the mean catch of the control.	29
Figure 4. Curves formed by flavivirus positive samples after nested PCR using Vasquez plus Moreou flavivirus primers.	31
Figure 5. Phylogenetic analysis of the NS5 genes of insect specific virus (ISV) isolates from *Ae. aegypti* from Busia and Kilifi. The sequences obtained in this study are designated in blue ink (Isolate). Viruses are identified by GenBank accession number.	33

Chapter 3

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya.	47
Figure 2. The BG sentinel traps were baited with socks and set up in Busia and Kilifi counties of Kenya. Assembly follows steps a-h.	50
Figure 3. The mean number and ± S.E of *Ae. aegypti* captured by the various BG sentinel traps baited with different baits in Kilifi and Busia counties. The different panels show comparisons at the two locations; Panel A –Kilifi and Panel B- Busia	54

Chapter 4

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya.	75
Figure 2. Representative GC/EAD profiles showing EAD- active components identified from; the feet- Panel 1 and trunk - Panel 2, of volunteers. Panel A- GC/EAD responses from F1 generation *Ae. aegypti* from Rabai, Kilifi.	84
Figure 3. The mean number ± S.E of *Aedes aegypti* captured by the various BG sentinel traps baited with different baits in Busia and Kilifi County**.**	90

Chapter 5

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya.	106
Figure 2. A set up of the Biogents trap in the field.	107
Figure 3. Plot of the principal coordinate analysis PCA generated using GenAIEx for *Ae. aegypti* samples from different localities	114
Figure 4. Haplotype dendrogram of 22 *Ae. aegypti* haplotypes from Busia, Kilifi and the 2 haplotypes of *Ae. albopictus* from the Genbank based on the neighbor-joining (NJ) method, computed using the Tamura-Nei model.	115
Figure 5. Haplotype dendrogram of *Ae. aegypti haplotypes* from Busia, Kilifi and the Genbank based on the neighbor-joining (NJ) method computed using the Tamura-Nei model. Bootstrap support values are recorded shown next to the branches.	116

List of Tables

Chapter 2

Table 1. List of Primers used in flavivirus testing of samples from Busia and Kilifi counties of Kenya	26
Table 2. Comparisons of mosquito collections by the various colored LED traps relative to the control (standard CDC) trap in Busia and Kilifi counties of Kenya.	30
Table 3. Comparisons of proportions of samples that tested viral positive by the various colored LED traps relative to the control (standard CDC) trap in Busia and Kilifi counties of Kenya	32

Chapter 3

Table 1. Comparisons of mosquito collections by BG sentinel traps baited with feet and trunk odors from volunteer 1, volunteer 2 and carbon dioxide in Kilifi county and from volunteer 2, volunteer 3 and carbon dioxide in Busia county relative to the control (Biogents commercial lure baited BG sentinel trap) trap.	55
Table 2. Comparisons of *Ae. aegypti* proportions per trap by sex and abdominal status with corresponding catch indices (CI).	56
Table 3. Main compounds identified in the volatiles released by the commercial BG-lure and trunk and feet of human volunteers captured on SPME and analyzed coupled GC-MS analysis.	58

Chapter 4

Table 1. Compounds in feet and trunk volatiles from 4 volunteers that elicited GC/EAD responses in female *Ae. aegypti*.	83
Table 2 . Comparisons of mosquito collections by BG sentinel traps baited with different odor baits relative to the BG sentinel trap baited with binary blend of nonanal and octanal in Busia and Kilifi.	87
Table 3. Comparisons of mosquitoes trapped with BG sentinel traps baited with different odor baits relative to the BG sentinel trap baited with hexanoic acid in Kilifi County during phase II of the study.	88
Table 4. Comparisons of *Ae. aegypti* percentage proportions per trap by sex and abdominal status with corresponding p values and catch indices (CI) during phase II of the study.	89

Chapter 5

Table 1. Genetic variability in *Ae. aegypti* populations from Busia and Kilifi.	111
Table 2. Summary of statistical analyses of the molecular polymorphism in *Ae. aegypti*	112
Table 3. Genetic variability in Lineage I and II of *Ae. aegypti* populations	113
Table 4. Genetic distance and gene flow based on population pair wise differences (F_{STs}) values for 6 *Ae. aegypti* populations in Busia and Kilifi	117
Table 5. Hierarchical analysis of the genetic Variation in *Ae. aegypti* samples from Busia and Kilifi	118

Chapter 1

General introduction, rationale of the study and Key Questions

Arboviruses - diseases that require blood-sucking arthropods as a host apart from the vertebrate host (WHO, 1995), have become the most important causes of re-emerging epidemic diseases in the world today (TDR/WHO, 2009). Amongst the arboviral diseases, dengue fever has been reported to cause more human morbidity and mortality than any other arthropod-borne viral disease (Gubler, 2004). An estimated 50-100 million dengue infections and several hundred thousand dengue hemorrhagic fever (DHF) cases occur each year in more than 100 endemic countries (WHO, 2012). In East Africa, unprecedented outbreaks of dengue have been reported in countries around the coast of the Indian Ocean including Kenya (Johnson *et al* 1982; Sang *et al.*, 2008) where an outbreak was reported as recently as May 2014 in Mombasa County (Standard newspaper, 2014).

Dengue fever is caused by infection with any of the 4 serotypes of dengue viruses (DENV-1, DENV-2, DENV-3, DENV-4) in the family *Flaviviridae*, genus *Flavivirus* (WHO, 2012). The disease is mainly transmitted by mosquitoes of the genus *Aedes* especially *Aedes aegypti* (Linnaeus) and *Aedes albopictus* (Skuse) that acquire it while feeding on the blood of an infected person (Halsted, 2008). After an incubation period of eight to ten days, an infected mosquito is capable of transmitting the virus for the rest of its life, during probing and blood feeding. They may also transmit the virus to their offspring transovarialy (via the eggs) (WHO, 2009).

Dengue infection is characterized with joint pain, fever and headaches, which are often mistaken for yellow fever as well as other diseases including influenza, measles, typhoid and malaria (Siler *et al.*, 1926; Halstead, 1997). Although dengue infection is rarely fatal, up to 90% of the population of an infected area can however, be

incapacitated during the course of an epidemic (Siler *et al*., 1926). Furthermore, mortality rates from dengue hemorrhagic fever (DHF) that attack children under 10 years of age and adults recovering from a dengue infection (Halstead,1988) can exceed 30% if the appropriate care is not available. Outbreaks of chikungunya which shares the same vectors *Ae. aegypti* and *Ae. albopictus* -, the same distribution, (Halsted *et al.,*1967) and the same disease symptoms (Deller & Rusell, 1968; Carey, 1971) with dengue virus have also been reported in the Indian Ocean islands of the Comoros, Seychelles, Reunion and Mauritius between 2004-2005 and in Lamu, Mombasa and Kilifi counties along the Kenyan coast (Powers & Logue, 2007; Sang *et al*., 2008).

Worldwide, *Ae. aegypti* is the main vector of dengue virus (DENV), chikungunya virus (CHIKV) and yellow fever virus (YFV) (Kow *et al*., 2001; Gubler, 2002). An adult *Ae. aegypti* is measures between three to four millimeters in length, discounting leg length and is totally black with white 'spots' on the body and head regions and white rings on the legs. The thorax is decorated with a white 'Lyre' shape of which the 'chords' are two dull yellow lines. The mosquito is currently present globally in tropical and sub-tropical regions although it originated in Africa (Scarpassa *et al*., 2008; Brown *et al.,* 2011) and has become a successful vector of viruses to humans. One unique character of *Ae. aegypti* is that unlike most other mosquitoes, it's a day biting mosquito, active during daylight for approximately two hours after sunrise and several hours before sunset.

A major characteristic believed to have contributed to the success of *Ae. aegypti* is its unique adaptation to close association with humans making it an efficient transmitter of viruses. Females *Ae. aegypti* are highly anthropophilic, they rest inside houses where they feed frequently preferentially on human blood to meet their energetic and reproductive needs (Scott *et al*., 1993a, 1993b). They also take the blood in multiple meals increasing the number of contact with the hosts thus increasing the probability of virus transmission (Scott *et al.* 1997). Actually, it doesn't take many mosquitoes to sustain unacceptable levels of viral transmission. This has made the entomological thresholds for dengue virus transmission to be quite low (Focks *et al*., 2003).

The adaptation of *Ae. aegypti* to breed in containers in and around human dwellings has also contributed immensely to their success as vectors. They thrive exceptionally well in urbanized areas where they not only have readily available containers to breed in like unused flowerpots, spare tires, untreated swimming pools, and drainage ditches (Christophers, 1960) but are also in close contact with their human hosts.

The other factor that has contributed to the success of *Ae. aegypti* is the ability to continually respond or adapt to environmental change. For example, recent research has reported that *Ae. aegypti* is able to undergo immature development in broken or open septic tanks resulting in the production of hundreds or thousands of *Ae. aegypti* adults per day (Burke *et al*., 2010). This is in contrast with what had been observed before that *Ae. aegypti* immature were never found in water containing sewage or in cesspools (Christophers, 1960).

The mosquito has also developed adaptations to be highly resilient in harsh environmental conditions and to rapidly bounce back to initial numbers after disturbances from natural phenomena (e.g., droughts) or human interventions (e.g., control measures). For example, their eggs have the ability to withstand desiccation (drying) and to survive without water for several months on the inner walls of containers (Christophers, 1960). In fact, if we were to eliminate all larvae, pupae, and adult *Ae. aegypti* at once from a site, its population could recover two weeks later as a result of egg hatching following rainfall or the addition of water to containers harboring eggs. With these adaptations, it is clear that *Ae. aegypti* is an efficiently adapted vector that must be given a top priority in dengue control interventions.

The increase in the emergence of dengue and chikungunya have been attributed to climate change (Chreiten *et al.,* 2007) urbanization (Gubler, 2008; Beatty, 2009) globalization (Ensenrik, 2008) and increased travel (WHO, 2012) amongst other factors. Consequently, the projected trends of continued global warming, urbanization and globalization will ensure that the incidences of these diseases will continue to increase if interventions are not forthcoming (Gubler, 2002; Alirol *et al*., 2010,). The problem is further exacerbated by the fact that at present there is no available treatment or vaccine

for both dengue and chikungunya (WHO, 2002; Gubler, 2011). This leaves vector surveillance, control and monitoring as the only method for managing dengue and chikungunya outbreaks. Efficient vector surveillance and monitoring tools are therefore required to prevent mortality and morbidity from these diseases.

Traditionally, dengue vector control programmes have been focused on using immature *Ae. aegypti* indices as a means of vector surveillance and control. However, existing immature indices are not sufficient to detect and prevent dengue outbreaks as they have a weak relation with transmission risk (Ooi *et al*., 2006). Only adult *Ae. aegypti* transmit viruses, surveillance and monitoring of adult host seeking mosquitoes would be the most effective means of gaining an accurate picture of the risk of infection in any specified area.

However, the currently available traps used in sampling *Ae. aegypti* are riddled with a myriad of weaknesses. The commonly used mosquito surveillance tool, the carbon dioxide baited Centers for Disease Control (CDC) and Prevention light trap, are functionally inadequate for sampling diurnal *Ae. aegypti* as they are not attracted to the incandescent bulb like the night biting mosquitoes, the anophelines and the culicines (Service, 1993). On the other hand, the alternative man landing catches (MLC) that most investigators of the two diseases resort to are ethically unacceptable exposing human baits / collectors to infective bites (Focks, 2003). The Biogents (BG) trap with its commercial lure developed to target *Ae. aegypti* (Geir *et al*., 2006; Owino *et al*., 2014) have also been reported to be less effective in sampling *Ae. aegypti* (Krockel *et al*., 2006). Developing new methodologies to collect adult *Ae. aegypti*, especially females, for surveillance purposes would therefore be a most valuable contribution to dengue prevention

Vision and color sensitivity play a principle role in adult *Ae. aegypti* biology, including location of hosts, food sources, mates, resting sites, and oviposition sites (Hawley *et al*., 1988; Hoel *et al*., 2011). Other studies have also shown that mosquitoes are attracted to transmitted light (Wilton & Fay, 1972; Browne & Bennett 1981). The diurnally active *Ae. aegypti* has previously been reported to have spectral sensitivity and color preference

(Browne & Bennett, 1981; Muir *et al.*, 1987). This ability could effectively be explored to develop efficient surveillance tools for monitoring and control of transmission of dengue and chikungunya viruses.

Super-bright light emitting (LEDs) diode traps have already been developed for trapping insects. The LEDs are compact bodies which emit a narrow bandwidth (350-700nm) of light of specific colors to attract insects, which is unlike the incandescent bulb that emits a broad spectrum of light. The LEDs have a greater intensity and run on significantly lower amounts of energy (ca. 0.125 ma/h vs. 150 ma/h for standard CM-47 bulb) than incandescent bulbs, resulting in substantial savings in battery life. Preliminary investigations showed that a preference might exist for some mosquito vectors based on observed differential attraction and preferences to specific wavelengths of the LED light traps. (Wilton & Fay, 1972; Burkett & Butler, 2005; Tchouassi *et al.*, 2012). The LED technology would therefore provide a cheaper and probably more effective means of monitoring diurnal flying vectors in developing countries. Furthermore, a previous study using red, green, blue, violet and a combination of blue-green-red (BGR) LED traps to sample Rift valley fever vectors in Ijara Kenya showed that *Aedes mcintoshi* (Huang) and *Aedes ochraceus* (Theobald) had a seasonal preference for BGR and blue LED traps (Tchouassi *et al.*, 2012).

Other studies have shown that at relatively close range, strong olfactory responses to human skin and breath odors facilitate the preference of blood seeking female *Ae. aegypti* to human hosts (Schreck *et al.*, 1990; Takken, 1991; Geier & Boeckh, 1999). Human odor contains volatile chemical substances that increase mosquito attraction in the laboratory (Schreck *et al.*, 1981; Eiras & Jepson, 1991, 1994) and in the field (Gillies & Wilkies, 1974). To date, many studies have shown that baiting traps with whole human odors increases the catch of mosquitoes (Constantini, 1993, 1996; Knols *et al.,* 1995, 1998). Furthermore, recent research suggests that potential mosquito attractant compounds identified could be formulated into attractants in the baits and used to trap mosquitoes in the field (Okumu *et al.*, 2010; Tchouassi *et al.*, 2013). One might even foresee the development of baits that might be used *en masse* to reduce the vector

population in a village, individuals' homesteads, houses and even bedrooms to divert mosquitoes away from the occupants.

Apart from behavioral ecology, mosquito population genetics has also been observed to be useful in vector control. For example, knowledge of the population genetics structure of *Ae. aegypti* in areas endemic for dengue have been observed to be vital in designing dengue suppression programs during the use of Wolbachia *pipientis* - a bacterium which has been shown to reduce vector competence of *Ae. aegypti* for dengue virus (Walker *et al*., 2011). Information on the population genetic structure helps in designing the logistics of field release of *Wolbachia* - infected mosquitoes as it helps in estimating how many mosquitoes to release, over what sized area and at what time of year (Olanratmanee *et al*., 2013).

Knowledge on the vector population structure has also been reported to be very important for effective vector control using insecticides as it gives information on insecticide susceptibility or resistance which could help in the prevention of deter insecticide resistance (Ocampo *et al*., 2004).Vector population structure could also give information on vector competence for dengue transmission (Gubler *et al*., 1979; Failloux *et al*., 1994) and help to determine the relatedness of geographic populations and associate this information with vector movements. This would help to analyze the risk of disease transmission (Ballinger-Crabtree *et al*., 1992).

Therefore, the goal of this study was to test the efficiency of various colored light emitting diode (LED) traps in trapping *Ae. aegypti* in the field and also to develop odor baits from human feet and trunk volatiles that could efficiently be used to trap *Ae. aegypti* in the field. The study was also aimed at giving a better understanding of the population genetic structure of *Ae. aegypti* in Busia and Kilifi, dengue and chikungunya virus endemic areas, which may contribute in improving vector surveillance and control.

Rationale of this study

Chikungunya and dengue are re-emerging mosquito-borne infectious diseases that are of increasing concern especially due to the projected increased global warming, rural to

urban migration, human travel around the world and expanding mosquito ranges increase the risk of spread. Dengue has caused increasing concern in tropical and subtropical regions and is emerging in areas where it has been absent for years, infecting millions every year (Guzman & Istúriz, 2010) and potentially increasing with climate change (Åstrom *et al*., 2013). Recently, chikungunya virus re-emerged in Asia and caused outbreaks in Italy and several Indian Ocean islands (Thiboutot *et al.,* 2010; Anyamba *et al*., 2012). Therefore, there is an urgent need to develop efficient monitoring tools to aide timely control campaigns before these diseases start causing deaths in humans.

At present, the only method of controlling or preventing dengue and chikungunya virus transmission is to combat the vector, *Ae aegypti* as there is currently no known treatment and vaccine for dengue fever (WHO, 2012) or chikungunya. Under these circumstances, it's important to monitor the vector populations in endemic areas to understand their ecology and population genetics structure before implementing appropriate and timely intervention. This calls for efficient sampling and surveillance tools that will give reasonably accurate measures of disease and vector abundance data to guide decisions on disease control measures.

Blood seeking mosquitoes locate their hosts by odors produced by the host (Schreck *et al*., 1990; Takken, 1991; Geier & Boeckh, 1999).The compounds in host odors responsible for the attraction can be identified and formulated into attractant odor blends that can effectively be used in the field to bait mosquito traps. Mosquitoes have also been observed to be attracted to specific wave lengths of light (Burkett *et al*., 1998; Burkett *et al*., 2005; Tchouassi *et al*., 2012). In addition, vector population genetics is essential for effective vector control and disease risk management because genetic traits of mosquito populations can be related to vector capacity (Failloux *et al*., 1994; Gubler *et al*., 1979) and/or insecticide resistance (Ocampo & Wesson, 2004).

The rationale of this study was to exploit the visual and olfactory cues provided by LEDs and human host skin odors respectively, as strategies to increase captures of *Ae. aegypti* in dengue and chikungunya endemic regions. Additionally, as the capacity of

vectors to transmit arboviral pathogens differs greatly between different species of the same genus or even populations of the same species, this study sought to compare the genetic differences among populations of *Ae. aegypti* in the two dengue and chikungunya endemic areas of Busia and Kilifi counties of Kenya. The ultimate goal of this project was thus to develop an efficient trapping tool to enhance trap captures of *Ae. aegypti* and to investigate the potential role of its genetic structure in improving the control of dengue and chikungunya fever viruses in Kenya.

Given the background presented above the key questions, the title of each chapter and associated research questions are presented below:

Key research questions

The key questions addressed in the present study include:

***Chapter 2*: Violet baited light emitting diode (LED) improves the sampling of adult *Aedes aegypti* in two dengue and chikungunya endemic zones in Kenya.**

Key research questions:

Q1: Do *Ae. aegypti* have a preference for any specific colored light emitting diode (LED)?

Q2: Can LED traps improve the sampling of flavivirus infected *Ae. aegypti* in dengue and chikungunya endemic zones in Kenya?

***Chapter 3:* Field evaluation of natural human odors and the Biogent-synthetic lure in trapping *Ae. aegypti*, vector of dengue and chikungunya viruses in Kenya.**

Key research question:

Q**:** Are natural human odors more effective than the Biogents-synthetic lure in trapping *Ae. aegypti*, vector of dengue and chikungunya viruses in Kenya.

Chapter 4: **Prospects for developing an improved monitoring system for *Ae. aegypti* vectors of dengue and chikungunya viruses in Kenya using compounds from the human skin.**

Key research questions:

Q1: What compounds from the human skin volatiles are electro-physiologically active to *Ae. aegypti*?

Q2: What compounds amongst the biologically active compounds are attractive in the field to Ae aegypti and at what concentrations?

Chapter 5: **Population genetic studies of *Ae. aegypti* in Busia and Kilifi counties of Kenya.**

Key research question:

Q1**:** What is the phylogeographic history of *Ae. aegypti* in Busia and Kilifi?

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Chapter 2

Potential of the violet baited light emitting diode (LED) in improving the sampling of adult *Aedes aegypti* in dengue and chikungunya endemic zones in Kenya

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Abstract

Background: A major challenge that scientists working in the public health sector face is the lack of an efficient sampling tool for *Aedes aegypti* the main vector of major pathogenic flaviviruses including yellow fever virus, dengue virus and West Nile virus, as well as of diverse insect specific flaviviruses. *Aedes* mosquitoes are less attracted than other mosquito genera to the commonly used Centers for Disease Control (CDC) and prevention light trap. Here, we document the comparison of the efficiency of Light Emitting Diodes (LED) emitting different colored lights against CDC light trap in sampling live adult *Ae. aegypti* for arboviral infection testing. Our study sites were two dengue and chikungunya endemic regions of Kenya, Kilifi and Busia counties of Kenya.

Methodology: Using Latin square designs, we compared the efficacies of LEDs emitting (a) blue (b) green (c) violet and (d) a mixture of blue, green and red lights (BGR) in trapping *Ae. aegypti* against (e) a control CDC light trap incandescent bulb) at the two sites. Specifically, we compared daily *Ae. aegypti* counts per trap modification using generalized linear models with Poisson error and identified *Flavivirus* and *Alphavirus* infections by reverse transcription-polymerase chain reaction (RT-PCR).

Results: In Busia, the violet LED captured significantly more *Ae. aegypti* than the control (CDC light trap incandescent bulb) [IRR=1.93, CI: (1.05-3.70), p=0.038], whereas the blue, green and the BGR LED traps captured fewer *Ae. aegypti* than the control. In Kilifi, all LEDs captured fewer mosquitoes than the control. Viral testing showed that the violet LED captured a significant higher proportion of *Flavivirus* infected *Ae. aegypti* than the control in Busia (p=<0.001). However, no *Alphavirus* infections were detected in any of the samples. Sequencing and phylogenetic analysis showed that the viruses were insect specific *flaviviruses* (ISF) that are closely related to the CFAV Surabaya and Kamiti viruses.

Conclusions: Violet colored LEDs in CDC light traps might have a potential in enhancing surveillance and monitoring of arboviral diseases transmitted by *Ae. aegypti.*

The apparent preference of arbovirus infected *Ae. aegypti* mosquitoes for violet colored LEDs should be investigated further and has the potential to be exploited in diverse vector control strategies.

Key words; *Aedes aegypti, Flaviviruses*, CDC light traps, Light emitting Diode bulbs

Background

Aedes aegypti, a mosquito that maintains close association with human populations, is a major vector of pathogenic flaviviruses that infect humans in the world today. It is the principal vector of the etiological agents of yellow fever and dengue fever (WHO, 2002;Tomori, 2004) and is also responsible for the recent chikungunya fever epidemics in countries in the Indian ocean area (Powers & Logue, 2007; Chreiten *et al*., 2007). Despite availability of an effective vaccine, yellow fever still remains a disease burden in Africa and parts of South America with over 200,000 cases per year resulting in approximately 30,000 annual deaths (Tomori, 2004). About 2.5 billion people are at risk for dengue, with over 50 million cases per year and over 500,000 cases of dengue hemorrhagic fever, the more serious manifestation of the disease (Gubler, 2002; WHO, 2008). The incidences of viral diseases spread by *Ae. aegypti*, for which mosquito management is currently the only prevention option, are on the increase (WHO, 2012). Thus, there is an urgent need to improve the surveillance and monitoring of these diseases and their vectors.

Recently, reports have emerged that *Ae. aegypti* is a major host of a new *Flavivirus* group termed as insect specific flaviviruses (ISFs), which replicate only in mosquitoes and are maintained in nature by vertical transmission from female mosquitoes to their progeny (Stollar & Thomas, 1975; Cook & Holmes, 2006; Hoshino *et al*., 2007). Despite their non-pathogenicity to humans and animals, ISFs have recently garnered increased attention due to research reports that these viruses may either enhance (Kent *et al*., 2011; Newman *et al*., 2011) or suppress (Bolling *et al*., 2012; Hobson *et al*., 2013) the transmission of pathogenic viruses like West Nile viruses.

One major challenge that entomologists targeting *Ae. aegypti* face is the lack of an adequate sampling tool for this mosquito species. The Centers for Disease Control and Prevention (CDC) light trap (Sudia & Chamberlain, 1962), which is a popularly used tool for capturing mosquitoes around the world, is inadequate in sampling *Ae. aegypti* as they are not attracted to the broad spectrum light produced from the incandescent bulbs of these trap (Service, 1963). The need to develop traps that can target *Ae. aegypti,* especially the virus infected ones, for effective surveillance and monitoring of pathogenic diseases can therefore not be overemphasized.

In recent years, technological advances have developed traps which use light emitting diode (LED) bulbs that unlike incandescent bulbs, emit super bright narrow bandwidth emissions of specific colors ranging from UV (350 nm) to infrared (700 nm) depending on the chemical composition of the LED (Constaedt *et al*., 2012). A previous study using red, green, blue, violet and a combination of blue-green-red (BGR) LEDs to sample Rift valley fever vectors in Ijara Kenya showed that *Aedes mcintoshi* and *Aedes ochraceus* had a seasonal preference for BGR and blue LEDs (Tchouassi *et al*., 2012). A solution would be provided if selected Light Emitting Diodes (LEDs) could be used to target *Ae. aegypti* which is usually underrepresented in the CDC light trap catches. A further viral testing on the collected mosquitoes would provide more insight on the diversity and the effect of viral infection on trap preference by the mosquitoes. The main objective of our study was to compare the attractiveness of different colored LEDs to *Ae. aegypti* relative to the CDC light trap in Busia and Kilifi counties of Kenya. We also tested for both *Flavi*- and *Alpha-virus* infections in *Ae. aegypti* collected by the various traps.

Methods

Study Sites

The study areas were Kilifi County at the Kenyan Coast and Busia County in Western Kenya (Figure 1). Previous seroprevalence studies had shown that dengue infection was prevalent in Malindi area of Kilifi, with chikungunya infection occurring in Busia

County (Mease *et al*., 2011). The most recent outbreak of chikungunya also occurred at the coast (Sergon *et al*., 2004).

Kilifi County has an average annual rainfall of 950 mm. The rainfall pattern is bimodal; the long monsoon rains (April - July) and the short rains (October- December). The annual temperatures range from a minimum of 21°C and a maximum of 32°C. Busia County on the other hand has an average annual rainfall of 1500 mm. The rainfall pattern is also bimodal; long rains (March - June) and short rains (October -December) with temperatures ranging from a minimum of 14°C to a maximum of 30°C.

In Busia County, traps were set up in villages in the rural areas namely Obekai (0 30.875 N, 34 12.293 E), Kamosin (0 31.530 N, 34 13.125E) and Kalwa (0 30.190 N, 3414.020E). These are locations that occur at approximately 1189m above the sea level (ASL). The main vegetation in these areas consists of large, tall eucalyptus trees that form thick canopies. The local inhabitants are mainly small-scale farmers growing maize, millet and cassavas as food crops while a few grow sugarcane and coffee as cash crops. They also keep animals mainly cattle, sheep, goats, pigs, chicken and guinea fowls.

In Kilifi County, traps were set up at two sites located in the urban area; Kenya Medical Research Institute (KEMRI) campus in Kilifi (3 37.800 S, 39 51.483 E) and Mnarani estate (3 38.368 S, 39 50.824 E) while the other site was in the Kaya Kauma forest (3 37.183 S, 39 44.167 E).These are locations that occur at approximately 30.5 m ASL. The inhabitants in the urban area mainly engage in small businesses or work in offices. They also grow maize, cassava and sweet potatoes and keep mainly goats.

The traps were set up during the wet seasons at both sites. In Kilifi, the traps were set up in April 2012 and June 2012 while in Busia they were set up in December 2012 and April 2013.

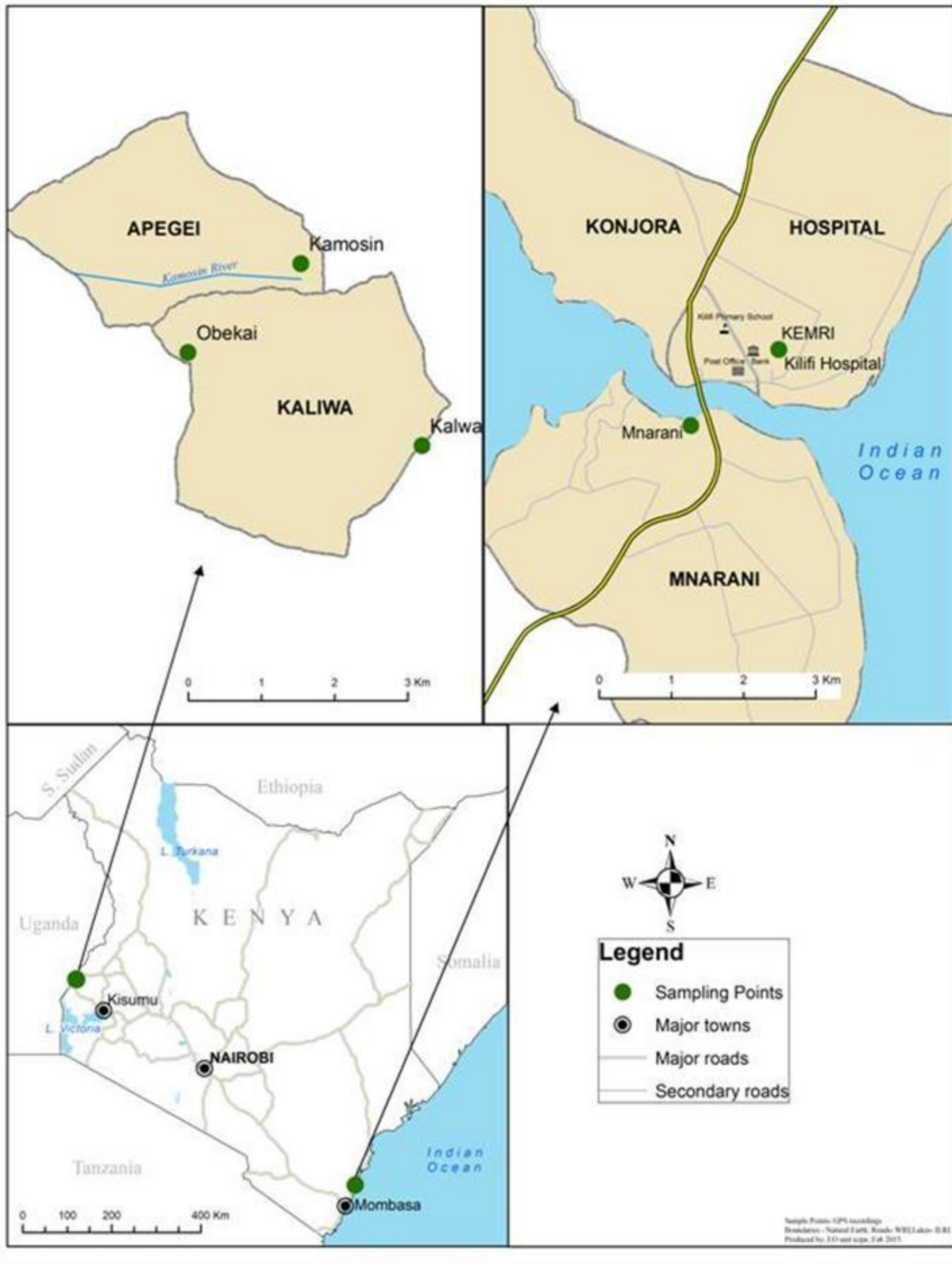

Figure 1. Trapping sites in Busia and Kilifi counties in Kenya

Study Design

Screening LED's

Latin square design was used to compare the efficacy of four Light Emitting Diodes (LEDs) in sampling *Ae. aegypti* against a standard CDC light trap. The LEDs were blue (2770B430, 430 nm), green (2770 G570, 570 nm), violet (2770UV, 390 nm) and a mixture of blue, green and red (BGR) (BioQuip Products 2321 Gladwick Street Rancho Dominguez, CA 90220, USA, while the control was a 1.5 watt incandescent bulb CDC light trap (John Hock). Each of the blue, the green and the violet LED had 8 LEDs of the same color (arranged in a circular alignment) to provide 360-degree coverage in the horizontal plane with each LED having a viewing angle of 45 degrees. The BGR LED trap contained 3 green, 3 blue and 2 red LEDs (BGR).

Five different sampling sites were randomly chosen both in Busia and in Kilifi. In Busia two traps were set in Obekai village, two in Kamosin village and one in Kalwa village. Whereas in Kilifi, two traps were set at the Kenya Medical Research Institute (KEMRI) grounds, two others at Kilifi hospital grounds and one trap was set in Mnarani village. A minimum distance of 100 meters was maintained between the traps. Each trap was hung half a meter above the ground and was baited with carbon dioxide in the form of dry ice dispensed from Bioquip) igloo coolers (BioQuip Products 2321 Gladwick Street Rancho Dominguez, CA 90220, USA) that were hung next to the traps [Figure 2]. All traps were set and left to run for 24 hours daily. To account for positional effects, traps were rotated every experimental day such that after five days all traps would have rotated once at each of the five sampling points. The sampling was done for a total of ten days. At the end of the 24 hour sampling period, trapped mosquitoes were collected from each trap and transported to the laboratory where they were freeze killed on dry ice and identified morphologically under dissecting microscopes to species level using appropriate keys (Edwards, 1941; Gillies & De meillon, 1968; Huang, 1981;Rueda, 2004).

Viral testing

Ae. aegypti mosquitoes were placed into pools (≤ 25 mosquitoes per pool) after which each pool was homogenized in 1.5 mL micro centrifuge tubes by adding one 4.5 mm copper bead and 500 μl of minimum essential medium (MEM) supplemented with 2% fetal calf serum, 2 mM glutamine, antibiotic mixture (fungizone, 100 U/ml penicillin, and 100 U/ml streptomycin) to each pool and homogenized in a Mini Bead Beater 16 (BioSpec, Bartlesville, OK). The homogenates were clarified by centrifugation at 4°C and 13,000 rcf for 10 minutes, and the resulting supernatants immediately processed further. Viral RNA was extracted from 200 μl of the mosquito homogenates in an automated MagNa Pure 96 extraction system (Roche Applied Science, Penzberg, Germany) using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche). The RNA extracts was immediately reverse transcribed (RT) into cDNA in 10 μl reaction volumes consisting of 0.5 μl of water, 2.0 μl of Buffer, 1 μL of dNTPs, 0.25 μl of inhibitor, 1.0 μl of random hexamer primers, 0.25 μl of High Capacity Reverse Transcriptase (Life Technologies, Carlsbad, California). Synthesis of cDNA was performed in 10 μl reaction volumes, using 5 μL of extracted RNA samples as templates that were incubated in the Dyad Peltier Thermal Cycler (BIO-RAD) at 25°C for 10 minutes followed by 55°C for 30 minutes and 85°C for 5 minutes.

For primary *Flavivirus* Polymerase chain reaction (PCR) amplifications, we used primers that flank the non-structural protein 5 (NS5) gene (1NS5F, 1NS5Re) (Vasquez *et al*., 2012) supplemented with a modified forward primer (1NS5Fb) (Moreau *et al*., 2007) (Table 1). This was done to enrich target genes for enhanced peak visualization in the subsequent nested PCR-HRM analysis. To minimize the degeneracy required for the nested short fragment universal-*Flavivirus* primers, we mixed four forward primer sequence variations (uni-Flavi-F a-d) and two reverse primer sequences (uni-Flavi-R a & b) (Table 1). The master mix for each sample (2μL) consisted of 1.5 μl of water, 5.0 μl of QIAGEN Multiplex PCR Master Mix (QIA mix), 1.0 μl of QIA solution, and 0.5 μl each of the forward and reverse universal-*Flavivirus* primer mixes.

Samples that tested positive for *Flavivirus* in this nested PCR-HRM assay, based on melting analysis of PCR products, were then amplified from primary PCR products using primers that amplify ~950 bp of the full non-structural protein 5 (NS5) gene (2NS5F, 2NS5Re) (Table 1). PCR products were forward and reverse sequenced at Macrogen (Seoul, Korea).

All *Flavivirus* reactions were performed in 10 µl reaction volumes containing 5.8 µl water, 2 µl 5x Hot FIREPol® EvaGreen® HRM Mix (no Rox, Solis Biodyne, Estonia), 0.3 µL NS5F primer, 0.3 µL modified NS5F primer, 0.6 µL NS5Re primer (Table 1), and 1 µl of cDNA template. The PCR cycling conditions consisted of 30 cycles at 95°C for 15 minutes, 94°C for 20 seconds, 48°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes.

For *Alphavirus* detection, singleplex PCR was run on the primary PCR products using Vir 2052 forward and reverse primers (Eshoo *et al*., 2007) (Table 1). The master mix for each sample (2 µl) consisted of 1 µl of water, 5 µl of 2 × MyTaq HS master mix (Bioline, London, UK), 1 µl of 50 µM SYTO-9 saturating intercalating dye (Life technologies), and 0.5 µl each of the forward and reverse universal-*alphavirus* primer mixes.

Phylogenetic analysis

Viral sequences were visually inspected and aligned in MEGA version 6.0 (Edgar & Robert, 2004)]. Sequence alignments were performed using MUSCLE in MEGA 6.0 (Tamura *et al*., 2013) using the default parameters of the program. The sequences were named as Isolate I and isolate II. Distance trees (dendograms) of only haplotypes from the sequenced samples and haplotypes of other cell fusing agent viruses from other parts of the world e. g Kamiti virus accession number(AB488430) and Surabaya virus accession (NC005064) were inferred using the neighbor-joining (NJ) algorithm in MEGA, following the Tamura-Nei model (Tamura & Nei, 2007). The tree was based on 1000 replicates. Specific parameters are available from the authors on request.

Figure 2. A set up of light traps. Panel A, standard CDC light trap. Panel B, light emitting diode (LED) CDC trap.

Statistical analyses

The statistical package R 3.1.0 was used (Core Team R, 2013). Mosquito counts were compared using generalized linear models, whereby the odds that mosquitoes chose a treatment (a colored light emitted by the different LEDs against the control (Incandescent bulb). The chi-square test was applied to evaluate differences between proportions of male and female *Ae aegypti* per trap and differences between proportions of *Flavivirus* positive and negative *Ae. aegypti* per treatment trap and the control. All statistical inferences were drawn on two-tailed distributions with $\alpha = 0.05$.

Table 1. List of Primers used in viral testing of samples from Busia and Kilifi counties of Kenya

Gene/Target Protein	Primer Name	Direction	Primer sequence
NS5,	1NS5Fb primer,	Forward	5'-CGCCGGATCCGCGGCCGCATGAGCG-3'
NS5	1NS5F primer	Forward	5'-GCATCTAYAWCAYNATGGG-3'
NS5	1NS5Re primer	Reverse	5'-CCANACNYNRTTCCANAC-3'
NS5	uni-Flavi-F a	Forward	5'-AGCCGYGCCATHTGGTATATGTGG-3'
NS5	uni-Flavi-F b	Forward	5'-AGYCGMGCAATHTGGTACATGTGG-3'
NS5	uni-Flavi-F c	Forward	5'- AGTAGAGCTATATCGTACATGTGG-3'
NS5	uni-Flavi-F d	Forward	5'-AGYMGHGCCATHTCGTWCATGTCC-3'
NS5	uni-Flavi-R a	Reverse	5'-GTRTCCCAKCCWGCTGTGTCGTC-3'
NS5	uni-Flavi-R b	Reverse	5'-GTRTCCCADAADGCDGTRTCATC-3'
NS5	2NS5F	Forward	5'-GCNATNTGGTNYATGTGG-3'
NS5	2NS5Re	Reverse	5'TRTCTTCNGTNGTCATCC-3'
NSP4	Vir 2052 F	Forward	5′-TGG CGC TAT GAT GAA ATC TGG AAT GTT-3′
NSP4	Vir 2052R	Reverse	5′-TAC GAT GTT GTC GTC GCC GAT GAA-3′

Results

Comparison of Aedes *trapping efficiency of CDC traps with different LED spectra*

A total of 345 *Ae. aegypti* were caught; 62 in Busia and 283 in Kilifi (Figure 3). Overall, the traps caught significantly higher numbers of *Ae. aegypti* in Kilifi than in Busia [χ_1^2 = 268.46, p<0.001].

In Busia, the violet trap collected significantly more *Ae. aegypti* than the control (CDC light trap) [IRR=1.93, CI: 1.05-3.70, p=0.038], while the blue, the green and the BGR LEDs collected fewer *Ae. aegypti* than the control [blue: IRR=0.330 CI= 11-0.86; green: IRR= 0.33, CI: 0.11-0.8, BGR: IRR= 0.130 CI: 02-0.47] (Figure 2). The order of trap performance was violet>control>blue=green>BGR (Table 2). When trap captures were compared in Kilifi, all the LEDs collected less *Ae. aegypti* than all the control with IRRs<1. The order of trap performance in Kilifi collection was control>RBG>violet>blue>green (Table 2).

Viral testing

Only six samples were identified as *Flavivirus* positive from HRM curves (Figure 4) while no alphaviruses were detected in the samples. The samples in which flaviviruses were detected were, EU 28, EU 34, EU 57, EU 58 EU 71 and EU 75. All the samples were trapped from Busia. The violet trap captured 4 positive samples - EU 28, EU 34, EU 57 and EU 58 while the CDC light trap and the green LED trap captured one positive sample each - EU71 and EU 75 respectively. The blue and the BGR did not capture any viral positive samples (Table 3). A comparison between the proportions of viral positive samples captured by the violet LED and the control showed that the violet LED captured a significantly higher proportion of viral positive *Ae. aegypti* than the control (CDC light) trap in Busia [p<0.001] while there was no significant difference between the viral positive proportions of the green LED and those of the control trap (Table 3) although the 969 bp NS5 gene sequences of samples EU58 group among

other ISFs isolated from East Africa such as Kamiti River virus and Nakiwogo Virus (Figure 5).

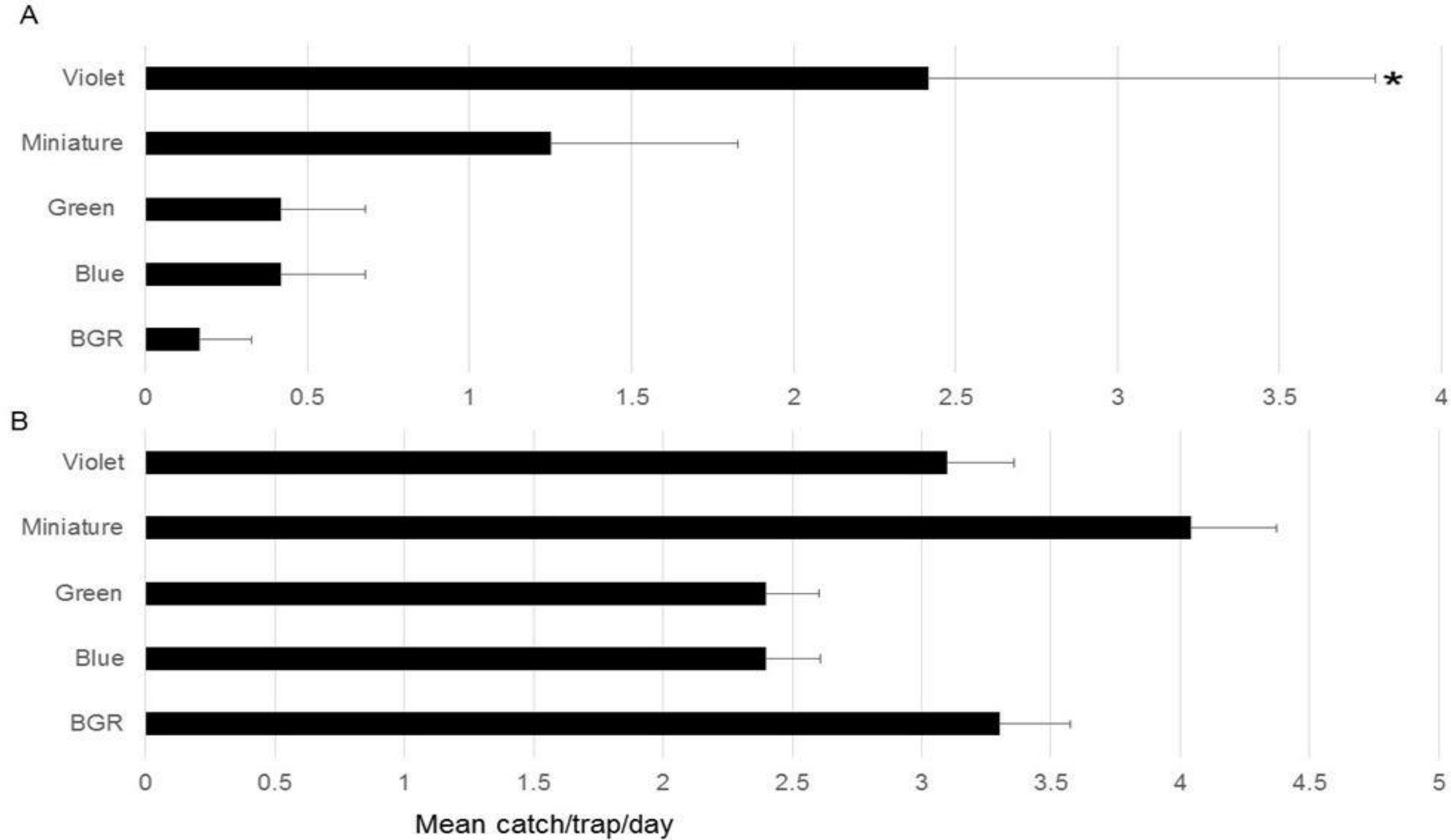

Figure 3. The mean number of *Ae aegypti* captured by the various light traps per night in Busia and Kilifi counties. Panel A – Busia, Panel B- Kilifi. Miniature-control (CDC light trap). Asterisks indicate that the mean catch of the LED bulbs significantly different from the mean catch of the control.

Table 2. Comparisons of mosquito collections by the various colored LEDs relative to the control (standard CDC light trap with incandescent (Miniature bulb) trap in Busia and Kilifi counties of Kenya

Site	Treatment	IRR(95%CI)	P value	Site	Treatment	IRR(95%CI)	P value
Busia	Blue light (6)	0.33(0.11-0.86)	0.033	Kilifi	Blue light (44)	0.603 (2.1- 8.95)	0.334
Busia	Green light (6)	0.33(0.11-0.86)	0.033	Kilifi	Green light (43)	0.589 (0.21- 1.67)	0.313
Busia	RBG (3)	0.13(0.02-0.47)	0.007	Kilifi	RBG (64)	0.822(0.29- 2.30)	0.705
Busia	Violet (30)	1.93(1.05-3.70)	0.038	Kilifi	Violet (56)	0.767(0.27- 2.15)	0.609

Estimated incidence rate ratio (IRR); confidence interval (CI) and corresponding P-values based on comparison to the control (standard CDC light trap with incandescent (Miniature bulb) following generalized linear model (GLM) with negative binomial error structure in R 3.1.0 software. The IRR for the control is 1; values above this indicate better performance while values below indicate under performance relative to the control. Values in parenthesis in the treatment column are the absolute number of mosquitoes caught by the various trap treatments. The control (standard CDC light trap with incandescent (Miniature bulb) catches; Busia (17), Kilifi (76).

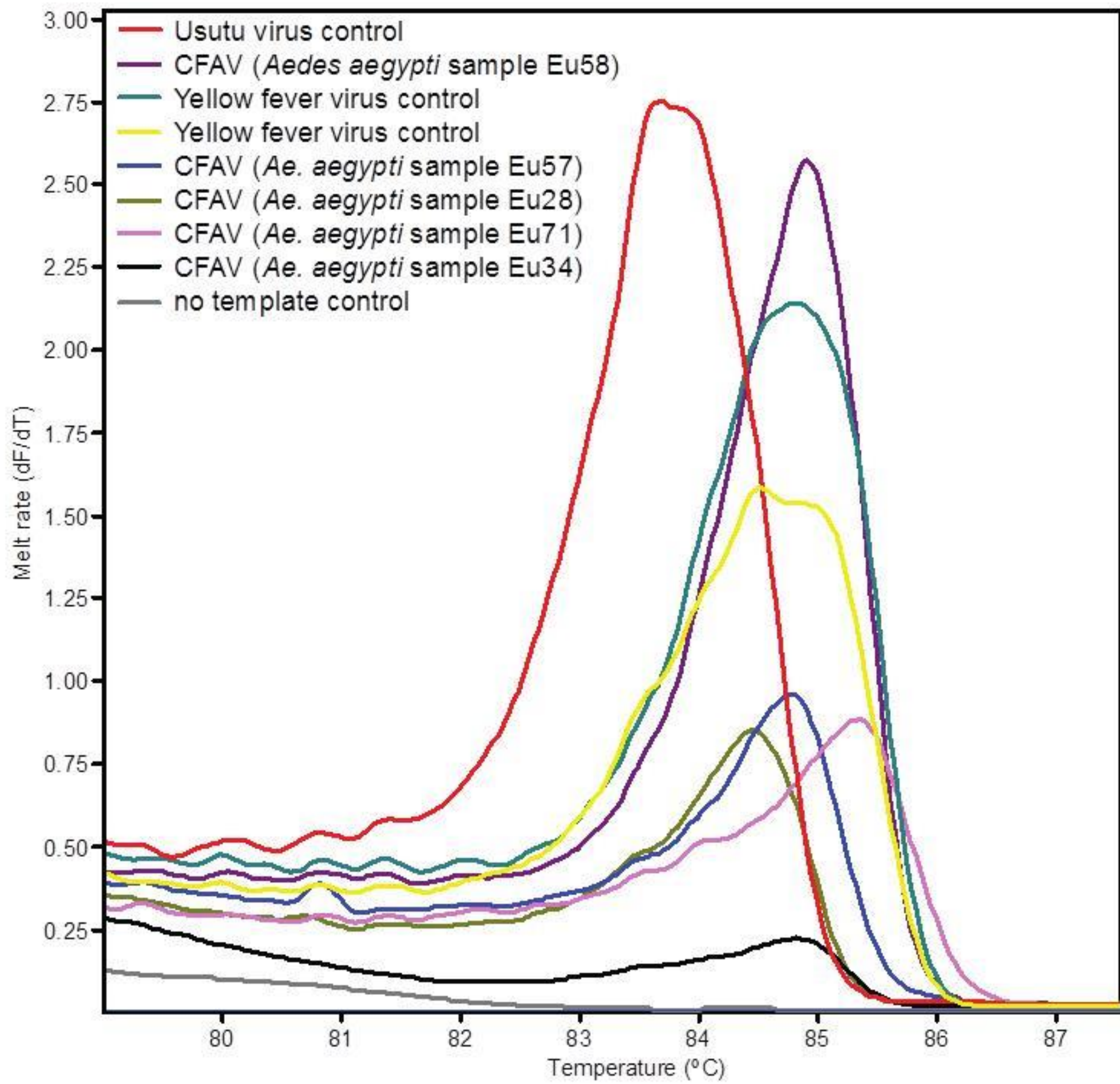

Figure 4. Melt rate curves formed by *Flavivirus* positive samples after nested PCR-HRM

Table 3. Percentages of samples that tested viral positive out of the number of samples tested per trap in Busia and Kilifi counties of Kenya

Bait	Site	No of samples	Percentages +	p-Values	Site	No of samples	Percentages +	p-Values
CDC light	Busia	8	12.5^{a}(1)	-	Kilifi	16	(0)0	-
Blue light	Busia	2	0	-	Kilifi	15	(0)0	-
Green light	Busia	4	12.5^{a}(1)	1	Kilifi	10	(0)0	-
BGR light	Busia	4	0	-	Kilifi	1	(0)0	-
Violet light	Busia	7	60^{b}(4)	<0.001*	Kilifi	12	(0)0	

Percentages following each other in the rows with different letters (a and b) are significantly different from each other. Asterisks on p values indicate significant difference with the control (standard CDC light trap with incandescent (Miniature bulb). The P-values are based on pair-wise comparison following chi-square goodness-of-fit in R 3.1.0 software.

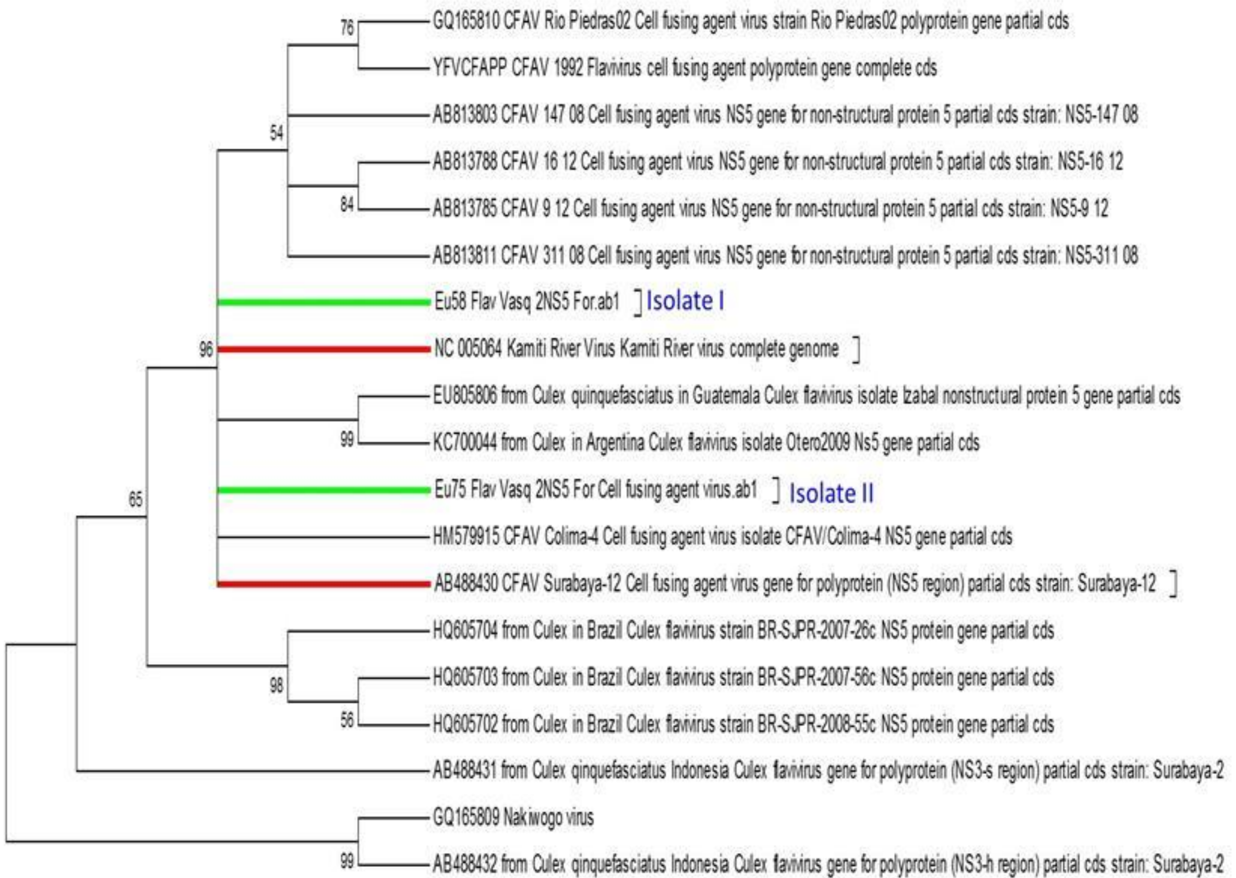

Figure 5. The phylogenetic analysis of haplotypes samples EU 58 and EU 75. The haplotypes are indicated as isolates and groups with other already identified ISF haplotypes, Kamiti virus - NC0056064 and Surabaya virus AB488430 with a bootstrap support of 96%indicating that they were closely related.

Discussion

Our results clearly showed that the violet light was the most preferred light trap by *Ae. aegypti* in Busia as it captured significantly higher numbers of *Ae. aegypti* than the CDC light trap and all the other LED bulbs. This is the first time according to our knowledge that an LED light has attracted more mosquitoes than the CDC incandescent bulb. Previous studies by Tchouassi *et al* 2012 (Tchouassi *et al*., 2012) while investigating whether LEDs could be effective substitutes for incandescent CDC light traps in the surveillance of important Rift Valley Fever (RFV) vectors in Ijara district Kenya, reported that no LED light outcompeted the CDC light traps in capturing these vectors.

The significantly higher captures of *Ae. aegypti* using violet light bulbs could be explained by a strong preference of ultraviolet light reported in previous electroretino-graphs (ERGs) studies that showed that *Ae. aegypti* (L.) have spectral sensitivities ranging from ultraviolet (323 nm) to orange-red (621 nm) with sensitivity peaks in both the ultraviolet (345 nm) and green (523 nm) wave-lengths (Muir *et al*., 1992). Furthermore, in other studies it was established that LED lights under some circumstances can attract substantially more insects than incandescent bulbs. For example, in a paired trap comparison in the tropical forests of French Guiana, 4-bulb LED combo configuration trap collected 30% more sand flies than the incandescent traps. In a second comparison in dry forest habitats in Colombia, 2 stacked LED lighting chips (16 LED bulbs) attracted 50% more sand flies than the incandescent (Constaedt *et al*., 2008).

We also observed that the light traps generally captured more *Ae. aegypti* mosquitoes in Kilifi than in Busia. This could have been due to the impact of microhabitat/ecological difference between our study sites. While Busia had a lot of tall trees providing heavy vegetation cover, Kilifi was less forested. Therefore, the tall trees that provide a heavy vegetation cover in Busia could have reduced the brightness of the LEDs and thus the distance from which they were attractive to mosquitoes. Barr *et al* (Barr *et al*., 1963) when using several colored light bulbs of different intensities to capture mosquitoes (*Anopheles* and *Aedes* species), determined that light intensity played a significant role

with higher intensity lights (100 W lamps) being more attractive than lower intensity lights (60 W and 25 W lamps). As such, the intensity of the light from the LEDs perceived by the mosquitoes in the two ecological different environments need to be considered.

Furthermore, we believe that as an urban area, a higher population density in Kilifi could have contributed to a higher abundance of *Ae. aegypti*. Previous studies observed that *Ae. aegypti* mosquitoes are highly domesticated and adapted to the urban environment (Chan, 1985) where its life cycle transpires mainly inside and around human residences (Kamgang *et al*., 2010) breeding in containers like water tanks, pots and flower vases (Christophers, 1960; Southwood *et al*., 1972). On the other hand, the Busia sampling sites which are rural would provide the opposite situation. A study in Brazil on the distribution of *Ae. aegypti* in a rural, suburban and urban areas showed that *Ae. aegypti* females and males were mostly captured in urban areas (56%) and indoors (78%), suggesting a preference by this species to rest inside houses and in areas with high human density, a behavior that favors vector-human contact (Lima-Camara, 2006). The same results were reported by Tsuda *et al*., 2006 who studied the distribution of *Ae. aegypti* and *Aedes albopictus* along an urban rural gradient in Thailand (Tsuda *et al*., 2006).

Our results also showed that all the viral positive mosquitoes were caught in Busia by mainly the violet trap. However, a longitudinal study is needed to establish whether this occurs across seasons or not. This would help to confirm whether there was a preference for the violet LEDs by virus infected *Ae. aegypti*. Previous reports established that during viral infection in mosquito vectors, the infection could occur not only in the salivary glands and the midgut, but also spreads and amplify in the neural tissue highly affecting the visual system (Linthicum *et al*., 1996; Salazar *et al*., 2007). Due to the fact that insect-specific viruses are phylogenetically closely related to and may represent earliest forms of pathogenic flaviviruses like dengue (Cook & Holmes, 2006; Hoshino *et al*., 2007) further studies would be necessary to establish whether pathogenic flaviviral infection alters color preference in virus infected mosquitoes because this has the potential to combat disease transmission. If infection with

pathogenic flaviviruses such as dengue, yellow fever and West Nile viruses could lead mosquito vectors to prefer certain colors, then colored traps may effectively be used in the surveillance and monitoring of the transmission of these diseases.

Phylogenetic analyses revealed that the ISFs from our study grouped together with previously described insect specific flaviviruses like the Kamiti virus which was also isolated in Kenya and was the first insect-only *Flavivirus* to be isolated in nature (Sang *et al*., 2005) and the cell fusing agent virus (CFAV) which was the first ISF to be ever isolated from laboratory reared *Ae. aegypti* (Cook & Holmes, 2006). This suggests that the ISFs just like the Kamiti virus might have a high infection rate in *Ae aegypti* and might be transmitted by vertical transmission in this mosquito species (Lutomiah *et al*., 2007). The close link of the viruses with other described ISFs group could also suggest possible interactions with other viruses that cause disease in humans, such as dengue virus. It has been observed before that despite their non-pathogenicity to humans and animals, insect-specific flaviviruses may either enhance (Kent *et al*., 2010; Newman *et al*., 2012) or suppress (Bolling *et al*., 2012; Hobson *et al*., 2013) the transmission of pathogenic viruses like West Nile viruses.

No alphavirus infected *Ae. aegypti* were detected in the samples from both of the two sites, Busia and Kilifi. This could be explained by the fact that probably no alpha viruses were circulating at that moment in both sites or could also be due to the fact that naturally more flaviviruses are transmitted and therefore detected in *Ae. aegypti* than alpha viruses. While 68 flaviviruses have been recognized, with an approximately one-third of which are medically important human pathogens only 27 alpha viruses have been recognized (Schmaljohn & McClain, 1996).

Conclusions/recommendations

The violet light has the potential to be used as an effective surveillance and monitoring tool for arboviral diseases transmitted by *Ae aegypti.* However, since we only got significant results from Busia that had actually lower densities of the vector, the response of *Ae. aegypti* to the violet light should be investigated further in different

regions and across seasons to establish its efficacy. Future studies should also concentrate on determining how insect-specific flaviviruses may interact with arboviruses like dengue and chikungunya in a co infected mosquito and how this may potentially impact vector competence.

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Chapter 3

Field evaluation of natural human odors and the biogent-synthetic lure in trapping *Aedes aegypti*, vector of dengue and chikungunya viruses in Kenya

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Abstract

Background

Methods currently used in sampling adult *Aedes aegypti*, the main vector of dengue and chikungunya viruses are limited for effective surveillance of the vector and accurate determination of the extent of virus transmission during outbreaks and inter - epidemic periods. Here, we document the use of natural human skin odors in baited traps to improve sampling of adult *Ae. aegypti* in two different endemic areas of chikungunya and dengue in Kenya – Kilifi and Busia Counties. The chemistry of the volatiles released from human odors and the Biogent (BG)-commercial lure were also compared.

Methods

Cotton socks and T-shirts were used to obtain natural human skin volatiles from the feet and trunk of three volunteers (volunteers 1 and 2 in Kilifi and volunteers 2 and 3 in Busia). Using Latin square design, we compared the efficacies of BG sentinel traps baited with carbon dioxide plus (a) no bait, (b) human feet volatiles, (c) human trunk volatiles each against (c) a control (Biogent commercial lure) at the two sites. Coupled gas chromatography-mass spectrometry (GC-MS) was used to identify and compare candidate attractants released by the commercial lure and human odors.

Results

Ae. aegypti captured in the trap baited with feet odors from volunteer 2 and trunk odors from the same volunteer were significantly higher than in the control trap in Busia and Kilifi respectively, [IRR= 5.63, 95% CI: 1.15 - 28.30, p= 0.030] and [IRR= 3.99, 95% CI: 0.95-16.69, p=0.049]. At both sites, *Ae. aegypti* captures in traps baited with either the feet or trunk odors from volunteers 1 and 3 were not significantly different from the control. Major qualitative differences were observed between the chemical profiles of human odors and the commercial BG-lure. Aldehydes, fatty acids and ketones dominated human odor profiles, whereas the BG-lure released mainly hexanoic acid.

Conclusions

Our results suggest that additional candidate attractants are present in human skin volatiles which can help to improve the efficacy of lures for trapping and surveillance of *Ae. aegypti*.

Keywords

Aedes aegypti, Dengue, Chikungunya, Human odor, Mosquito, Traps

Background

Aedes aegypti is one of the most important disease vectors worldwide. It is the principal vector of dengue (Gubler, 1989), chikungunya (Halsted *et al.*, 1967) and yellow fever (Monath, 1989) viruses. Among arboviral diseases, dengue fever has been reported to cause more human morbidity and mortality than any other arthropod-borne viral disease (Gubler, 2002; Gubler, 1997). It is estimated that each year, 50–100 million dengue infections and several hundred thousand cases of dengue hemorrhagic fever (DHF) occur, depending upon epidemic activity (WHO, 2006; 2009). In the past 10 years, there have been sporadic outbreaks of chikungunya fever along the Kenyan coast and the Indian Ocean islands of the Comoros, Seychelles, Reunion and Mauritius (Powers& Logue, 2007; Chreiten *et al.*, 2007; Sergon *et al.*, 2008; Sang *et al.*, 2008). Additionally, in Kenya, a dengue outbreak was reported in Mandera County in September 2011 (Standard newspaper, 2011) and more recently in Mombasa County in May 2014 (Standard newspaper, 2014).

The increase in the emergence of dengue and chikungunya fever has been attributed to climate change (Chreiten *et al.*, 2007) urbanization (Gubler *et al.*, 1997; 2002; Alirol *et al.*, 2009, Beatty *et al.*, 2011] and globalization (Gubler *et al.*, 1997; 2002; Ensenrik, 2007), amongst other factors. Consequently, the projected trends of continued global warming, urbanization and globalization will ensure that the incidence of these diseases will increase, especially if interventions are not forthcoming (Gubler, 2002; Alirol *et al.*, 2009). Presently, there is no registered

vaccine for prevention of dengue and chikungunya viruses which makes vector control the only available target for disease control and prevention. Under the circumstances, it is important to monitor the viruses and vector populations in endemic areas to understand their ecology before implementing appropriate and timely intervention. This therefore, calls for efficient surveillance and monitoring tools that will give reasonably accurate measures of disease and vector abundance data to guide decision on disease control measures.

The simplest and most effective sampling method for adult *Ae. aegypti* has been human-landing collections (Service, 1993; Focks, 2003). Although effective in determining the exact anthropophilic species composition, human attack rate, and potential for disease transmission, renders this method inappropriate because it exposes the collectors to a degree of risk to infection and is also labor intensive. On the other hand, the popularly used mosquito surveillance trap, the Centers for Disease Control and Prevention (CDC) light trap (Sudia & Chamberlain, 1962) is virtually ineffective in sampling the day biting *Ae. aegypti* as it targets nocturnal host seeking species (Service, 1993).

Odor-baited traps provide an effective means for monitoring insect populations. A recent study demonstrated the efficacy of an odor-baited trapping system for mosquito vectors of Rift Valley Fever virus (Tchouassi *et al.*, 2012; 2013). The Biogent (BG) sentinel trap baited with synthetic human skin compounds consisting of lactic acid, ammonia, and hexanoic acid (caproic acid) was used for sampling *Ae. aegypti* (Geir *et al.*, 2006). However, considerable reports have suggested that synthetic odors (Canyon &Hii, 1997; Jones *et al.*, 2003; Schoeler *et al.*, 2007) or extracted human component blends (Bernier *et al.*, 2007) do not attract *Ae. aegypti* at a level comparable to natural human odors. Evaluating the effectiveness of the BG synthetic lure against natural human odors at different sites would therefore be critical for its wide scale use in disease vector control, especially *Aedes sp* vectors of chikungunya and dengue viruses.

In this study our objective was to compare the attractiveness of the commercial BG lure with natural human odors from two different sources, feet and trunk, in trapping *Ae. aegypti* in the field. We carried out this study in two dengue and chikungunya virus endemic areas in Kenya. Since mosquitoes are attracted to volatiles released

from the different treatments, we compared the composition of these volatiles in order to identify the candidate attractants from the different treatments.

Methods

Study sites

The study areas were Kilifi County at the Kenyan Coast and Busia County in Western Kenya (Figure 1). Previous seroprevalence studies had shown that dengue infection was prevalent in the Malindi area of Kilifi, with chikungunya infection occurring in Busia County (Mease *et al.*, 2011). The most recent outbreak of chikungunya also occurred at the coast (Sergon *et al.*, 2004)

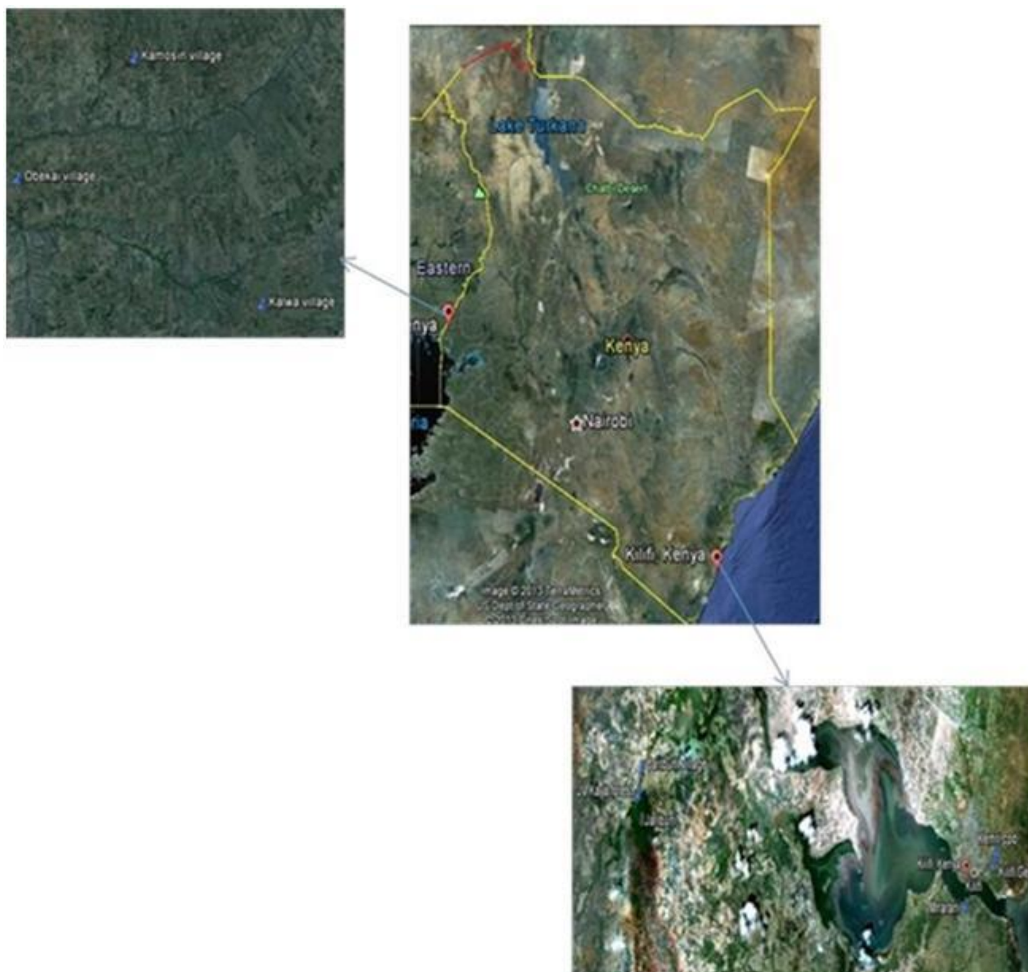

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya

Kilifi County has an average annual rainfall of 950 mm. The rainfall pattern is bimodal; the long monsoon rains (April - July) and the short rains (October-December). The annual temperatures range from a minimum of 21 °C and a maximum of 32 °C. Busia County on the other hand has an average annual rainfall of 1500 mm. The rainfall pattern is also bimodal; long rains (March - June) and short rains between (October -December). The temperatures range from a minimum of 14 °C and a maximum of 30 °C.

In Busia County, traps were set up in villages in the rural areas namely Obekai (30.875 N, 34 12.293 E), Kamosin (0 31.530 N, 34 13.125E) and Kalwa (0 30.190 N, 3414.020E). These are locations that occur at approximately 1189 m above the sea level (ASL). The main vegetation in these areas consists of large, tall eucalyptus trees that form thick canopies. The local inhabitants are mainly small-scale farmers growing maize, millet and cassava food crops while a few grow sugarcane and coffee as cash crops. They also keep a few animals mainly cattle, sheep, goats, pigs, chicken and guinea fowls.

In Kilifi county, traps were set up at two sites located in the urban area; Kenya medical research institute (KEMRI) campus in Kilifi (3 37.800 S, 39 51.483 E) and Mnarani estate (3 38.368 S, 39 50.824 E), while the other site was in the Kaya Kauma forest (3 37.183 S, 39 44.167 E). These are locations that occur at approximately 30.5 m ASL. The inhabitants in the urban area mainly engage in small businesses or work in offices. They also grow maize, cassava and sweet potatoes and keep a few animals, mainly goats.

The traps were set up during the wet seasons at both sites. In Kilifi, the traps were set up in April 2012 and June 2012 while in Busia they were set up in December 2012 and April 2013.

Study design

A Latin square design was used. At each sampling location, Kilifi or Busia, the efficacy of the BG sentinel trap baited with carbon dioxide plus (i) the BG commercial lure, (ii) cotton socks or T-shirts worn by two volunteers in Kilifi and two volunteers in Busia and (iii) no bait, were set.

Odor collection and mosquito sampling with odor-baited traps

Odors were obtained from the feet and trunk of three male volunteers (volunteers 1, 2 and 3) aged between 25–50 years. Trunk and feet odors from volunteer 1 were used as baits in Kilifi and that from volunteer 3 in Busia while that from volunteer 2 were used in both Kilifi and Busia. The same individuals were involved throughout the study. Socks and T-shirts worn by volunteers 1 and 2 were used to sample mosquitoes in Kilifi while those worn by volunteers 2 and 3 were used to sample mosquitoes in Busia. The volunteers were requested to put on new, clean, 100% cotton socks and T-shirts (Lux Industries Ltd 39 K.K Tagare st, Kolkata-700-007) for 18 hrs daily to trap odors from their feet and trunk for nine 12 days. After 18 hrs each day, the volunteers removed the socks and T-shirts which were used to bait BG sentinel traps by hanging them on the rails of the BG sentinel trap inner structure as shown in (Figure 2). During this period and prior to wearing the socks and T-shirts, the volunteers were provided with an odorless soap daily for bathing and were requested to avoid the use of deodorants and/or perfumes.

Figure 2. The BG sentinel traps were baited with socks and set up in Busia and Kilifi counties of Kenya. Assembly follows steps a-h

Mosquito sampling with odor baited traps

Four different sites were randomly chosen around homesteads after obtaining oral consents from the homestead heads. Four BG sentinel traps baited with the commercial lure, socks or T-shirts worn by the different volunteers and no baits were randomly set up at each of the four sites with a distance of at least one hundred (100 m) between traps. The traps were hung at 0.2 m above the ground and attached to each was a Bioquip igloo that dispensed carbon dioxide in the form of dry ice (Figure 2). To account for positional effects, traps were rotated every experimental day. This was repeated for 12 days.

Because some sites were at a distance of up to 40 km apart in both Busia and Kilifi, traps were set up at each site at different times of the day and left to run for 24 hrs. Mosquitoes were then collected and transported to the laboratory where they were freeze-killed and identified under a dissecting microscope to species level using morphological keys (Edwards, 1941; Gillies & De Meillon, 1969; Huang & Ward,

1981; Rueda, 2004). Mosquitoes were categorized as engorged when blood fed or gravid based on observation of their abdominal condition as described in the WHO Manual (WHO, 2003). Daily mosquito counts per trap were recorded for each mosquito species.

Collection and analysis of volatiles

In order to analyze and compare the composition of volatiles released by the commercial lure and the human odors, headspace volatiles from the commercial BG-lure and from the three volunteers' feet and trunks were collected using solid phase micro- extraction (SPME) technique for 6 hrs at room temperature. Odors were also trapped and analyzed from unused 100% cotton socks and T-shirts, which acted as control. The odors were adsorbed on 75 µm carboxen-poly dimethyl siloxane (CAR/PDMS) and 50/30 µm Divinyl benzene/ Carboxen/ Poly dimethyl siloxane (DVB/CAR/PDMS) (Supelco: Sigma-Aldrich Pty Ltd, Bellefonte, USA) fibers. The fibers were each conditioned at 270 °C for 1 hr before use.

After extraction the SPME fibers were injected into the gas chromatography - mass spectrometry (GC-MS) and mass selective detector (MSD) system consisting of a model HP 7890A gas chromatograph, a 5975 Mass spectrometer with a triple Axis detector and an Agilent ChemStation data system. The GC column was a Carbowax HP-20 with 20% Carbowax stationary phase (30 m × 250 µm × 0.25 µm film thickness). The carrier gas was helium with a column head pressure of 8.8271 psi and flow rate of 1.2 ml/min. Inlet temperature was 220 °C and mass selective detector temperature was 230 °C. The oven temperature was held at 35 °C for 5 min, a rise of 10 °C min^{-1} to a final temperature of 220 °C, which was held for 20.5 min. The identity of compounds in the volatiles was determined by comparison with references from mass spectral libraries (NIST05, Agilent Technologies [NIST database, G1033A, revision D.05.01, ChemStation data system (G1701EA, version E.02.00) and SPME analysis of a mixture of the authentic compounds. Each compound in the authentic mixture was 100 ng/ul.

The chemicals were; hexanoic acid, hexanal, octanal, nonanal, decanal, 6-methyl-5-hepten-2-one, geranylacetone (Sigma-Aldrich Chemie GmbH, Germany), 3-methylbutyric acid and 2-methylpropionic acid (Sigma-Aldrich Corporation, 3050

Spruce Street, St. Louis, Missouri 63103 USA). Purities of the compounds ranged between 95% and 99%.

Data analysis

The daily mosquito counts in the different traps were subjected to negative binomial regression following the generalized linear models (GLM) procedures in R 3.1.0 (Core team R 2014). The BG commercial lure baited trap was used as the reference category. The incidence rate ratios (IRR) - a likelihood measure that mosquito species chose other treatments instead of the control - and corresponding P-values were estimated. The chi-square test was applied to evaluate differences between proportions of male and female *Ae. aegypti* per trap and differences between proportions of fed and gravid mosquitoes per a treatment trap and the control. The tests were performed at 5% significance level.

Ethics statement

The study was approved by the national ethics review committee based at the Kenya Medical Research Institute (KEMRI) and informed consent was obtained from each of the participants.

Results

Mosquito sampling with odor baited traps

A total of 1,989 *Ae. aegypti* were collected, 1,805 in Kilifi and 184 in Busia. Overall, we found a significant variation in trap captures of *Ae. aegypti* based on location [$X^2 = 332.35$, d.f = 1, $p < 0.001$], with higher trap captures recorded in Kilifi than Busia for the same number of days [IRR = 9.81, 95% CI: 5.8-16.6, $p < 0.001$].

In Kilifi, the trap baited with trunk volatiles from volunteer 2 trapped a significantly higher number of *Ae. aegypti* than the control (Figure 3), [IRR = 3.99, 95% CI: 0.95-16.69, $p = 0.049$], while the trap baited with trunk volatiles from volunteer 1 and the trap baited with carbon dioxide only captured fewer of this mosquito species than the control [IRR = 0.92, 95% CI: 0.22 - 3.87] and [IRR = 0.691, CI: 0.16 - 2.92]

respectively (Table 1). At the same site, the trap baited with feet volatiles from volunteer 2 captured more *Ae. aegypti* than the control trap (Figure 3), [IRR = 2.43, 95% CI: 0.71 - 8.29], while both the trap baited with feet volatiles from volunteer 1 and the trap baited with carbon dioxide only captured fewer *Ae. aegypti* than the control trap [IRR =0.86, 95% CI: 0.25 - 2.93] and [IRR = 0.32, 95% CI: 0.09 -1.10] respectively (Table 1).

In Busia, the traps baited with foot odor from volunteer 2, foot odor from volunteer 3 and carbon dioxide only captured more *Ae. aegypti* than the control (Table 1), with the trap baited with feet volatiles from volunteer 2 trapping significantly more *Ae. aegypti* than the control trap (Figure 3) [IRR = 5.63, 95% CI: 1.15 - 28.30, p = 0.030]. The same trend was observed when traps baited with the same volunteer's trunk volatiles were compared with the control (Figure 3). The order of performance was; volunteer 2 [IRR = 3.00, 95% CI: 0.18 - 6.68], carbon dioxide only [IRR =1.16, 95% CI: 0.192- 6.98] and volunteer 3 [IRR = 1.11, 95% CI: 0.51-17.61] (Table 1].

When proportions of male and female *Ae. aegypti* captured per trap were compared, a significantly higher number of males were captured by the trap baited with trunk odors from volunteer 1 [$p < 0.001$, $X^2 = 20.92$, d.f = 1] (Table 2). A further comparison between the proportions of fed and gravid *Ae. aegypti* per treatment trap and the control trap showed that traps baited with foot odors from volunteer 1, volunteer 2 and volunteer 3 captured more gravid *Ae. aegypti* than the control (Table 2).

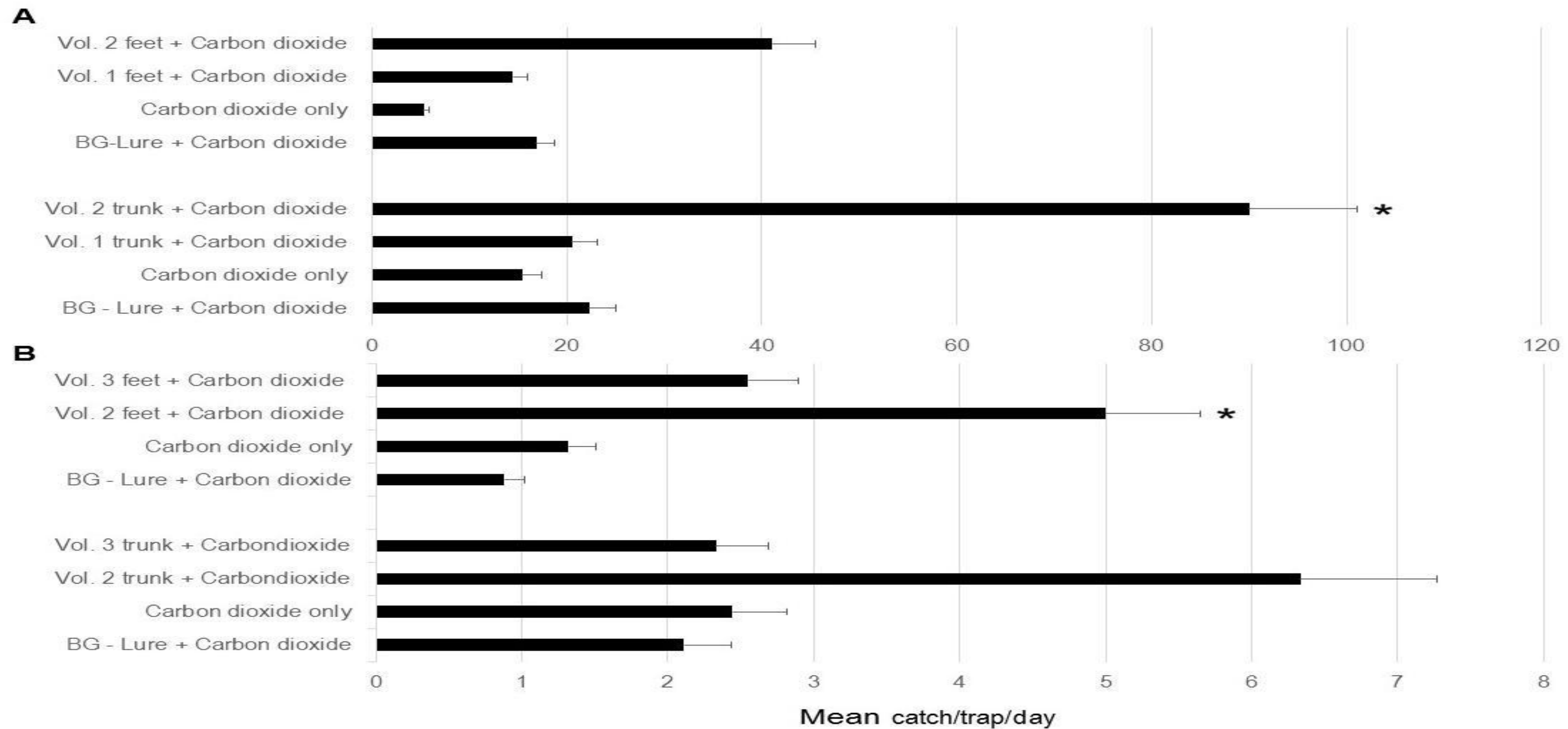

Figure 3. **The mean number/day and ±SE of Aedes aegypti captured by the various BG sentinel traps baited with different baits in Kilifi and Busia counties**. The different panels show comparisons at the two locations; Panel A- Kilifi and Panel B-Busia. Asterisks indicate that the mean catch of the trap is significantly different from the mean catch of the control trap (Biogent's commercial lure baited trap). Error bars indicate standard error of the mean.

Table 1 Comparisons of mosquito collections by BG sentinel traps baited with feet and trunk odor from volunteer 1, volunteer 2 and carbon dioxide in Kilifi county and from volunteer 2, volunteer 3 and carbon dioxide in Busia county relative to the control (Biogents commercial lure baited BG sentinel trap) trap

Site	Treatment	IRR(95%CI)	P value	Treatment	IRR(95%CI)	P value
Kilifi	Carbon dioxide	0.69(0.16-2.92)	0.602	Carbon dioxide	0.32(0.09-1.10)	0.064
Kilifi	Volunteer 1 trunk odor	0.92(0.22-3.87)	0.906	Volunteer 1 feet odor	0.86(0.25-2.93)	0.790
Kilifi	Volunteer 2 trunk odor	3.99(0.95-16.69)	0.049*	Volunteer 2 feet odor	2.43(0.71-8.29)	0.143
Busia	Carbon dioxide	1.16(0.19 - 6.97)	0.867	Carbon dioxide	1.50(0.28-8.04)	0.627
Busia	Volunteer 2 trunk odor	3.00(0.52-17.61)	0.203	Volunteer 2 feet odor	5.63(1.15-28.30)	0.030*
Busia	Volunteer 3 trunk odor	1.10(0.18 -6.67)	0.909	Volunteer 3 feet odor	2.87(0.57-14.80)	0.192

Estimated incidence rate ratio (IRR); confidence interval (CI) and corresponding P-values based on comparison to the BG lure following generalized linear model (GLM) with negative binomial error structure and log link in R 3.1.0 software. The IRR for the control is 1; values above this indicate better performance while values below indicate under performance relative to the control. Asterisks on p values indicate significant differences with the control.

Table 2 Comparisons of *Ae aegypti* proportions per trap by sex and abdominal status with corresponding catch indices (CI)

Bait	Total	♂ Proportion	♀ Proportion	P-values	Fed Proportion	CI	P-values	Gravid Proportion	CI	P- values
BG-Lure	191	52.4[a]	47.6[a]	0.412	5.5	1	-	2.2	1	-
Carbon dioxide only	166	45.8[a]	54.2[a]	0.153	2	0.3	0.191	3.3	1.3	0.951
Volunteer 2 Socks	415	41.9[b]	58.1[a]	<0.001	0.8	0.2	0.040*	1.2	1.3	1
Volunteer 2 T-shirt	858	52.0[a]	48.0[a]	0.112	0.2	0.2	0.001*	1.2	2.5	1
Volunteer 1 Socks	130	43.9[a]	56.1[a]	0.061	0	0	<0.001*	2.7	1	1
Volunteer 1 T-shirt	185	62.2[b]	37.8[a]	<0.001	0	0	<0.001*	0	0	0.252
Volunteer 3 Socks	23	43.5[a]	56.5[a]	0.562	0	0	<0.001*	7.7	4.5	0.043*
Volunteer 3 T-shirt	21	0[b]	100[a]	<0.001	0	0	<0.001*	0	0	0.256

Proportions following each other in the rows with different letters (a and b) are significantly different from each other. Asterisks on p values indicate significant difference with the control. The P-values are based on pair-wise comparison following chi-square goodness-of-fit in R 3.1.0 software.♂-Male *Ae. aegypti*, ♀- female *Ae. aegypti.*

Although data analysis was only limited to *Ae. aegypti,* other mosquito species including *Culex quinquefasciatus*, *Culex annulioris, Anopheles gambiae* and *Anopheles funestus* also occurred in large numbers in the traps at both sites. There were also small numbers of *Anopheles coustanii* in both Kilifi and Busia, *Mansonia uniformis, Mansonia africana*, *Eretmapodites chrysogaster group*, *Culex poicilipes, Coquillettidia faseri*, *Aedes metallicus*, *Aedes woodi* and *Aedes bromeliae* in Kilifi

Analysis of volatiles

The BG-lure, trunk and feet of human volunteers all released volatiles that attracted *Ae. aegypti* into traps. Analysis of the volatiles showed major qualitative and quantitative differences in the chemical profiles between trunk and foot odors and the commercial lure. Aldehydes and fatty acids dominated the volatiles released by human odors, which varied between individual volunteers, whereas hexanoic acid was the major component released by the BG lure (Table 3)

Table 3 Main compounds identified in the volatiles released by the commercial BG-lure and trunk and feet of human volunteers captured on SPME and analyzed coupled GC-MS analysis

Volatile source	Major compounds in percentages
BG-Lure	Hexanoic acid 73%
Volunteer 1, 2 & 3 trunks	Decanal (8% -33%)
	Hexanal (8 - 32%)
	6-methyl-5-hepten-2-one(15 - 28%)
	Nonanal (2 - 26%)
	Geranylacetone (3 - 13%)
	Hexanoic acid (4 - 9%)
Volunteer 1, 2 & 3 feet	Hexanoic acid (7-36%)
	Octanal (3 – 18%)
	Nonanal (7 - 17%),
	Hexanal (3 -15%)
	3-methylbutyric acid (7 - 9%)
	2-methylpropionic acid (2-9%)

Discussion

We observed that *Ae. aegypti* captures in Kilifi were generally higher than in Busia. Several factors could have played a role in this difference. First, *Ae. aegypti* is a known container breeding mosquito (Christophers, 1960; Southwood *et al.*, 1972) and since the sampling sites in Kilifi were mainly in an urban area, there is the likelihood for the mosquito to find more of this type of breeding site in this area. On the other hand, the Busia sampling sites which are rural would provide the opposite situation. Secondly, Kilifi being an old urban center, with older and abundant houses that could serve as suitable breeding sites for this mosquito species. Walker *et al.*, 2011 (Walker *et al.*, 2011) observed that older houses with mature vegetation, and objects collected in the yard tended to have higher densities of *Ae. aegypti* eggs than newer houses. Third, previous studies of *Ae. aegypti* in the Kenyan coast observed that they are highly anthropophillic and domesticated in behavior, where their life cycle transpires mainly inside and around human residences (Tabachnik & Powell, 1978; Mcdonald, 1977). They are therefore more likely to be attracted to human odors than the inland populations of Busia. Furthermore, *Ae. aegypti* mosquitoes have been observed to be highly adapted to urban rather than rural areas. They have a preference to rest inside houses and for areas with high human density, a behavior that favors vector-human contact (Lima-Camara, 2006; Tsuda *et al.*, 2006). Therefore, as an urban area, a higher population density in Kilifi could have contributed to a higher abundance of *Ae. aegypti.*

Climatic differences between the two sites could also have contributed to the observed variation. Busia receives an average annual rainfall of 1500 mm and is cooler with a minimum temperature of 14 °C and a maximum of 30 °C compared to an average annual rainfall of 950 mm and higher temperatures with a minimum of 21 °C and a maximum of 32 °C in Kilifi. Previous studies reported that while adequate amounts of rain will create natural water bodies and fill artificial habitats, providing females with opportunities to lay their eggs, excessive rain may flush the immature stages, especially the eggs, from their habitats causing a population crash of *Ae. aegypti* (Koenraadt & Harrington, 2008). Higher temperatures increased the developmental rate of *Ae. aegypti*

(Yussoff *et al*., 2012), thus Kilifi which is relatively warmer than Busia would favor the breeding of higher densities of *Ae. aegypti* than Busia

The traps baited with natural human odors from the feet and trunk, especially from volunteer 2 captured significantly more *Ae. aegypti* than the control trap baited with the synthetic commercial lure. Similar results were observed when the efficacy of the BG-sentinel trap baited with the commercial lure was compared with human landing/biting collections, a gas-powered CO_2 trap, and a Fay-Prince trap, in monitoring adult populations of *Ae. aegypti* in field tests in the city of Belo Horizonte, Brazil (Krockel *et al*., 2006). Furthermore, human odors were found to be significantly more attractive than a synthetic three-component blend consisting of L-Lactic acid, acetone and dimethyl disulfide during competitive bioassays that simultaneously compared the attractiveness of *Ae. aegypti* to two treatments in a dual port olfactometer (Bernier *et al*., 2007). The presence of additional fatty acids such as 2-methylpropionic acid and 3-methylbutyric acid, the four aldehydes; hexanal, octanal, nonanal and decanal and the two ketones 6-methyl-5-hepten-2-one and geranylacetone in human odors but not the BG-lure, suggests that these compounds likely played a role in the attractiveness of human odors over the BG-lure. Indeed, previous studies had shown that some of these compounds, including 2-methylpropionic acid, 3-methylbutyric acid, hexanal, octanal, nonanal and decanal are attractants of other mosquito species such as the malaria mosquito *Anopheles gambiae* (Mukabana *et al*., 2012; Nyasembe *et al*., 2012,) and mosquito vectors of Rift Valley Fever virus (Tchouassi *et al*., 2013).

We found individual variation in the attractiveness of volunteers to mosquitoes based on our field captures. This observation is supported by our chemical analysis of volatiles collected from the different individuals, which showed qualitative and quantitative differences in specific components. This result is similar to previous studies of volatiles of mammalian odors in mosquito attraction (Lindsay *et al*., 1993, Geier *et al*., 1996). For example, the difference in the attraction of different individuals to host seeking *Ae. aegypti* has been attributed to the difference in the quantity of lactic acid present on their skin (Geier *et al*., 1996). Individuals with higher amounts of lactic acid on their skin attracted more mosquitoes, while adding lactic acid to the skin rubbings of individuals

who were less attractive made them more attractive to mosquitoes. Inter-individual variation in body odor has also recently been attributed to the aggregation of different communities of micro biota on the skin. Individuals with lower bacteria diversity and with a significantly higher abundance *of Leptotrichia spp.*, *Delftia spp.* and *Actinobacteria Gp3 spp* of bacteria on their skin are highly attractive to *Anopheles gambiae s.s*. while individuals with a higher microbial diversity and a higher abundance of *Pseudomonas spp* or *Variovorax spp.* of bacteria on their skin are poorly attractive (Verhulst *et al*., 2009).

The fact that traps baited with natural human skin odors collected significantly more male *Ae. aegypti* than the trap baited with the Biogent's lure is striking. This suggests that having a trap that is efficient in capturing male *Ae. aegypti* would help dengue and chikungunya fever control programs because it has been established that although male *Ae. aegypti* are not blood feeders they are usually infected with dengue and chikungunya viruses via transovarial transmission (Thenmozi *et al*., 2000, Thavara et *al*., 2009). Recent studies document that male mosquitoes play an important role in the prevalence and maintenance of these diseases in the environment through venereal transmission of chikungunya virus from male to female *Ae. Aegypti,* which then transmits it to possible vertebrate hosts (Mavale *et al*., 2010).

Lastly, the observation that traps baited with volatiles from the feet of volunteers not only captured more gravid *Ae. aegypti* than the control trap but also some blood fed ones increases their potential usefulness in dengue and chikungunya fever surveillance. Gravid mosquitoes are a high priority in arboviral surveillance programs. Conceivably, gravid mosquitoes would have already been exposed to virus infection through previous feeding, hence serving as likely indicators of virus activity (Allan *et al*., 2010). On the other hand, blood-fed mosquitoes give information regarding the feeding preference, seroconversion status of that host, and infectivity level of the reservoir host (Kay *et al*., 2007), which immensely helps researchers in understanding the ecology of arboviruses spread by mosquitoes. Additionally, testing of blood fed mosquitoes helps to understand the interaction mechanisms between host, vector and possible reservoirs, and to

identify and evaluate the role of potential bridge vector species in transmission of pathogens of public health importance (Reiter, 1983).

Conclusions

Our data indicate that traps baited with natural skin volatiles are more efficient than traps baited with the Biogent synthetic lure in sampling *Ae. aegypti*. However, the efficacy of human odors varies between individuals (Knols *et al*., 1995; Logan *et al*., 2008; Schreck *et al*., 2006) and hence causes variation in trap captures. Additional studies will be required to determine the specific compound(s) that increase the attractiveness of human odors and subsequent trap captures for development and evaluation.

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Chapter 4

An improved odor bait for monitoring populations of *Aedes aegypti*-vectors of dengue and chikungunya viruses in Kenya

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Abstract

Background

Effective surveillance and estimation of the biting fraction of *Aedes aegypti* is critical for accurate determination of the extent of virus transmission during outbreaks and inter-epidemic periods of dengue and chikungunya fever. Here, we describe the development and use of synthetic human odor baits for improved sampling of adult *Ae. aegypti*, in two dengue and chikungunya fevers endemic areas in Kenya; Kilifi and Busia counties.

Methods

We collected volatiles from the feet and trunks of two female and two male volunteers aged between 25 and 45 years. We used coupled gas chromatography-electroantennographic detection (GC/EAD) analysis to screen for antennally-active components from the volatiles and coupled GC-mass spectrometry (GC/MS) to identify the EAD-active components. Using randomized replicated designs, we compared the efficacies of Biogents (BG) sentinel traps baited with carbon dioxide plus either single or blends of the identified compounds against the BG sentinel trap baited with carbon dioxide plus the BG commercial lure in trapping *Ae. aegypti*. The daily mosquito counts in the different traps were subjected to negative binomial regression following the generalized linear models procedures.

Results

A total of ten major EAD-active components identified by GC/MS as mainly aldehydes and carboxylic acids, were consistently isolated from the human feet and trunk volatiles from at least two volunteers. Field assays with synthetic chemicals of the shared EAD-active components identified from the feet and trunk gave varying results. *Ae. aegypti* were more attracted to carbon dioxide baited BG sentinel traps combined with blends of aldehydes than to similar traps combined with blends of carboxylic acids. When we assessed the efficacy of hexanoic acid detected in odors of the BG commercial lure and volunteers plus carbon dioxide, trap captures of *Ae. aegypti* doubled over the trap

baited with the commercial BG lure. However, dispensing aldehydes and carboxylic acids together in blends, reduced trap captures of *Ae. aegypti* by ~45%-50%

Conclusions

Our results provide evidence for roles of carboxylic acids and aldehydes in *Ae. aegypti* host attraction and also show that of the carboxylic acids, hexanoic acid released at low rates is a more effective lure for the vector than the BG commercial lure.

Keywords

Aedes aegypti, dengue, chikungunya, attractant, electrophysiology, mosquito, traps.

Background

Arboviral diseases such as dengue and chikungunya fever transmitted by *Ae. aegypti* are emerging and resurging causing global concern (; Kyle & Harris, 2008; WHO, 2012). The global incidence of dengue has risen rapidly in recent decades and the disease is now endemic in more than 100 countries in Asia, Africa, and the Americas. Infections from arboviral diseases have also risen and are now estimated at 50–100 million infections every year, with 21,000 fatalities (WHO, 2009). This puts some 3.6 billion people, that is, half of the world's population, mainly in the urban centers of the tropics and subtropics at risk (Gubler, 2002; Beatty *et al*., 2011). Cases of chikungunya outbreaks have also increased (WHO, 2006). In 2004-2005, widespread outbreaks of chikungunya occurred along the Kenyan coast and four island countries in the Indian Ocean including Comoros, Seychelles, Reunion and Mauritius (WHO, 2006; Sang *et al*., 2008). A year later, the outbreak spread to the Indian subcontinent (Rezza *et al*., 2008)] and to south of Italy in 2006 (WHO, 2011) .Outbreaks have also been reported in Central and Latin America as recently as in September 2014 where the epidemic is reported to have overwhelmed hospitals and cut economic productivity (Latino fox news, 2014).

Presently, dengue and chikungunya fevers have no treatment or vaccine (WHO, 2014). This has left vector control as the only available measure for prevention even though major progress has been made in developing a vaccine against dengue/severe dengue (WHO, 2014). In addition, disease monitoring for both dengue and chikungunya depends on vector collection and abundance tracking. In our previous work (Owino *et al*., 2014), we tested the responses of *Ae. aegypti* to human feet and trunk odors captured in cotton socks and T-shirts in field assays using the Biogents sentinel traps in Busia and Kilifi Counties of Kenya. We found that *Ae. aegypti* responses to the human odors varied with the volunteer and body part and also with the study site. We also analyzed odors from the human volunteers and the BG lure by GC/MS and observed major qualitative differences between the chemical profiles. Aldehydes, fatty acids and ketones dominated human odor profiles, whereas the commercial BG-lure originally comprising of lactic acid, ammonia, and hexanoic acid (caproic acid) (Geier *et al*., 2006)

released mainly hexanoic acid. Our results suggested that some of the human volunteers who participated in this study could be sources for the identification and development of more potent lures than the BG-lure for *Ae. aegypti.* Here, we report the identification of attractants from human feet and trunk odors for *Ae. aegypti* and field evaluation of improved odor baits for sampling adults of this mosquito species.

Methods

Study sites

Field studies were carried out in Kilifi County at the Kenyan coast and Busia County in Western Kenya (Figure 1). An outbreak of dengue was reported in Malindi, Kenya in 1982 (Johnson *et al*., 1982) and previous seroprevalence studies have shown that dengue infection was prevalent in Malindi area of Kilifi, with chikungunya infection occurring in Busia County (Mease *et al*., 2011).

Kilifi County experiences a bimodal kind of rainfall- the long monsoon rains (April-July) and the short rains (October-December) that averages annual rainfall of 950 mm. The temperatures range from a minimum of 21°C and a maximum of 32°C. Busia County on the other hand has an average annual rainfall of 1500 mm. The rainfall pattern is also bimodal; long rains (March-June) and short rains (October-December). Temperatures range from minimum of 14°C and maximum of 30°C.

In Busia County, traps were set up in villages in the rural area namely Obekai (0 30.875 N, 34 12.293 E), Kamosin (0 31.530 N, 34 13.125 E) and Kalwa (0 30.190 N, 34 14.020E). These locations occur at approximately 1189 m above sea level (asl). The main vegetation in these areas consists of large, tall eucalyptus trees that form thick canopies. The local inhabitants are mainly small-scale farmers growing maize, millet and cassava as food crops while a few grow sugarcane and coffee as cash crops. They also keep a few animals mainly cattle, sheep, goats, pigs, chicken and guinea fowls.

In Kilifi county, traps were set up at three sites located in the urban area namely Kenya Medical Research Institute (KEMRI) campus, Kilifi hospital (3 37.800 S, 39 51.483 E)

and Mnarani estate (3 38.368 S, 39 50.824 E). These locations occur at approximately 30.5 m asl. The inhabitants in the urban area mainly engage in small businesses or work in offices. They also grow maize, cassava and sweet potatoes and keep a few animals mainly goats.

The traps were set up during the wet seasons at both sites. In Busia, traps were set up in November 2013 and in Kilifi in December 2013 and April 2014.

Odor collection from trunk and feet of Volunteers

Four adult volunteers, 2 males and 2 females, between the ages of 25 and 45 years were identified and enrolled to participate in the study after obtaining informed consent. The two males had participated in our previous study and one of the males was more attractive than the other (Owino *et al*., 2014). The volunteers were each requested to put on clean new cotton T shirts and clean new cotton socks (Lux Industries Ltd 39 K.K Tagarest, Kolkata-700-007) issued to them by the researchers for 18 hrs. The worn socks and T shirts from the volunteers were individually wrapped in at least 4 layers of aluminium foil and stored in cool boxes (10°C) for immediate transportation to the laboratory for odor trapping using the volatile entrainment system as described below.

Headspace trapping of odors trapped in worn socks and T shirts

The socks and T shirts obtained from the volunteers were held in tightly sealed volatile collection jars (ARS, Gainesville, FL, USA) and odors collected on Super Q adsorbent (30 mg, Alltech, Nicholasville, KY) traps for 24 hr. The Super Q filters were eluted with 150 µl dichloromethane Sigma-Aldrich Corporation (3050 Spruce Street, St. Louis, Missouri 63103 USA) and stored at -80°C until use.

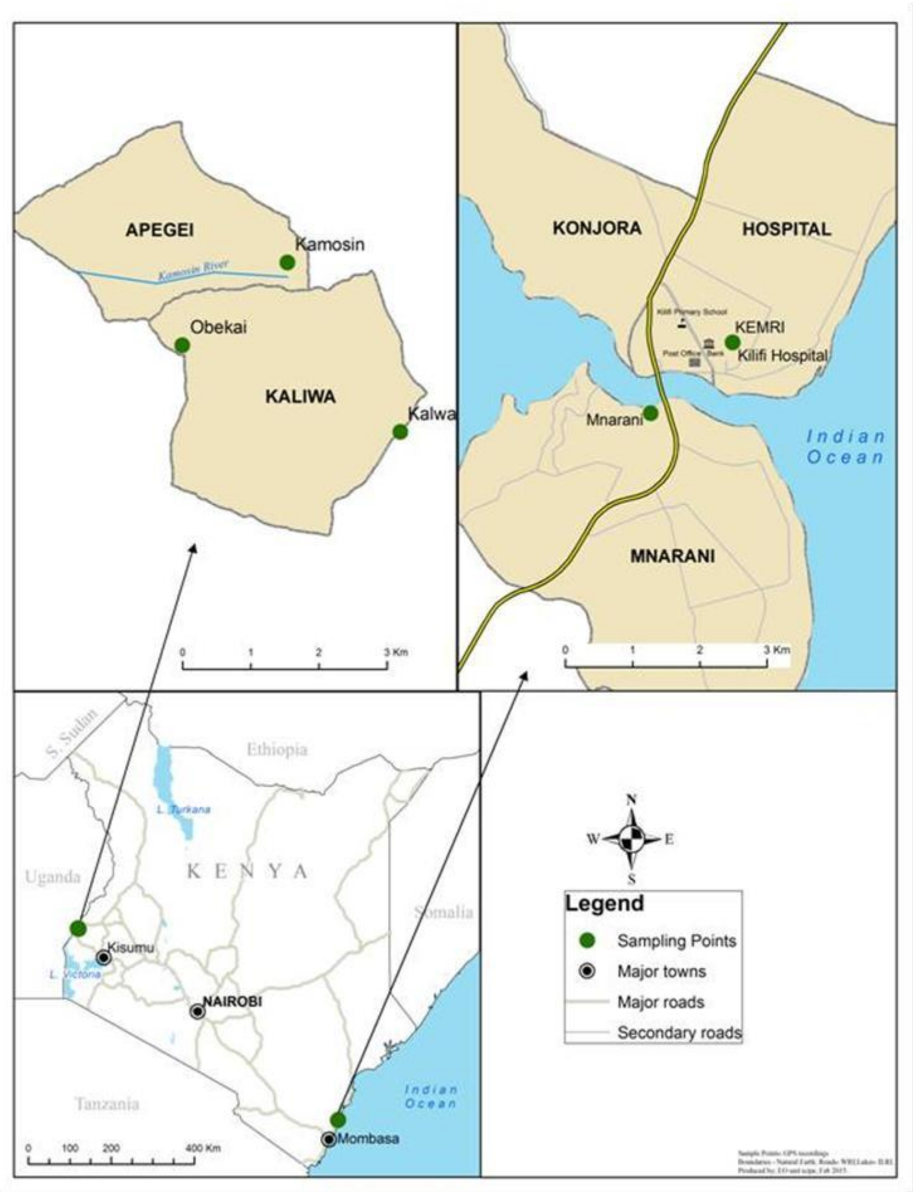

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya.

Mosquitoes

Mosquitoes used in this study were obtained from two different populations; (i) An inbred generation reared at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi, established in 2001 from blood-fed and gravid *Ae. aegypti* caught at Rabai, Kilifi County, and (ii) A first filial (F1) generation of *Ae. aegypti* established from eggs collected from Rabai, Kilifi in 2013 and reared in a separate

insectary at *icipe's* Duduville campus. In both cases, *Ae. aegypti* were reared at a mean temperature and relative humidity of day, 28°C, 70% RH and night, 26°C, 80% RH; and a reversed circadian rhythm of light (15:01-3:00) and darkness (3:01-15:00). The newly emerged adult females were maintained on glucose (6% solution *ad libitum*) (Sigma®) continuously available on filter paper and no blood meal. On the experimental days the mosquitoes were deprived of glucose for 6 hrs before the experiments.

Gas chromatography/electroantennographic detection (GC/EAD)

Volatiles collected from the feet and trunk of volunteers were analyzed by coupled GC/EAD analysis using a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an HP-5 column (30 m × 0.25 mm ID × 0.25 µm film thickness, Agilent, Palo Alto, California, USA). Nitrogen was used as the carrier gas at 1.2 ml/min. Volatiles were analyzed in the splitless mode at an injector temperature of 280°C venting at 0.8 min. The oven temperature was held at 35°C for 5 min, then programmed at 10°C/min to 280°C and maintained at this temperature for 10 min. The column effluent was split 1:1 after addition of make-up nitrogen gas for simultaneous detection by flame ionization detector (FID) and EAD. For EAD detection, silver-coated wires in drawn-out glass capillaries (1.5 mm I.D.) filled with Ringer saline solution (Kugel, 1977) served as reference and recording electrodes.

Antennal preparations were made by decapitating 4-7 days old females of *Ae. aegypti* at the base of the head and slicing off the tip of the last antennal segment with a scalpel under a dissecting microscope. The antenna was then mounted on to the micromanipulator such that the base of the head was connected to the reference electrode, and the cut tip of the antenna was connected to the recording electrode. The analog signal was detected through a probe (INR-II, Syntech, Hilversum, the Netherlands), captured and processed with a data acquisition controller (IDAC-2, Syntech, the Netherlands), and later analyzed with soft- ware (EAG 2000, Syntech) on a personal computer. An aliquot (5µl) of the Super Q-trapped volatile extract from each volunteer's feet and trunk was analyzed using fresh female antennae in at least three replicate runs.

Coupled gas chromatography/mass spectrometry (GC/MS)

GC/MS analysis of volatiles was carried out on an Agilent system (Agilent Technologies, Inc., Santa Clara, CA, USA) consisting of a 7890A gas chromatograph, a 5975C Mass spectrometer with a triple Axis detector and an Agilent ChemStation data system. The GC column was an HP-5 MS fused silica capillary (30 m × 0.25 mm ×0.25 μm film thickness) (J&W, Folsom, CA, USA). The carrier gas was helium with a column head pressure of 8.827psi and flow rate of 1.2 mL/min. Inlet temperature was 270°C and MSD detector temperature was 280°C. The oven temperature was held at 35°C for 5 min and then increased at 10°C/ min to a final temperature of 280°C, which was held for 10.5 min. The identity of each component in the extracts of the volatiles was determined by comparison with references from mass spectral libraries (NIST05, Agilent Technologies [NIST05, Agilent Technologies NIST database, G1033A, revision D.05.01, ChemStation data system (G1701EA, version E.02.00). An aliquot (1 μl) of the volatile extract from each volunteers' feet or trunk and of synthetic authentic compounds was injected into the GC-MS for analysis.

GC/EAD-active components were identified both by comparing their mass spectral data with those recorded in the Mass Spectral Library NIST 2005 and by co-injection with authentic standards.

Chemicals

Hexanal, heptanal, hexanoic acid, octanal, nonanal, decanal and undecanal were obtained from (Sigma-Aldrich Chemie (GmbH, Germany) while propionic acid, 3-methylbutyric acid, and 6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone) were sourced from Sigma-Aldrich Corporation (3050 Spruce Street, St. Louis, Missouri 63103 USA). Purities of the compounds ranged between 95% and 99%. The BG lure used in this study was purchased from Biogent, with an expiry date of December 2015. It mainly contains lactic acid, hexanoic acid and ammonia (Geier *et al.*, 2006).

Field testing of EAG-active compounds

Experiment 1

Study design

For field testing in both Kilifi and Busia, mosquitoes were collected using six BG sentinel traps baited with carbon dioxide plus (i) Blend 1; 3-methylbutyric acid and propionic acid each at 0.05mg/µl at a ratio of 1:1 (ii) Blend 2; nonanal and octanal each at 0.05mg//µl at a ratio of 1:1(iii) Blend 3; nonanal, octanal, 3-methylbutyric acid and propionic acid each at 0.05mg/µl dispensed separately at a ratio of 1:1:1:1 (iv) BG-lure (v) worn socks and (vi) worn T shirts.

Traps baited with human odors

Odors were obtained from the feet and trunk of a male volunteer aged 32 years old in Busia and a male volunteer aged 30 years old in Kilifi. Both of them had donated odors for the GC/EAD tests. The volunteers were requested to put on new, clean, 100% cotton socks and T shirts (Lux Industries Ltd 39 K.K Tagarest, Kolkata-700-007) to trap odors from their feet and trunk for 18 hrs daily for a period of 12 days. New socks and T shirts were provided daily. The volunteers were also provided with odorless soap to bathe with daily and requested to avoid the use of deodorants and perfumes. The socks and T shirts once removed by the volunteers were wrapped in at least 4 layers of aluminium foil and stored in cool boxes at 10 °C and transferred into the laboratory and then into -80 °C freezer until use. The worn socks and T shirts were used daily to bait BG sentinel traps by hanging them on the rails of the BG sentinel trap inner structure as described in Owino *et al.*, 2014 (Owino *et al.*, 2012).

Traps baited with synthetic chemicals

Preliminary trials to determine the possible range of attractive doses of, nonanal, octanal, propionic acid, 3-methylbutyric acid and hexanoic acid, were conducted in the field at *icipe*'s Nairobi campus. These chemicals were identified as the consistent EAD-active components that were most commonly shared amongst the different volunteers. Concentrations of individual compounds, including 0.005, 0.01, 0.02 and 0.05mg/µl were evaluated in three replicate trials. Trap captures showed that the optimal attractive dose of nonanal and octanal to *Ae. aegypti* was 0.05 mg/µl while hexanoic acid, 3-methylbutyric acid and propionic acid were effective between 0.01 and 0.05mg/µl/ [data not shown]. Hexanal and decanal did not show strong attraction to *Ae. aegypti* at the tested concentrations.

To obtain stock concentrations, 100 mg of each EAD-active compound was diluted in 1 ml of hexane. Ten milligrams (10% of the concentration of individual component) of the antioxidant, 2, 6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT, Aldrich) was then added to the aldehyde stocks to prevent oxidization to their respective corresponding fatty acids. To bait the traps, 50 µl of each compound was transferred from the stock and diluted in hexane to make 100 µl. The solution was adsorbed on cotton wicks measuring 5 mm x 30 mm wrapped in a nylon stocking material measuring 12 mm x 30 mm. The cotton wicks and BG-lure were then inserted into the odor pockets of the BG sentinel traps. Each compound was dispensed from its own cotton wick.

Mosquito sampling

At each of the study sites, Kilifi and Busia, six different locations were randomly chosen around homesteads. Traps were set up at approximately 100 m away from the nearby house (occupied or unoccupied). The six BG sentinel traps baited as described above were randomly set up at each of the six locations with a distance of at least 100 m between traps. The traps were hung at 0.2 m above the ground and attached to each was a Bioquip igloo that dispensed carbon dioxide in the form of dry ice (Owino *et al.*, 2014). To offset any positional bias, traps were rotated every experimental day. The traps were set up at 9.00 am and left to run until 5.00 pm. Trapped mosquitoes were

collected and transported to the laboratory where they were freeze-killed and identified under a dissecting microscope to species level using morphological keys (Edwards, 1941; Gillies & De meillon, 1968; Huang &Ward, 1981; Rueda, 2004).

Experiment 2

Study design

Comparison of the efficacies of various EAD-active carboxylic acids in attracting *Ae. aegypti* at the two field sites, Kilifi and Busia (Experiment 1), showed that only hexanoic acid strongly attracted this mosquito species. Previously, we had detected it in the odors released from the BG lure (Owino *et al*., 2014). It was therefore selected for further evaluation in Kilifi which compared to Busia had a higher density of *Ae. aegypti* (see Results section). This experiment was carried out in five locations in Kilifi. Mosquitoes were collected in the field using five BG sentinel traps baited with carbon dioxide plus either (i) hexanoic acid at 0.05 mg/µl (ii) Blend 2; octanal and nonanal each at 0.05 mg/µl at a ratio of 1:1 (iii) Blend 4; hexanoic acid, nonanal and octanal each at 0.05mg/µl at a ratio of 1:1:1 (iv) BG-Lure (v) carbon dioxide only. The compounds were dispensed from rubber septa which were inserted into the odor pockets of the BG sentinel traps instead of the cotton wicks wrapped in Nylon materials like in experiment 1. In traps baited with more than one compound, each compound was prepared individually as already described and dispensed separately from rubber septa. The average release rate of the hexanoic acid was 0.7 mg/hr over the 7 hr trapping period while the average release rate of hexanoic acid from the BG lure was calculated as 1.9 mg/hr over the same period. The release rates were calculated based on GC/MS peak area comparison with those of authentic standards.

Mosquito sampling

The five BG sentinel traps were randomly set up at each of the five locations just as described in experiment 1 above after which captured mosquitoes were freeze- killed and identified to species using appropriate keys (Edwards, 1941; Gillies & De meillon, 1968; Huang &Ward 1981; Rueda, 2004).

Data analysis

The daily mosquito counts in the different traps were subjected to negative binomial regression following the generalized linear models (GLM) procedures in R 3.1.0 [21]. The trap baited with the BG commercial lure was used as the control and the reference category in both field experiments 1 and 2. The incidence rate ratios (IRR), a likelihood measure that mosquito species chose other treatments instead of the reference category, and corresponding P-values were estimated. The Pearson's chi-square test was applied to evaluate differences between proportions of fed and gravid mosquitoes per treatment trap against the reference category. The tests were performed at 5% significance level.

Ethics statement

The study was approved by the national ethics review committee based at the Kenya Medical Research Institute (KEMRI) and informed consent was obtained from each of the participants. Different sampling locations were randomly chosen around homesteads after obtaining oral consents from the heads of the homes.

Results

GC/EAD and GC/MS analyses of volatiles

A total of 21 EAD-active components were identified from the odor collections from the four volunteers, with most of them identified based on selected ion monitoring because they were present in low levels (Table 1). Of these, 10 were common to the trunk and feet odors of at least two of the volunteers consistently eliciting GC/EAD responses from either *Ae. aegypti* obtained from the Rabai, Kilifi F1 generation or the inbred laboratory reared population (Fig. 2). Antennal responses were stronger using the F1 generation than the x generation of laboratory-reared population of *Ae. aegypti* (Fig. 2). The components which consistently elicited EAD activity in odors were identified by GC/MS as the aldehydes; hexanal, heptanal, octanal, nonanal, decanal, undecanal, and the carboxylic acids; propionic acid, 3-methylbutyric acid, hexanoic acid and the ketone, 6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone) (Table 1). Three additional

compounds that co-eluted with the solvent (Fig. 2, panel 1B) were unidentified. Minor EAD-active components identified from the odors of the different volunteers, were 2-methylbutyric acid, pentanoic acid,1-octen-3-ol, 6-methyl-5-hepten-2-one, 3,7-dimethyl-1-6-octadien-3-ol (linalool), 2-ethylhexanoic acid, [*E*]- or [*Z*]-2-nonenal, nonanoic acid, hexadecanoic acid and octadecanoic acid. Except for 2-nonenal which was identified based on comparison of its mass spectrum with library data, all the other components were identified based on library data and co-injection with authentic standards.

Table 1. Major and minor GC/EAD active compounds in the volunteer's feet and trunk odors.

3-methylbutyric acid (3)	7.7
Heptanal (4)	9.1
Hexanoic acid (5)	10.8
Octanal (6)	11.2
Nonanal (7)	13.0
Decanal (8)	14.6
Undecanal (9)	16.2
6,10-dimethyl-5,9-undecadien-2-one (Geranyl acetone	18.1
Minor EAD-active compounds	
2-methylbutyric acid	7.9
Pentanoic acid	8.7
1-octen-3-ol	10.8
6-methyl-5-hepten-2-one	10.9
3,7-dimethyl-1,6-octadien-3-ol (Linalool)	13.0
2-ethylhexanoic acid	13.3
[*E*] or [*Z*]-2-nonenal	14.0
Nonanoic acid	15.5
Dodecanal	17.5
Hexadecanoic acid	23.5
Octadecanoic acid	25.6

RT-retention time

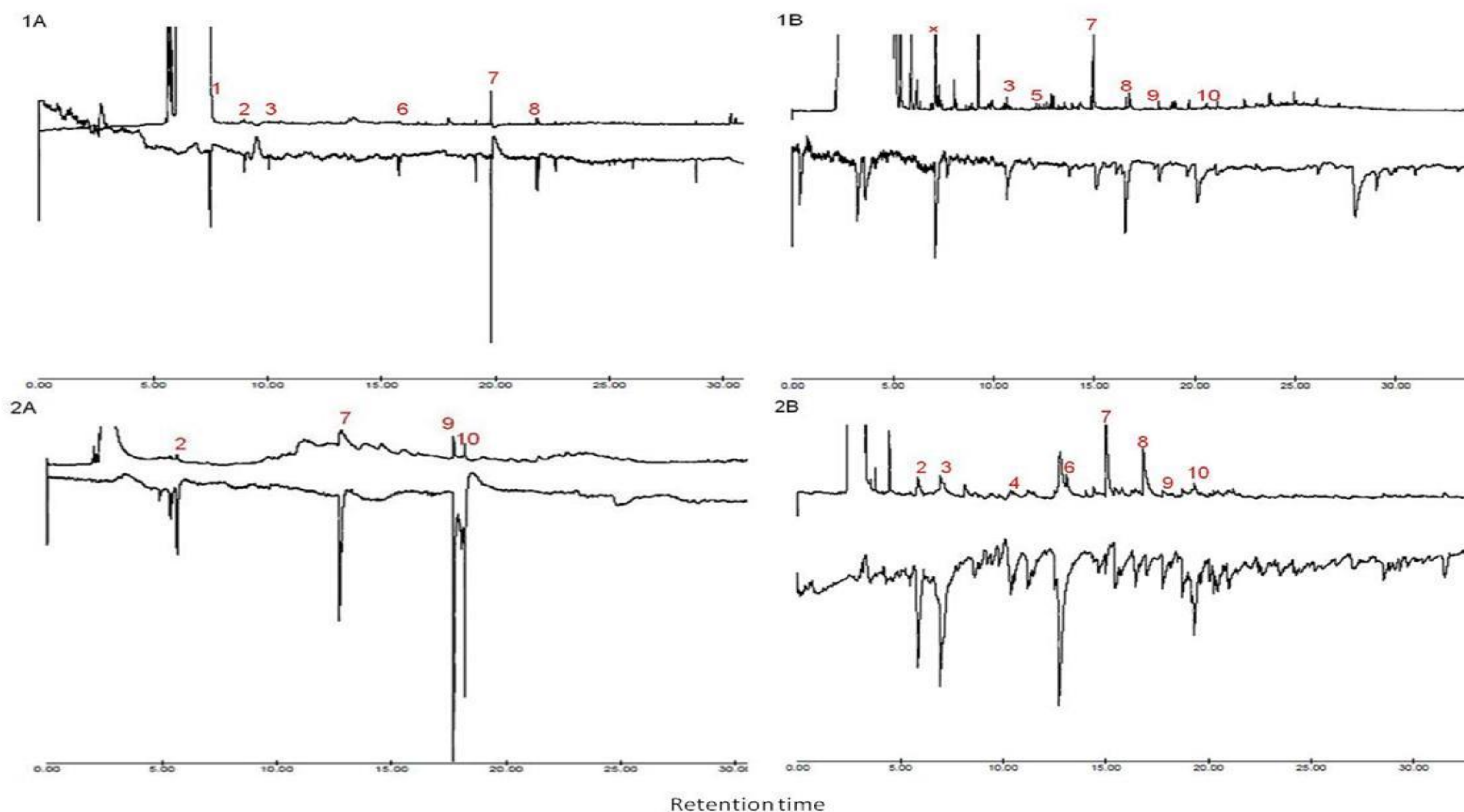

Figure 2. Representative GC/EAD profiles showing EAD- active components identified from; the feet- Panel 1 and trunk - Panel 2, of volunteers. Panel A- GC/EAD responses from F1 generation *Ae. aegypti* from Rabai, Kilifi. Panel B – GC/EAD responses from inbred generation *Ae. aegypti* from Rabai, Kilifi. EAD-active components; 1- propionic acid, 2- hexanal, 3-methylbutyric acid, 4- heptanal, 5- hexanoic acid, 6- octanal, 7- nonanal, 8- decanal, 9- undecanal, 10- 6,10-dimethyl-5,9-undecadien-2-one (geranyl acetone)

Field tests

Experiment 1

Of the total 2,954 *Ae. aegypti* captured, a significant number (~3-fold more) was captured in Kilifi (n=2,153) than in Busia (n=801) [p< 0.001]. The trap baited with the binary aldehyde blend of nonanal and octanal (Blend 2) plus carbon dioxide captured 1.3-fold more *Ae. aegypti* than similar traps baited with the BG commercial lure [IRR=1.3, 95% CI: 0.61-2.75, p=0.49] (Figure 3). In contrast, the trap baited with the binary carboxylic acid blend comprising 3-methylbutyric acid and propionic acid (blend 1) plus carbon dioxide- captured only 0.6-fold of *Ae. aegypti* compared to captures by the trap baited with the commercial BG lure. However, when the aldehydes and the carboxylic acids were dispensed together (Blend 3), there was a 45 % reduction in trap captures [IRR=0.75 95% CI: 0.43- 1.55 p=0.40] (Table 2). The same trap capture pattern was found in Kilifi where the overall order of trap performance was Blend 2 (nonanal + octanal) > volunteer 2 feet odors > BG lure > volunteer 2 trunk odors = Blend 3 (nonanal + octanal +3-methylbutyric acid + propionic acid) > Blend 1(3-methylbutyric acid + propionic acid) (Table 2).

Experiment 2

In the second study carried out in Kilifi which was carried out based on the results from Expt. 1, whereby *Ae. aegypti* was found to be more abundant than in Busia, a total of 6, 239 *Ae. aegypti* were trapped. The trap baited with carbon dioxide and hexanoic acid captured 2.2-fold more *Ae. aegypti* than the trap baited with carbon dioxide and the BG lure [IRR=2.2, 95% CI: 0.82-5.87, p=0.109] (Figure 3). However, similar traps baited with hexanoic acid dispensed together with nonanal and octanal, only captured 0.95-fold more *Ae. aegypti* than traps baited with the BG lure (Table 3) showing a 50% reduction of trap captures relative to captures by the hexanoic acid baited trap. The hexanoic acid baited trap also captured more *Ae. aegypti* than all the other traps, with trap performance in the order of hexanoic acid > Blend 2 (nonanal + octanal) > BG–lure > blend 4 (hexanoic acid and nonanal+ octanal) > carbon dioxide only (Table 3). Comparison of trap captures showed a significantly higher proportion of female than

male *Ae. aegypti* in all the traps (Table 4). A further comparison of captures for fed and gravid mosquitoes per trap showed that the trap baited with hexanoic acid and carbon dioxide captured significantly higher proportions of fed p=0.047 *Ae. aegypti* than the BG commercial lure plus carbon dioxide baited trap. It also captured 1.2-fold more gravid *Ae. aegypti* than the trap baited with the BG lure (Table 4).

Table 2 . Comparisons of *Ae. aegypti* captured by BG sentinel traps baited with different odor baits relative to the control (BG sentinel trap baited with the BG commercial lure) in Experiment 1 in Busia and Kilifi

Site	Treatment	IRR(95%CI)	P value	Site	Treatment	IRR(95%CI)	P value
Busia	Blend 3	0.75(0.43-1.55)	0.40	Kilifi	Blend 3	0.91(0.35-2.41)	0.858
Busia	Blend 2	1.3(0.61-2.75)	0.49	Kilifi	Blend 2	1.23(0.47-3.24)	0.665
Busia	Blend 1	0.62(0.29-1.35)	0.23	Kilifi	Blend 1	0.61(0.23—1.6)	0.307
Busia	Volunteer 1 feet odors	1.12(0.53-2.4)	0.76	Kilifi	Volunteer 2 feet odors	1.09(0.42-4-2.8)	0.849
Busia	Volunteer 1 trunk odors	1.01(0.48-2.15)	0.97	Kilifi	Volunteer 2 trunk odors	0.91(0.35—2.41)	0.858

Estimated incidence rate ratio (IRR); confidence interval (CI) and corresponding P-values based on comparison to the control (BG lure baited trap) following generalized linear model (GLM) with negative binomial error structure and log link in R 3.1.0 software. The IRR for the control is 1; values above this indicate better performance while values below indicate under performance relative to the control. Blend 1; propionic acid and 3-methylbutyric acid, Blend 2; nonanal + octanal, Blend 3; Blend 1 + Blend 2.

Table 3. Comparisons of *Ae. aegypti* trapped by BG sentinel traps baited with different odor baits relative to the control (BG sentinel trap baited with the BG commercial lure) in Experiment 2 in Kilifi County

Site	Treatment	IRR(95%CI)	P value
Kilifi	Carbon dioxide only	0.57(0.21 – 1.52)	0.255
Kilifi	Blend 2	1.33 (0.50 -3.57)	0.552
Kilifi	Blend 4	0.95(0.36- 2.56)	0.931
Kilifi	Hexanoic acid	2.2 (0.82- 5.87)	0.109

Estimated incidence rate ratio (IRR); confidence interval (CI) and corresponding P-values based on comparison to the BG lure following generalized linear model (GLM) with negative binomial error structure and log link in R 3.1.0 software. The IRR for the control is 1; values above this indicate better performance while values below indicate under performance relative to the control.

Table 4. Comparisons of *Ae. aegypti* catch percentages per trap by sex and abdominal status with corresponding p values and catch indices (CI) in Experiment 2.

Bait/Mosquito count	**BG Lure**	**No bait**	**Blend 1**	**Blend 2**	**Hexanoic acid**
Total	1028	587	985	1377	2262
♀ **Percentage**	55.4	61.8	60	67.4	54.2
♂ **Percentage**	44.6	38.2	40	32.6	45.8
Fed percentage	0.9	0.3	0.9	1.5	4.9
CI	1	0.3	1	1.6	5.4
P-value	-	1	1	1	0.047*
Gravid percentage	4.7	0	0.7	3.2	5.8
CI	1	0	0.15	0.7	1.23
P-value	-	0.11	0.07	0.804	0.97

Catch percentages, Catch indices (CI) and corresponding p values. Asterisks on p values indicate significant difference of the catch percentage with the catch percentage of the control (trap baited with the BG commercial lure). The P-values are based on pair-wise comparison following chi-square goodness-of-fit in R 3.1.0 software.♂-Male *Ae. aegypti*, ♀- female *Ae. aegypti*.

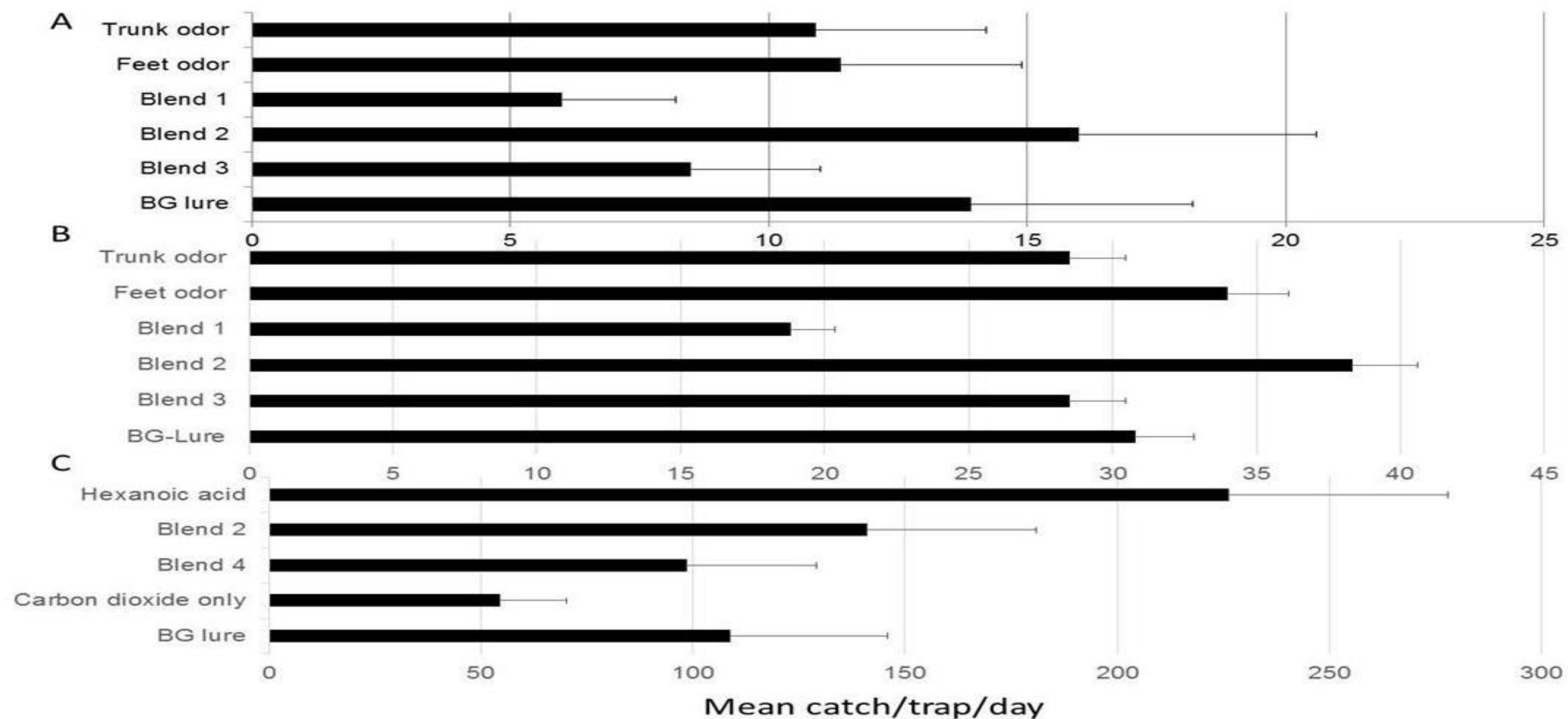

Figure 3. The mean number ± S.E of *Aedes aegypti* captured by the various BG sentinel traps baited with different baits in Busia and Kilifi County. Blend 1; Acids - propionic + 3-methylbutyric acid, Blend 2; Aldehydes - nonanal + octanal, Blend 3; Blend 1 + Blend 2, Blend 4; Blend 2 + hexanoic acid. The different panels show comparisons at the two locations; Panel **A** - Experiment 1 in Busia, Panel **B -** Experiment 1in Kilifi **and Panel C** - Experiment 2 in Kilifi. Error bars indicate standard error of the mean.

DISCUSSION

This study investigated volatiles released from the feet and trunk of human volunteers and isolated predominantly aldehydes and carboxylic acids as the electrophysiologically-active components using antennae of *Ae. aegypti*. To the best of our knowledge, of the 21 EAD-active components detected, propionic acid, 3-methylbutyric acid, 2-methylbutyric acid, pentanoic acid, hexanoic acid, 2-ethylhexanoic acid, nonanoic acid, undecanal, hexadecanoic acid and octadecanoic acid are being reported for the first time as detected by antennae of *Ae. aegypti*. Electrophysiological activity for some of these aldehydes and carboxylic acids from human skin odors have previously been reported for various mosquito species. For example octanal and nonanal, identified from the human feet, trunk and armpit were reported to elicit electrophysiological response in antennae of *Ae. aegypti* (Ghaninia *et al*., 2008; Logan et al., 2008) and *Aedes mcintoshi* (Tchouassi *et al*., 2013), a major vector of Rift Valley fever virus, and *Culex quinquefasciatus* (Syed & Leal 2009)], the major vector of West Nile virus in bird headspace volatiles, respectively. Carboxylic acids were reported to elicit EAG responses in *An. gambiae* (Cork & Park, 1996) and *Cx. quinquefasciatus* (Puri *et al*., 2006). These findings emphasize the importance of aldehydes and carboxylic acids in host seeking behavior of *Ae. aegypti.* Both aldehydes and carboxylic acids have previously been reported as common residues on human skin (Curran *et al*., 2005; Zhang *et al*., 2005). They play a vital dose dependent role in the balance of attraction and inhibition to host seeking *Ae. aegypti* (Bernier *et al*., 2002; Curran *et al*., 2005; Zhang *et al*., 2005). For example, individuals with relatively higher concentrations of aldehydes, especially nonanal, were less attractive to *Ae. aegypti*. (Schreck *et al*., 1990; Bernier *et al*., 2002). Also, identified as EAG-active in the present study is the ketone geranyl acetone, reported previously to elicit electrophysiological activity in *Ae. aegypti* (Logan *et al*., 2010). Notably, these EAD-active components varied between volunteers, and also varied between body parts with carboxylic acids detected mainly in the feet odors while aldehydes were dominant in the trunk odors. Qualitative differences in odors released between different individuals and also from their body parts have been reported previously (Owino *et al*., 2014; Ghaninia *et al*., 2008). The origin of

human-specific volatiles emanating from different body regions has been attributed to the aggregation of diverse communities of micro biota (Braks *et al*., 1999; Logan *et al*., 2010; Verlhust *et al*., 2009, 2011), which differ both in quality and quantity between different individuals and are responsible for driving the attraction of mosquitoes to different host individuals [Braks *et al*., 1999; Penn *et al*., 2006). Although it was apparent that there was no difference in the EAD-active compounds that both the F1 and the inbred generations of *Ae. aegypti* detected, antennae of the F1generation detected the compounds more strongly than the inbred population. This suggests that inbreeding may lead to partial loss of antennal sensitivity in agreement with the findings using tsetse fly antennae to isolate EAD-active compounds from odors of vertebrate hosts (Gikonyo *et al*., 2002).

In the field evaluation of odors, we found that traps baited with the binary blend comprising octanal and nonanal, each dispensed at 0.05mg/µl, captured more *Ae. aegypti* than all the other traps including the traps baited with natural human odors (worn socks and worn T-shirts) in both Busia and Kilifi. Similar results showing high attractiveness of aldehydes to mosquitoes have been reported before where a bait formulated from four aldehydes (heptanal, octanal, nonanal and decanal) combined with CO_2 doubled to tripled trap captures of a CDC trap without a light bulb compared to a control trap baited with CO_2 alone (Tchouassi *et al*., 2013). It has also been reported that traps baited with nonanal alone significantly captured more *Cx. quinquefasciatus* than traps baited with no odors (Syed & Leal, 2009). Together, these results greatly improve upon our knowledge of odor-based technologies for trapping mosquitoes (Logan *et al*., 2008; Nyasembe *et al*., 2012) and represent a significant advancement in attempts to develop synthetic lures, which would effectively compete against humans for host seeking mosquitoes in field settings. They also suggest that it is possible to formulate synthetic odor blends that are highly attractive to *Ae. aegypti* without including all the physiologically-active components found in natural human odors. Thus, odor baits may represent a future potential control tool for mass trapping to reduce vector population around houses in disease endemic villages.

An interesting observation that we made in the field based on our trap captures was that of antagonism that appeared to result into a spatial repellency effect between aldehydes and carboxylic acids when dispensed side by side. This was in sharp contrast to our expectations that trap captures with a blend of, the attractive carboxylic acid, hexanoic acid and the attractive binary aldehyde blend of nonanal and octanal was 45% less attractive than that of hexanoic acid alone. Similar antagonistic and spatial repellent effect on mosquitoes by synthetic human odor blends have been observed before however, mainly in laboratory assays (Logan *et al*., 2009; Kline *et al*., 2009). For example, it was observed that linalool when used alone, attracts mosquitoes to a trap; however, when used with CO_2, or with l-octen-3-ol, both of which are mosquito attractants on their own (Takken & Kline, 1989), reduced mosquito collection size by as much as 50% (Kline *et al*., 2009). It could also be argued that the carboxylic acids especially hexanoic acid and the aldehydes (nonanal and octanal) are attractants on their own but act as inhibitors when combined with each other. These findings are in line with a previous study that observed that in the absence of gaseous lactic acid, N, N-diethyl-meta-toluamide (DEET) attracted mosquitoes but when mixed with the already attractive lactic acid, DEET reduced mosquito captures (Dogan *et al*., 1999). Evidently, the presence of the inhibitor severely impedes the ability of mosquitoes to detect odors that would normally be highly attractive.

We also observed that in Experiment 2, carried out in Kilifi, traps baited with hexanoic acid and carbon dioxide captured more mosquitoes than any other trap including the trap baited with carbon dioxide and the BG commercial lure which also contains hexanoic acid as one of its components (Geier *et al*., 2006). This difference could be associated with the different concentrations and release rates of hexanoic acid in our bait compared to that of the BG lure. The release rate of our trap baited with hexanoic acid was 0.7 mg /ml, ~3- fold less than that released by the BG lure at 1.9 mg/ml. Previous studies report that the effectiveness of hexanoic acid depends on its release rate. For example, at 0.3 ml/min, hexanoic acid had little effect on the attractiveness of lactic acid while increasing it to 100-fold at 30 ml/min, significantly increased attraction of lactic acid to *Ae. aegypti*. At a 1000-fold increase, 300 ml/min caused a significant decline in attraction (Bosch *et al*., 2000). These results are in line with our previous

findings where we observed that human trunk and feet odors that were more attractive to *Ae. aegypti* than the BG commercial lure in field bioassays contained between 2-18-fold less hexanoic acid than that present in the BG commercial lure (Owino *et al*., 2014).

Our results also showed that in Experiment 2, traps baited with carbon dioxide and hexanoic acid captured higher proportions of blood fed, gravid and male *Ae. aegypti* than all the other traps. This makes hexanoic acid superior bait in the surveillance and monitoring of these arbovirus vectors. Blood fed mosquitoes provide information on the interactions between host, vector and possible reservoirs, and helps to identify and evaluate the role of potential bridge vector species in the transmission of pathogens of public health importance (Allan *et al*., 2010). They also give information regarding the feeding preference, seroconversion status of that host, and infectivity level of the reservoir host, (Kay *et al*., 2007) which immensely helps researchers to understand the ecology of arboviruses spread by mosquitoes. Furthermore, gravid mosquitoes are a high priority in arboviral surveillance programs because they are likely to be already exposed to virus infection through previous feeding, hence are likely indicators of virus activity (Reiter, 1983). Lastly, it has been established that although male *Ae. aegypti* are not blood feeders they can be infected with dengue and chikungunya viruses via transovarial transmission (Thenmozhi *et al*., 2000; Thavara *et al*., 2009). They can also transmit the viruses venereally to the females who can then transmit it to humans (Mavale *et al*., 2010). Therefore, a trap that is more efficient in capturing male *Ae. aegypti* would be more helpful in dengue and chikungunya fever control programs.

Conclusions

We conclude that natural human skin odor is a good source for identifying attractant compounds that could be used to improve the existing commercial lures for effective surveillance of *Ae. aegypti* in the field. However, blend composition and release rate are critical to determining vector behavioral response. It is clear that some compounds such as hexanoic acid when released alone at a slow rate are an effective lure to *Ae. aegypti* than when released in blends or at higher rates. Future work should therefore focus on

the areas of release rates and effective formulation of hexanoic acid combined with carbon dioxide as a potent lure for monitoring populations of *Ae. aegypti.*

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Authors' contributions

EAO RS BT conceived and designed experiments. EAO conducted the experimental work. EAO analyzed the data. EAO RS CLS CPL CM BT wrote the manuscript. All authors approved the final version for submission.

Competing interests

The authors declare that they have no competing interests.

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Chapter 5

Aedes aegypti in Busia and Kilifi counties of Kenya: Population genetics

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Abstract

Background

In the last decade, a high number of outbreaks of dengue fever have been reported along the Kenyan coast. Prevalence of chikungunya fever has also been detected in western Kenya. Since the control of these two diseases relies mainly on vector control, knowledge of the genetic structure of the vector population on the Kenyan coast and within western Kenya is critical for control and prevention strategies. We investigated the genetic population structure of *Aedes aegypti*, the main vector of dengue and chikungunya fever in Kilifi at the Kenyan coast and Busia in western Kenya.

Methodology

Population genetic analyses were conducted using samples of *Ae. aegypti* from Busia and Kilifi counties of Kenya. A 653-bp region of the mitochondrial DNA cytochrome oxidase I (*COI*) gene was used in the analyses.

Results

The cluster analyses revealed two groups (lineages I and II) existing sympatric ally in both Kilifi and Busia counties. It also revealed that lineage I populations are probably related to the Moyo strain (East African strain) while lineage II was closely related to the Liverpool strain (West African strain). Genetic distances (pair wise F_{ST}), and AMOVA did not indicate genetic differentiation between the *Ae. aegypti* populations captured in Busia and those captured in Kilifi.

Conclusions

The lack of genetic differentiation between *Ae. aegypti* captured in Busia and those captured in Kilifi suggest high gene flow between *Ae. Aegypti* in the two regions. This could probably be due to the intense traffic on the northern corridor, a major route of

travel and trade exchange between the Kenyan coast where Kilifi is and western Kenya where Busia is situated at the border of Kenya and Uganda.

Key words: Population genetics, genetic structure, genetic differentiation

Background

In the last decade dengue outbreaks have become very frequent worldwide suggesting a change in the epidemiology (WHO, 2012). In East Africa, unprecedented outbreaks of dengue have been reported in countries around the coast of the Indian Ocean including Kenya where an outbreak was reported as recently as May 2014 in Mombasa County (Standard newspaper, 2012). Outbreaks of chikungunya which shares the same vector, *Aedes aegypti* (Diallo *et al.*, 2003), has a similar distribution, (Halsted, 1967) and disease symptoms (Carey, 1971; Deller, 1968) with that of dengue virus have also increased with outbreaks reported as recently as September 2014 in Central and Latin America (Latino Fox News, 2014). The epidemic is reported to have overwhelmed hospitals and cut economic productivity (Latino Fox News, 2014). Both dengue and chikungunya have no vaccine or treatment and therefore, their prevention relies mostly on the control of the mosquito vector *Ae. aegypti* (WHO, 2009).

Knowledge of the genetic structure of a vector population has become critical and useful in designing control and prevention strategies for vector-borne diseases in the world today (Urdaneta & Failloux, 2011). Information on the genetic structure of a vector population may help in designing control strategies and determining appropriate control limits necessary to disrupt pathogen transmission (Olanratmarene *et al.*, 2013). This information may also help in analyzing the risk of disease transmission (Ballinger-Crabtree *et al.*, 1992; Huber *et al.*, 2000) as estimating genetic exchanges between vector populations, provide estimates of their abilities to harbor and transmit viruses. Furthermore, understanding genetic structure patterns and gene flow among *Ae. aegypti* populations may be useful in tracking and even preventing the movement of associated genetic traits such as vector competence and other characteristics of epidemiological importance in *Ae. aegypti* populations (Tabachnik, 1991).

The mitochondrial DNA whose genome is maternally inherited and very rarely undergoes recombination (Ballard & Whitlock, 2004) has become a useful marker worldwide for studying intraspecific (Avise, 1994; Walton *et al*., 2000). This is because it has a more linear or clonal evolution than nuclear DNA and its coding genes also display a more rapid rate of evolution. Population genetic studies on *Ae. aegypti* conducted using mitochondrial markers have provided information on the convergence and divergence amongst various populations of the vector around the world (Moore *et al*., 2013). They have also provided vital information on vector population structures and their effect on arbovirus transmission efficiency at the micro- and macro-geographic levels (Urdaneta *et al*., 2008).

Knowledge of the population genetic structure and the dispersal patterns via gene flow of *Ae. aegypti* in areas prone to dengue and chikungunya epidemics would therefore be of importance in understanding their roles as pathogen carriers. In Kenya, previous population genetic studies on *Ae. aegypti* include an investigation of two subspecies of *Ae. aegypt*i, a dark, sylvan form, and a lighter domestic form found closely associated with man, which occur sympatric ally but show a restricted gene flow in the Rabai Kilifi county of Kenya (Tabachnik *et al*., 1979). Here, we analyzed the population genetic structure of *Ae. aegypti* samples from two dengue and chikungunya endemic areas in Kenya, Busia and Kilifi counties (Mease *et al*., 2011), using sequences of the mitochondrial DNA cytochrome oxidase subunit I (*COI*) gene.

Methods

Mosquito collection in the field

Samples of *Ae. aegypti* were collected from Busia and Kilifi counties of Kenya. The collections in Busia were done in the month of November 2013 and those in Kilifi were done in December 2013. Sampling in both places was done during the rainy season. The geographic locations are shown (Figure 1). An average annual rainfall of 1500 mm characterizes the climatic conditions of Busia. The rainfall pattern is bimodal; long rains (March-June) with the short rains (October- December). Temperatures range between

14°C and 30°C. The climate in Kilifi County has an average annual rainfall of 950 mm. The rainfall pattern is also bimodal; the long monsoon rains (April-July) with the short rains (October- December). Temperatures range from a minimum of 21°C to a maximum of 32°C.

Mosquito sampling

In both Kilifi and Busia, mosquitoes were collected using three Biogents (BG) sentinel traps baited with BG lure. Three different sites were randomly chosen around homesteads after obtaining verbal consent from the heads of the homes. The three BG sentinel traps were randomly set up at each of the three sites with a distance of at least one hundred (100 m) between traps. The traps were hung at 0.2 m above the ground and attached to each was a BioQuip igloo (BioQuip Products, 2321 Gladwick Street, Rancho Dominguez, CA 90220, USA) that dispensed carbon dioxide in the form of dry ice (Figure 2). Trapping was done for 9 days at each site. Due to the fact that in both Busia and Kilifi some sites were at a distance of up to 40 km apart, traps were set up at each site at different times of the day and left to run for 24 hours. Mosquitoes were then collected and transported to the laboratory where they were freeze-killed and identified under a dissecting microscope to species level using the appropriate morphological keys (Edwards, 1963; Huang & Ward, 1981; Rueda, 2004).

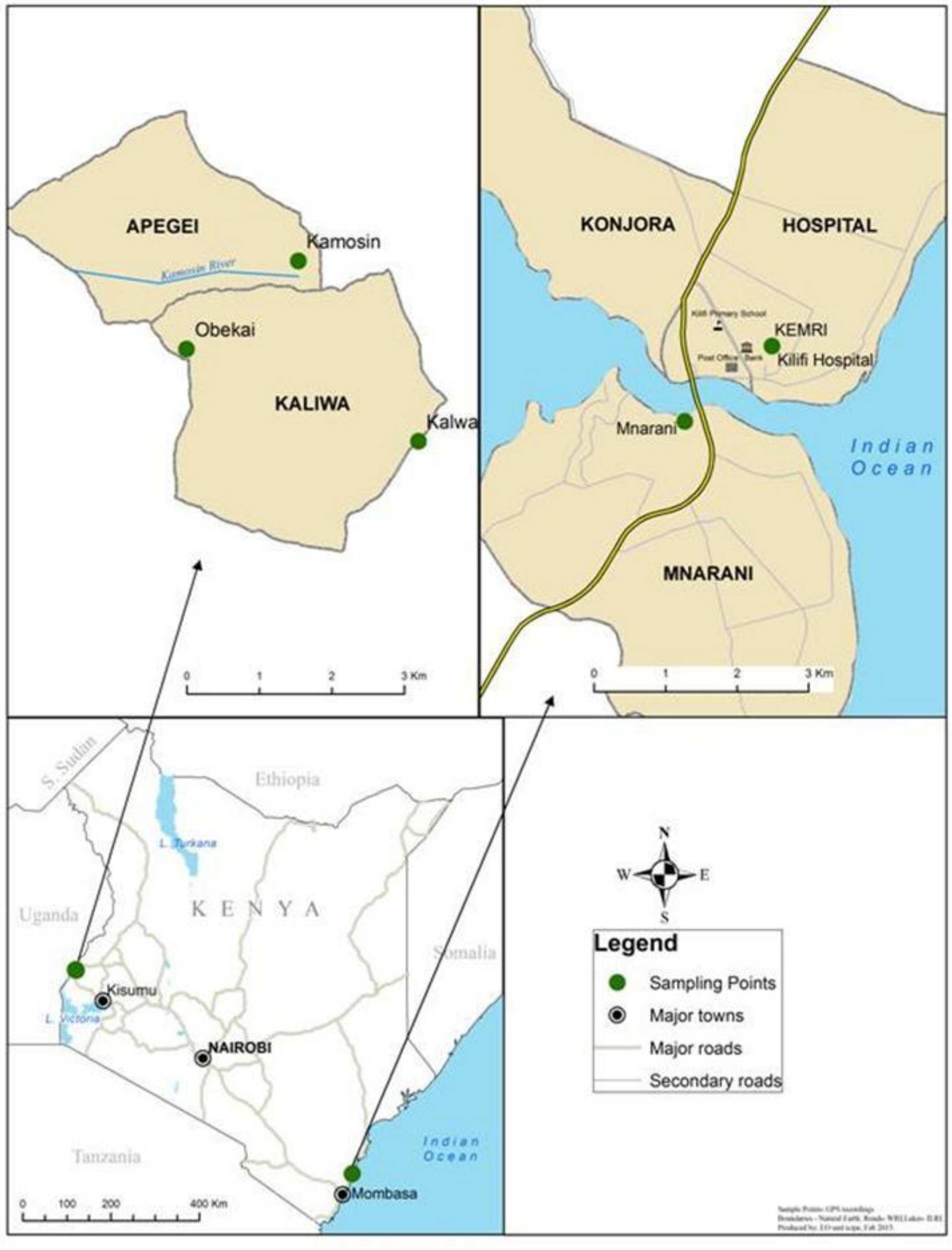

Figure 1. The study sites; Kilifi district in the coast and Busia district in western Kenya.

Figure 2. A set up of the Biogents trap in the field.

DNA extraction and PCR preparation

Genomic DNA was extracted from 22 female *Ae. aegypti* mosquitoes each placed individually in 1.5ml microcentrifuge tubes using QIAGEN DNeasy extraction kits (Qiagen Inc., Valencia,CA,USA), following the manufacturers specifications. The extracted DNA served as DNA template in subsequent polymerase chain reaction (PCR) assays with primers based on *COI* sequences of insect species. The primer pairs LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') (Folmer *et al.*, 1994) were used to amplify a

653 bp fragment of the *COI* gene. The PCR mixture was made in a volume of 15µl: 1 µl of genomic DNA, 0.5 µl forward primer (LCO 11490), 0.5µl reverse primer (HCO2198), 5.85 µl PCR $H_2 0$, 3µl of 1 ×HF amplification buffer, 0.3µl of 0.2nM dNTPs, 0.45µl DMSO and 0.2 µl pol. PCR reaction was performed in the BIORAD thermo cycler. The PCR amplification involved an initial hold of 98°c for 30 sec, followed by 40 cycles of 98°c for 10 sec denaturation, 48°c for 30 sec annealing, 72°c for 40 sec extension and 72°c for 7 min final hold.

Gel electrophoreses, Purification of PCR product and sequencing

The PCR products were electrophoresed for 45 min at 90 V using 1.5 percent agarose (TopVision^{TM} Agarose, Fermentas) gels stained with ethidium bromide. Electrophoresis was done using Cleaver Scientific Limited's runVIEW (Model; RUNVIEW-B). Amplicons for *COI* were purified prior to sequencing using the Exosap Pure kit (according to the manufacturer specifications). Purified amplicons were cycle sequenced using version 3.1 of the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA). Sequencing reactions were purified by sodium acetate precipitation, denatured and then run on a 3730xl DNA Analyzer (Applied BioSystems, Inc.).

Sequence analysis

The *COI* sequences were visually inspected and aligned in MEGA version 6.0 (Tamura *et al.*, 2013). Sequence alignments were performed using MUSCLE (Edgar & Robert, 2004) in MEGA 6.0 using the default parameters of the program. The data set was trimmed to 653bp and thereafter imported into other programs for sequence analyses. The *COI* gene sequences were translated to ensure that no stop codons occurred and the mutational frequency at first, second and third base position was recorded to further rule out the possibility of nuclear mitochondrial pseudogenes (numts) in the mitochondrial dataset (Hlaing, 2009). The *COI* sequences were named according to the area from which they were sampled. In Busia there were three sampling sites, Kalwa, Kamosin and Obekai. The samples were therefore named according to the sites they came from thus; samples from Kalwa were named as KAL 1-4, samples from Kamosin,

named as KAM 1-4 and samples from Obekai as OBK 1 & 2. In Kilifi, there were also three sampling sites, Kilifi hospital, KEMRI Kilifi and Mnarani. So the sequences were named as Hosp 1-4, Kem 1-4 and MNR 1-4.

Polymorphism and diversity

Initial estimates of DNA sequence polymorphism based on the full in group sequence data set were computed using DnaSP 5.10 .01 (Rozas, 2003) following which genetic variability measurements based on the locations and neutrality tests such as Tajima's *D* test, Fu and Li's *D* and *F* tests, Fu's F_S test, and Strobeck's *S* statistic were analyzed. The genetic variability parameters included the number of variable sites, nucleotide diversity, haplotype number and diversity. The neutrality tests Tajima (Tajma, 1989) and Fu and Li (Fu, 1993) tests were used to test the hypothesis that all mutations are selectively neutral (Kimura, 1983). Tajima's *D* is based on the differences between the number of segregating sites and the average number of nucleotide differences (Tajma, 1989). The *D* and *F* tests, proposed by Fu and Li, are based on molecular polymorphism data (Fu, 1993,) Fu's F_S test (Fu, 1997)and Strobeck's *S* statistic (Strobeck, 1997) assess the haplotype structure based on the haplotype frequency distribution and were used as additional neutrality tests. The indices D and Fs were examined based on 1000 coalescent simulations with consideration of the recombination rate using DnaSP. The expectations of these statistics are nearly zero in a constant population size; significant negative values indicate a sudden population subdivision on recent population bottlenecks.

Phylogeny

A principal component analysis (PCA) was performed in GenAIEx 6.4 (Peakall & Smouse, 2006) to cluster the sequence data into genetically homogeneous populations. The data for the PCA was generated after constructing a table of mean evolutionary divergence estimates over sequence pairs within sites for *Ae. aegypti* in MEGA (Tamura *et al*., 2007) (Table 1). Distance trees (dendograms) of only haplotypes from this study and haplotypes of *Ae. aegypti* from Moyo-R strain, origin from Kenya, East Africa (Accession: AF380835), Liverpool strain; origin from West Africa (Accession:

AY056596) and the sylvan form of *Ae. Aegypti*, the formosus strain (Accession: AY056597) (Appendix II) were inferred using the neighbor-joining (NJ) algorithm (Saitou & Nei, 1987) in MEGA, following the Tamura-Nei model (Tamura & Nei, 1993). Sequences of *Aedes albopictus* from Turkey (Accession: JQ412506.1) and India (Accession: KC970276.1) were included as out groups in phylogenetic analysis. The relationships between the observed haplotypes were also assessed by median-joining networks using MEGA 6. Phylip data files (PHY) were created with DnaSP (Rozas *et al.*, 2003) and imported into Network v 4.6.1.2 (Fluxus –Technology, www.fluxus-engineering.com). Networks were calculated with the median-joining algorithm using maximum parsimony post-processing (Tamura *et al.*, 2004).

Genetic structure

Genetic differentiation (F_{ST}) and hierarchical analyses in the program AMOVA were estimated in Arlequin 3.5.1.3 software (Excoffer, 2006). The significance level of F_{ST} values was determined by a permuting test between localities (10,100 permutations). AMOVA analyses were performed at several levels: within Busia, within Kilifi, between Busia and Kilifi, and among all samples (non-grouped).

Results

Polymorphism and diversity

A total of 22 haplotypes were recovered from the 22 *COI* genes. There was a total of 43 variable sites; 19 singleton variable sites and 24 parsimony informative sites even though some of the singletons could have been as a result of Taq PCR error during sequencing (Simard *et al.*, 2007).

The average haplotype diversity (*h*) was 1±0.23, ranging from 1 ± 0.18 (KAM) to 1 ± 0.50(OBK). The average nucleotide diversity (π) was 0.02±0.00, ranging from 0.01±0.00 (KAM) to 0.03±0.00 (MNR). The average number of nucleotide differences (*K*) was 12.75, with the highest values for MNR (Table 1).

For OBK, KAM, KAL, Kem and MNR, Tajima's *D* and Fu and Li's *D* and *F* neutrality tests showed positive and non-significant values while for HSP the values were negative but still not significant. Therefore for all the samples the Tajima's *D* and Fu and Li's *D* and *F* neutrality tests values accepted a neutral model suggesting no possible balancing selection or population subdivision (Table 2). Fu's F_S test, which is more powerful for detecting population expansion, showed positive values for all samples but not significant. Also, Strobeck's *S* test was positive but not significant for all samples. Thus, none of the tests indicated population expansion. In the combined analyses of all the samples, Fu and Li's *D* and *F* neutrality tests showed negative but no significantly different values, Fu and Li's *D* = -1.08, P > 0.10) and Fu and Li's *F* =-1.02, P > 0.10) indicating that the mtDNA diversity *of Ae. aegypti* at both sites is not the result of a single rapid expansion.

Table 1. Genetic variability in *Aedes aegypti* populations from Busia and Kilifi.

	Statistical analysis				
Population	**Haplotypes observed**	**NS**	**K**	**Haplotype diversity (h±SD**	**Nucleotide diversity (π±SD+**
OBK	H1,H2	16	16.000	1.00±0.5	0.02±0.00
KAM	H3,H4,H5,H6	13	7.500	1.00±0.18	0.01±0.00
KAL	H7,H8,H9,H10	21	12.667	1.0± 0.18	0.02±0.00
KEM	H11,H12,H13,H14	19	10.833	1.00± 0.18	0.02±0.00
HSP	H15,H16,H17,H18	15	14.833	1.00± 0.18	0.01±0.00
MNR	H19,H20,H21,H22	26	11.11688	1.00± 0.18	0.03±0.00

NS, number of variable sites; *K*, average number of nucleotide differences.

OBK-Obekai (Busia), KAM-Kamosin (Busia), KAL – Kalua (Busia), kem-KEMRI(Kilifi)HSP – Kilifi hospital (Kilifi), MNR – Mnarani (Kilifi)

Table 2. Summary of statistical analyses of the molecular polymorphism in *Ae. aegypti*

	Statistical analysis				
Population	**Tajmas D**	**Fu and Li's D**	**Fu and Li's F**	**Fu's Fs**	**Storbeck's S**
OBK	-	-	-	-	-
KAM	0.40	0.40	0.40	0.142	1.00
KAL	0.27	0.40	0.39	0.72	1.00
KEM	0.14	0.29	0.27	0.56	1.00
HSP	-0.33	-0.33	-0.34	0.27	1.00
MNR	0.34	0.34	0.35	0.89	1.00
Total population	-0.39	1.08	-1.02	-13.60	1.00

OBK-Obekai (Busia), KAM-Kamosin (Busia), KAL – Kalua (Busia), kem-KEMRI(Kilifi)HSP – Kilifi hospital (Kilifi), MNR – Mnarani (Kilifi)

Phylogeny

Analyses were carried out on 653bp of the mtDNA sequences from 22 *Ae. aegypti* individuals from Busia and Kilifi. The principal component analysis (PCA) based on the 22 *COI* sequences yielded 2 components (Figure 3). The first two principal axes contributed 68.23% (the first axis 56.87 and the second axis 11.36) of the total variance and showed partial separation of these populations into two clusters. The clustering observed by PCA was confirmed in the neighbor joining tree of the 22 haplotypes plus an outer group of *Ae. albopictus* (Figure 4) and another haplotype dendogram based on neighbor joining tree of the 22 haplotypes plus the haplotype of the East African Moyo-R strain (origin from Kenya, East Africa), the haplotype of the Liverpool strain (origin from West Africa) and the haplotype of the sylvan form of *Ae. aegypti* (the formosus strain) (Figure 5). Between the two lineages, the average number of nucleotide differences (*K*)

was 11.117, whereas the nucleotide divergence (*D*) was 0.02. Within the first lineage, there were 25 polymorphic sites, and within the second lineage, there were 138 polymorphic sites (Table 3). Both lineages were detected at both sites showing overlapping tendencies. However samples from Busia formed the higher proportion (67%) of samples in lineage I while samples from Kilifi formed the higher percentage (69%) of samples in lineage II.

Table 3. Genetic variability in Lineage I and II of *Aedes aegypti* populations

	Statistical analysis				
Population	**Haplotypes observed**	**NS**	**K**	**Haplotype diversity (h±SD)**	**Nucleotide diversity (π±SD+)**
Lineage I	H1,H2	25	8.444	1.000±0.052	0.0129±0.00639
Lineage II	H3,H4,H5,H6	138	27.551	1.000±0.030	0.043±0.0026

NS, number of variable sites; *K*, average number of nucleotide differences.

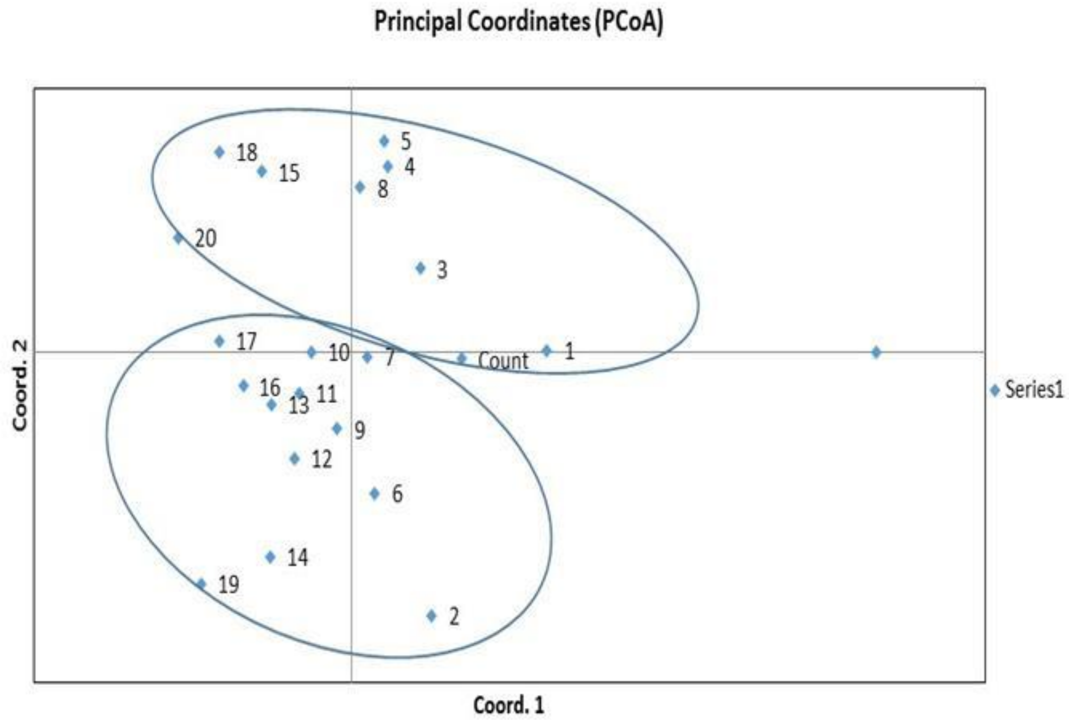

Figure 3. Plot of the principal coordinate analysis PCA generated using GenAlEx for *Ae. aegypti* samples from different localities

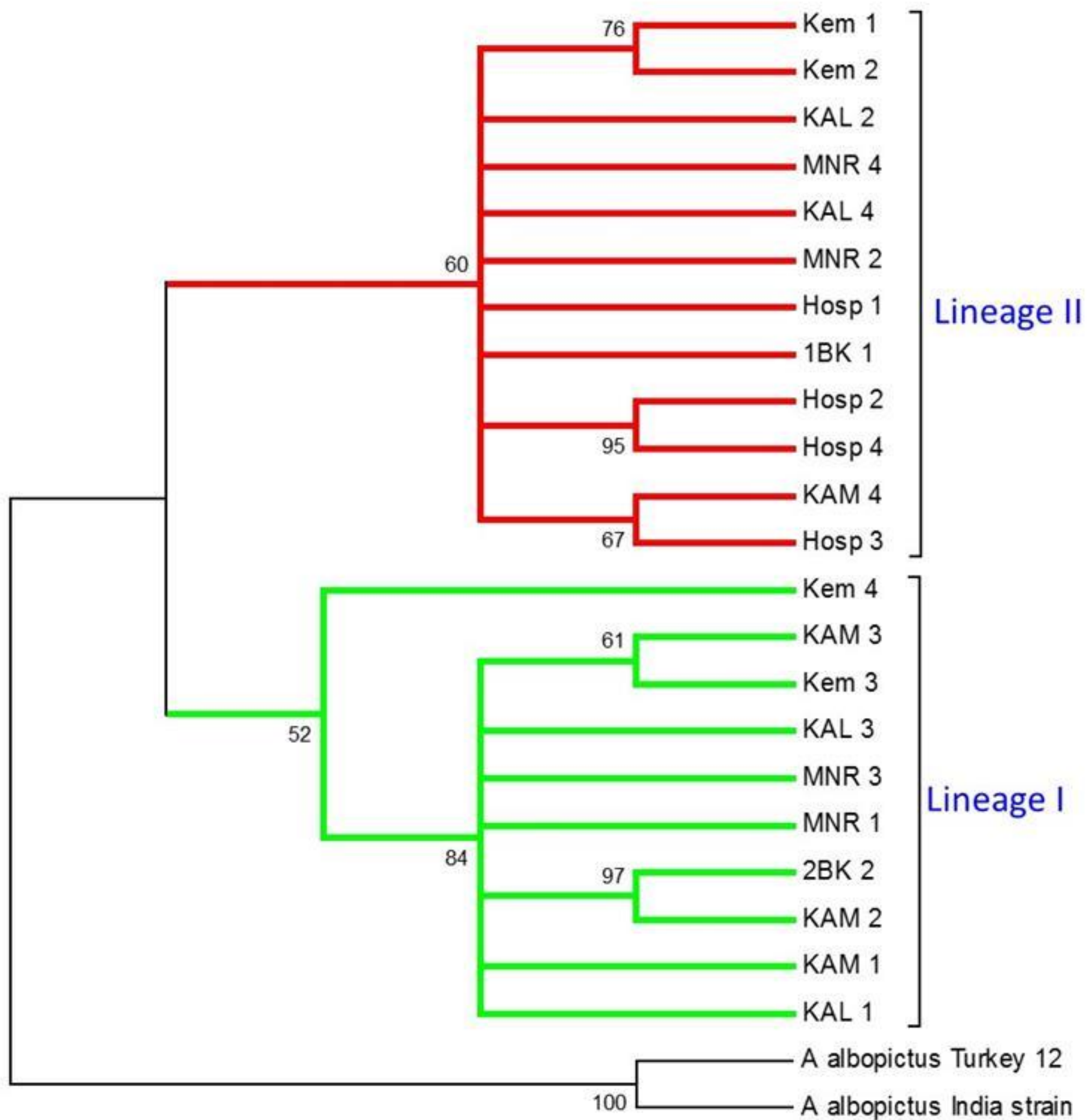

Figure 4. Haplotype dendrogram of 22 *Ae. aegypti haplotypes* from Busia, Kilifi and the 2 haplotypes of *Ae. albopictus* from the Genbank based on the neighbor-joining (NJ) method, computed using the Tamura-Nei model. Bootstrap support values are recorded shown next to the branches. All ambiguous positions were removed for each sequence pair. Samples KAL 1, KAL 2, KAL 3, KAL 4, KAM 1, KAM 2,KAM3, KAM 4 1BK1 and 2BK 2 were sampled from Busia while samples kem 1, kem 2, kem 3, kem 4, MNR 1, MNR 2, MNR 3, MNR 4,Hosp 1, Hosp 2, Hosp 3 and Hosp 4 were sampled from Kilifi.

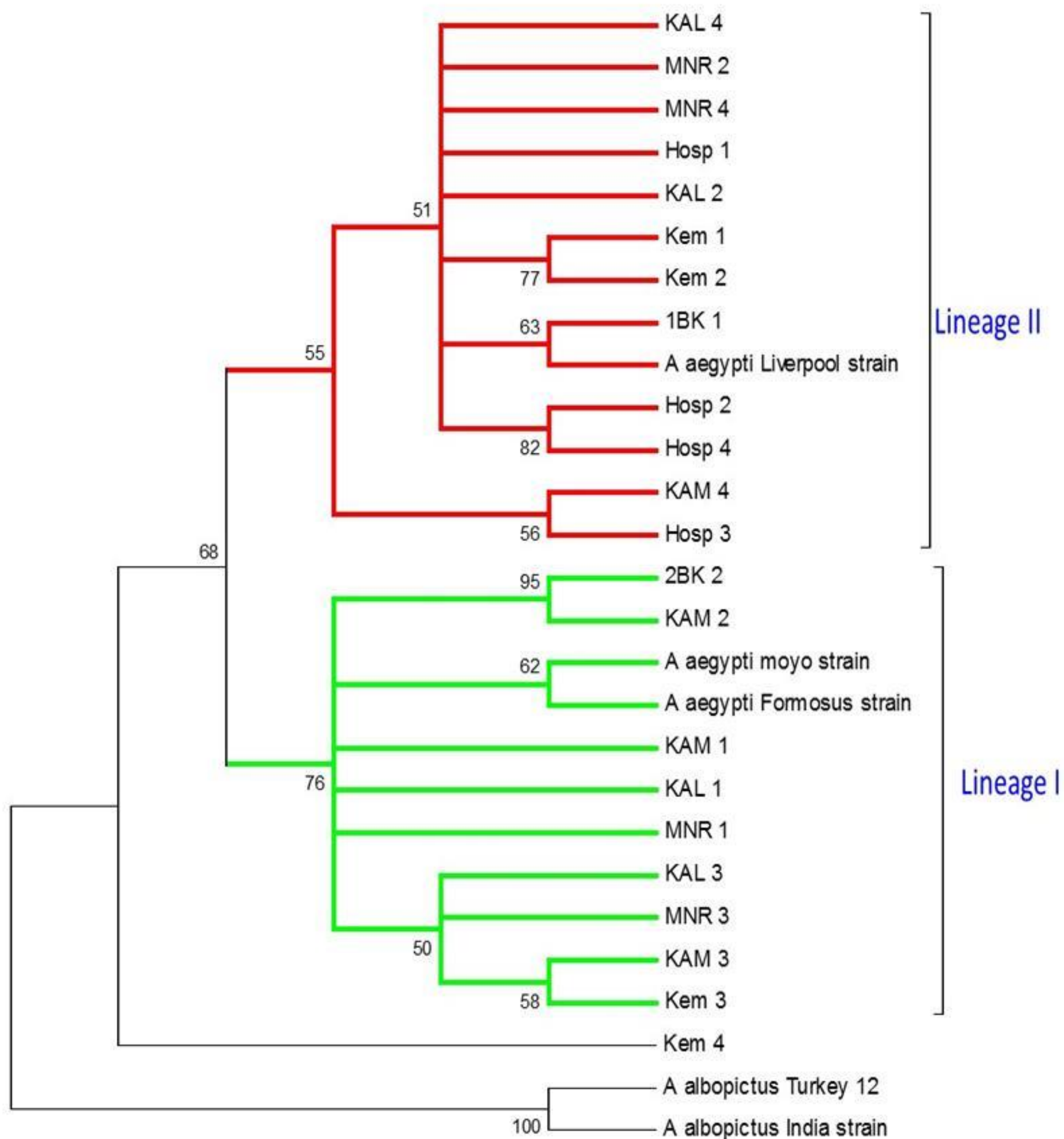

Figure 5. Haplotype dendrogram of *Ae. aegypti haplotypes* from Busia, Kilifi and the Genbank based on the neighbor-joining (NJ) method computed using the Tamura-Nei model. Bootstrap support values are recorded shown next to the branches. All ambiguous positions were removed for each sequence pair. Samples KAL 1, KAL 2, KAL 3, KAL 4, KAM 1, KAM 2,KAM3, KAM 4 1BK1 and 2BK 2 were sampled from Busia while samples kem 1, kem 2, kem 3, kem 4, MNR 1, MNR 2, MNR 3, MNR 4,Hosp 1, Hosp 2, Hosp 3 and Hosp 4 were sampled from Kilifi.

Genetic structure of mosquito populations in the two sites

Genetic differentiation showed a large range (F_{ST} =−0.20354– 0.28589). The pair wise comparison between KAM and HSP showed significant difference after Bonferroni correction (Table 4). The sample from HSP showed the highest number of significant comparisons of all (F_{ST} = 0.03058–0.28589), followed by the samples from KEM (F_{ST}= -0.11640–0.11450) and MNR (F_{ST} = - 0.20354–0.11560).

The 5 AMOVA tests were not statistically significant for all hierarchical levels (Table 5). Within Busia and Kilifi, the most variation (109.88% and 95.7% respectively) occurred within samples; however, there were no significant differences (FST = 0.04298 and 0.09882) among samples. Between the samples captured in Busia and Kilifi, a small percentage ~2.69% (F_{CT}= 0.02687; $P < 0.30462$) of the total variance was due to differences between them while a negative percentage (~ 1.48%) with no significant proportion of the variance was found among samples within the two regions. Again, the most variance was found within samples (98.79%) when all samples were considered.

Table 4. **Genetic distance and gene flow based on population pair wise differences (F_{ST}) values for 6 *Aedes aegypti* populations in Kilifi and Busia**

	OBK	**KAM**	**KAL**	**kem**	**HSP**	**MNR**
OBK	0.00000					
KAM	-0.14286	0.00000				
KAL	-0.20354	-0.02857	0.00000			
kem	-0.11066	0.11450	-0.11640	0.00000		
HSP	0.03058	0.28589*	0.02439	0.04348	0.00000	
MNR	-0.20354	0.03286	-0.13287	-0.03051	0.11560	0.00000

Asterisk indicate significant values after Bonferroni correction at P<0.05.

OBK-Obekai (Busia), KAM-Kamosin (Busia), KAL – Kalua (Busia), kem-KEMRI(Kilifi)HSP – Kilifi hospital (Kilifi), MNR – Mnarani (Kilifi)

Table 5. Hierarchical analysis of the genetic Variation in Aedes aegypti samples from Busia and Kilifi

Group of samples	Source of variation	Degrees of freedom	Variation%	Fixation index	P value
Within Busia	Among samples	2	-9.88		
	Within samples	7	109.88	F_{ST}= 0.09882	0.891
Within Kilifi	Among samples	2	4.3		
	Within samples	9	95.7	F_{ST}=- 0.04298	0.227
Between Kilifi/Busia	Between regions(Among groups)	1	2.69	F_{CT} =0.02687	0.305
	Among samples (populations) within groups/regions	4	1.48	F_{SC} = -0.01521	0.585
	Within all samples (populations)	16	98.79	F_{ST} =0. 01207	0.481

Discussion

Our data indicate the existence of 2 genetic lineages occurring sympatricaly in both Kilifi and Busia counties of Kenya. These observations could be compared with those of Tabachnik *et al*., 1979 Tabachnik, 1982) who observed 2 genetically distinct groups of *Ae. aegypti* occurring sympatricaly in Rabai Kilifi County. They also observed that the two distinct groups are morphologically and behaviorally distinct; A dark, sylvan form and a lighter, domestic form which is found associated with man. However, in our study we were not able to show that the two lineages were morphologically and behaviorally different, although since our study site in Busia was mainly in a rural forested area while Kilifi was mainly in urban area, we could infer that Lineage I which was the dominant group in Busia was likely to be the sylvan form while lineage II which was dominant in Kilifi was likely to be the domestic form.

We also observed that the haplotypes clustered in lineage I show close relationships with the Moyo strain from Kenya while haplotypes clustered in lineage II show close relationships with the Liverpool strain from West Africa. These lineages likely evolved from the ancestral population, presumably in North Africa, and later on dispersed all over the world (Tabachnik, 1991)through intensive trade between different countries in the world i.e West African countries, countries in the Asian continent and countries in the American continent, that may have favored passive dispersion of *Ae. aegypti* through accidental transportation of eggs, larvae or adults which resulted in multiple introductions. Furthermore, the observation that lineage II mosquitoes had a close relationship with strains from Latin America support the hypothesis of Powell and others (Huber *et al*., 2002) who on the basis of isozyme data, suggested that the *Ae. aegypti* populations of South America, the United States, and the Caribbean are genetically related to those from East Africa.

Another important observation was that the genetic diversity of *Ae. aegypti* sampled in both Busia and Kilifi was high as evidenced from the high estimates of the haplotype diversity with overall low frequency of shared haplotypes as there was no shared haplotypes amongst the 22 mosquito samples. These results are likely due to high gene

flow with other populations (Kamgang *et al*., 2011) at both sites brought about by factors like intensive urbanization especially in Kilifi, favorable social and environmental factors and abundance of blood sources fostering the proliferation of *Ae. aegypti* in these areas and consequent increase of gene flow among subpopulations with subsequent increase in genetic variation (Wallis *et al*., 1984; Paupy *et al*., 2005). Another reason for high polymorphism is that *Ae. aegypti* is known to be engaged in the transovarial maintenance and spread of dengue and chikungunya. Transovarial transmission of symbionts is known to influence polymorphism in insect populations as they confer some selective advantage on their hosts thereby favoring them by natural selection (Russell & Moran, 2006; Martinez *et al*., 2014). Selective pressure as a result of transovarial maintenance of the dengue virus in this mosquito could play a role in population sub-structuring or polymorphisms.

Another important observation was that samples from Busia which was mainly a rural area showed a higher nucleotide difference (k) and nucleotide diversity (pi) than samples from Kilifi which was mainly an urban area. This could be explained by the fact that population differentiation has been observed to be shaped by the type, the density and the location of the breeding sites, as well as the human density in an area (Huber *et al*., 2002). Huber *et al*, 2002 observed that populations of *Ae. aegypti* in urban centers are panmictic because there are abundant larval breeding sites and an abundance of humans for adults to feed upon. In contrast, populations on the outskirts became differentiated largely through the processes of genetic drift because larval breeding sites are not as abundant (Huber *et al*., 2002). Another explanation could be that in Busia, *Aedes aegypti* dispersal could have been limited by gardens, cultivated fields, rivers and ponds separating habitations leading to less gene flow. Conversely, in Kilifi which was an urban area the houses were closer to each other and the number of productive breeding sites was likely to be much higher allowing for easier dispersal and thus higher gene flow.

Lastly, Tajima's *D*, and Fu and Li's *D* and *F* neutrality tests were negative and non-significant for both populations in Busia and Kilifi suggesting that there were no population subdivision and expansion. AMOVA tests also demonstrated non-significant

genetic structure for all hierarchical levels with the least variance being recorded between samples from Kilifi and Busia and the most variance recorded within samples in Busia and within Kilifi. This lack of considerable genetic differentiation between mosquito populations of the two sites could have been because all the haplotypes in these populations had similar frequencies. The results also indicate extensive gene flow between Busia and Kilifi populations which could have been caused by human transportation (Failloux *et al*., 1997; Huber, 2004) as *Aedes aegypti* has a short flight range (about I0-800m) in its entire lifetime (Trips & Hausserman, 1986) that can't explain the extensive gene flow. The intense traffic on the northern corridor, a major route of travel and trade exchange between the Port of Mombasa at the Kenyan coast and Busia at the border of Kenya and Uganda, could be considered to have been responsible for mosquito dispersal and the genetic exchange.

Conclusions

The presence of genetically distinct lineages occurring sympatricaly in both Kilifi and Busia counties could imply differences in response to vector control measures and vector competence between the two lineages. This observation could explain the difference in response to LED lights and in infection with ISF's in chapter 2 of this thesis whereby the *Ae. aegypti* in Busia that majorly fell in lineage I were significantly attracted to the violet LED's and more infected by ISF's than those in Kilifi that majorly fell in lineage II. However, it would be important to mention that since our results were drawn from a small sample size and our gene fragments were sequenced only in one direction, further investigations by probably increasing the sample size and sequencing the gene fragments in both directions would be necessary for confirmatory purposes especially bearing in mind that some of the singletons could be as a result of PCR Taq error during sequencing (Simard *et al*., 2007).

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General Conclusions and Recommendations

Effective surveillance of populations of mosquitoes that transmit arboviruses is important in the control of outbreaks. Vector surveillance programs of *Aedes aegypti*, the main dengue and chikungunya vectors, mainly target the immature stages (Sivagnaname *et al*., 2012). However, the immature stage surveillance does not correlate to transmission risks and consequently is not effective in preventing outbreaks and control of pathogen spread into new areas (Ooi *et al*., 2006). Surveillance should therefore concentrate on adult mosquitoes as they are the ones that are directly involved in disease transmission.

However, the currently available tools for sampling adults have inadequacies in one way or the other. While *Ae. aegypti* are not attracted to the incandescent bulb of the carbon dioxide (CO_2) baited CDC light trap, man landing catches (MLC) that most health workers in the area of dengue control resort to is labor intensive and exposes the catcher/bait to infective bites making it unethical. The need for an effective trap for *Ae. aegypti* can therefore not be over-emphasized. Our study aimed to explore ways of improving the existing tools for efficient surveillance and monitoring of *Ae. aegypti* using both visual and odor cues. In addition we also tested for any flavivirus infection of the sampled *Ae. aegypti* from both Busia and Kilifi and evaluated the genetic structures of the *Ae. aegypti* populations from the two sites.

Light has been observed to be an attractant to mosquitoes (Wilton &Fay, 1972; Browne & Bennett, 1981). Investigations have also proven that vision and color sensitivity play a principle role in host location by adult *Ae. aegypti* (Muir *et al*., 1992). Therefore, in an effort to improve *Ae. aegypti* sampling using light, chapter 2 investigated if CDC light traps with Light Emitting Diodes emitting blue, green, violet and a mixture of blue, green and red lights (BGR) could be effective substitutes to the CDC light trap with the incandescent bulb and if so, determine the best color trapping *Ae. aegypti* in the field in Kilifi and Busia. Chapter 2 also investigated the flavivirus infection rate of *Ae. aegypti* from the two sites . We observed that the violet LED trapped significantly more *Ae aegypti* and significantly more flavivirus infected *Ae. aegypti* in Busia than the standard CDC light trap. However, this trend was not observed in Kilifi. We would therefore recommend further investigations to establish

the efficiency and potential of violet LEDs as effective surveillance tools for *Ae. aegypti.* We recommend more field investigations perhaps in different field sites and not just cross sectional but longitudinal studies across different seasons of the year.

Mosquitoes have been observed to be attracted to their human hosts via the odors produced by the hosts (Schreck *et al*., 1990; Takken 1991; Geier & Boeckh, 1999). Previous studies have also observed that human host odor baits can effectively be used in sampling mosquitoes in the field (Costantini, 1993, 1996; Knols *et al.,* 1998). This was the subject of chapter 3 where we compared the efficacy of Biogent sentinel traps baited with natural human odors against commercial odor bait the Biogents (BG) lure derived from human skin volatile compounds. We observed that traps baited with human odors captured more mosquitoes than the BG lure. An analysis of the chemical profile of the human volatiles and the commercial lure revealed major qualitative differences between the chemical profiles of human odors and the commercial BG-lure. Aldehydes, fatty acids and ketones dominated human odor profiles, whereas the BG-lure released mainly hexanoic acid. We therefore conclude that additional candidate attractants are present in human skin volatiles which can help to improve the efficacy of lures for trapping and surveillance of *Ae. aegypti*. We recommend further investigations to identify the biologically active chemical compounds from the human volatiles.

Current science is concentrating on identification of attractant compounds in human volatiles and formulation and testing of the synthetic odor baits in the field (Okumu *et al*., 2010, Tchouassi *et al*., 2013). This formed the subject for chapter 3 where we identified the biologically active compounds in human skin volatiles and tested their efficacy as attractants as single compounds or in blends in sampling *Ae. aegypti* in the field. We consistently isolated thirteen EAD-active components from four volunteers by GC/EAD analysis. A further identification of the compounds by GC/MS revealed that they were mainly aldehydes, carboxylic acids and ketones. Field assays of some attractant compounds either as single compounds or as blends revealed that a binary blend of aldehydes, nonanal and octanal, trapped 3-fold more *Ae. aegypti* than a binary blend of the carboxylic acids, isovaleric acid and propanoic acid in Busia. The binary blend of aldehydes also captured more *Ae. aegypti* in Kilifi than the binary blend of carboxylic acids. However, combining the binary blend of

carboxylic acids with the binary blend of aldehydes reduced trap captures by 45%. Interestingly, traps baited with hexanoic acid which is also a carboxylic acid significantly trapped more *Ae. aegypti* than the control trap baited with carbon dioxide only in Kilifi. However, traps baited with a mixture of hexanoic acid and the aldehydes octanal and nonanal captured, fewer *Ae. aegypti* than the trap baited with hexanoic acid alone. We therefore conclude and recommend that when one is formulating attractant baits its critical to note that some compounds that might be highly attractive to *Ae. aegypti* mosquitoes as single compounds might produce antagonistic and spatial repellence effects when mixed together. However, we would like to urge for further bioassays to identify the olfactory role played by the other EAD active compounds identified but not tested in our study as this might lead to more effective and efficient baits or repellents. One might even foresee a push pull system where on one side there would be strong repellents that would repel *Ae. aegypti* to traps baited with strong attractant baits on the other side. This would lead to mass trapping of *Ae. aegypti* in dengue and chikungunya endemic areas and thus the reduction of mortality and morbidity from these diseases.

Currently, it has been observed that the genetic structure of a mosquito population can determine important epidemiological factors like their vector competence (Gubler *et al*., 1979, Failloux *et al*., 1994) and susceptibility to insecticides (Ocampo *et al*., 2004). Therefore, it was critical to study the genetic structure of the mosquito populations in the two dengue and chikungunya endemic areas as this would directly help in the control of diseases. This subject formed our chapter 4 where by using 653-bp region of the mitochondrial DNA cytochrome oxidase I (*COI*) gene we observed two groups (lineages I and II) existing sympatricaly in both Kilifi and Busia counties. Lineage I populations were observed to be likely related to the Moyo strain (East African strain) while lineage II was closely related to the Liverpool strain (West African strain). We also observed no genetic differentiation between the *Ae. aegypti* populations captured in Busia and those captured in Kilifi. We therefore conclude that there was high gene flow between *Ae. aegypti* in the two regions which could be due to the intense traffic on the northern corridor, a major route of travel and trade exchange between the Kenyan coast where Kilifi is and western Kenya where Busia is situated at the border of Kenya and Uganda. However, since this suggests that the mosquito populations in the two regions could have similar vector competence and

probably insecticide resistance, we would also like to recommend that future research should concentrate on investigating vector competence and insecticide resistance of *Ae. aegypti* from Busia and Kilifi in order to better understand the epidemiological aspects and use of insecticides as a vector control method.

Overall, we believe that our work has improved on the knowledge on the role of olfactory and visual cues in the control of pathogens transmitted by mosquitoes. It has represented a detailed investigation on the development and use of natural and synthetic human skin volatiles as odor baits in trapping *Ae. aegypti* for the surveillance and monitoring of dengue and chikungunya viruses. It has also given information on light preferences of *Ae. aegypti.* However, it's very clear from our work that although *Ae. aegypti* responds to light cues, the response to human odor cues is much stronger as shown by our data especially in Kilifi where the odor baits attracted up to 9 folds more *Ae. aegypti* than the light cues. In addition, our work has given details on the genetic structure of *Ae. aegypti* populations in Kilifi and Busia counties of Kenya. Genetic structure of a mosquito population has been observed to be important epidemiologically as it determines vector competence and susceptibility of the mosquito population to control measures like insecticides.

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