Vector potential of *Aedes* (*Stegomyia*) spp. populations and risk of dengue and yellow fever virus transmission in three Kenyan cities

By

Sheila Biloh Agha

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Department of Zoology and Entomology

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Supervisors:

Prof A.D.S. Bastos, Prof R. Sang and Dr D.P Tchouassi

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Sheila Biloh Agha

Supervisors:

Prof. Armanda D.S. Bastos
Mammal Research Instute (MRI)
Department of Zoology and Entomology
University of Pretoria
Private Bag 20, Hatfield 0028, South Africa
Email: ADBastos@zoology.up.ac.za/

Prof. Rosemary Sang
Arbovirologist and Head: Viral Hemorrhagic Fever (VHF) Reference Lab
Centre for Virus Research, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya
Email: rsang@icipe.org / Rosemary.Sang@usamru-k.org

Dr. David P. Tchouassi
Scientist: Behavioural and Chemical Ecology Department
International Centre of Insect Physiology and Ecology (*icipe*)
P.O. Box 30772-00100, Nairobi, Kenya
Email: dtchouassi@icipe.org

Declaration

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

Signature:

Date: November 2017

Disclaimer

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

Thesis Summary:

Dengue (DENV) and Yellow fever (YFV) viruses are medically important flaviviruses that are transmitted by *Aedes* mosquito vectors of the subgenus *Stegomyia* especially *Aedes aegypti* and *Aedes bromeliae,* respectively, in East Africa. Urbanization has been identified as a major driver in the emergence of these diseases because of the permissive environment it creates promoting the convergence of susceptible human hosts and local vectors. However, while dengue outbreaks have mainly been reported in urban and semi-urban areas of East Africa, yellow fever outbreaks remain limited to the sylvatic cycle. Urban outbreaks may be facilitated by adaptation of the viruses to sylvatic vectors. In Kenya recurrent dengue outbreaks remain limited to the city of Mombasa and do not occur in the other major cities of Kisumu and Nairobi, despite unplanned urbanization being a problem across Kenya. The risk of the spread of dengue to other major cities as well as the emergence of an urban yellow fever outbreak in Kenya therefore remains unknown and deserves public health attention. This scenario emphasizes the need for risk assessment studies to inform public health decisions on cost effective vaccination for yellow fever and vector control for dengue. With no knowledge of the level of yellow fever risk in the major urban areas of Kenya, or how the dengue risk levels in Mombasa compare to those of other areas of contrasting endemicity (Kisumu and Nairobi), this study initially performed an intensive house-to-house survey of natural and artificial water holding containers to assess the degree of infestation with mosquito immatures (larvae and pupae) using standardized immature sampling tools. Based on estimated *Stegomyia* indices of the major *Aedes* (*Stegomyia*) species encountered, the risk of transmission of DENV/YFV in these three cities of Kenya was established and included seasonal trends (long-rains, short-rains and dry seasons). Based on the container type and the number of immatures collected, the preferred breeding sites of the major vectors were identified and characterized for targeted vector control. Also, following an intensive sampling aided by carbon dioxide baited BG-Sentinel traps, the seasonal abundance and diversity of the host-seeking adult vector populations were compared for the three cities. Using prokopack aspirators, the preferred resting sites of the major vector *Ae. aegypti* were identified and compared across the different cities. Furthermore, the 12S gene target extensively used for blood meal identification was used to determine the host feeding preference of wild-caught, blood-fed *Ae.*

aegypti mosquitoes collected in all three cities. The ability of *Ae. aegypti* populations to transmit strains of DENV/YFV at selected temperatures of 22°C, 28°C and 31°C (representing the minimum/maximum average monthly temperatures), was assessed and the vectorial capacity for DENV transmission estimated and compared for the three cities. Following vector competence studies, the genetic difference of *Ae. aegypti* specimens found to be susceptible and non-susceptible for YFV were compared by analyzing sequence variation in mitochondrial cytochrome oxidase subunit I (*COI*) gene, a genome target widely used in molecular evolution studies to differentiate between closely related mosquito species. Findings from this study showed that *Ae. aegypti* and *Ae. bromeliae* were the major *Stegomyia* species in all three cities. While the immatures of *Ae. aegypti* preferentially occurred in artificial water-holding containers like jerricans, drums, used/discarded containers and tyres, those of *Ae. bromeliae* utilized more natural sites such as tree holes and leaf axils. These identified breeding sites could be made the focus of targeted vector control. Based on established vector index thresholds, the risk of DENV transmission was high in Kilifi (outskirts of Mombasa) and Kisumu, and low-to-medium in Nairobi, while the risk of YFV transmission was low-to-medium in Kilifi and Kisumu, and low in Nairobi. *Ae. aegypti* was the most abundant host-seeking *Stegomyia* species, and found to be significantly more abundant in Kilifi and Kisumu than Nairobi, and during the long-rains (April-June). Low occurrence of *Aedes bromeliae* was observed and the abundance varied neither by season nor by city. *Ae. aegypti* displayed differences in resting habits with populations in Kilifi being comparably endo- and exophilic compared to Kisumu and Nairobi (mainly exophilic), suggesting possible genetic difference in the mosquito populations. Further laboratory experiments to assess how dengue risk levels in Kilifi compare to Kisumu and Nairobi revealed that all three populations of *Ae. aegypti* could transmit the DENV serotype-2 and virus transmission increased with an increase in temperature. Interestingly, blood meal analysis suggested that *Ae. aegypti* mosquitoes in Kilifi and Nairobi were more anthropophagic compared to Kisumu. Overall, the estimated potential for DENV transmission (vectorial capacity) in Kilifi was 9- and 14-fold higher than in Kisumu and Nairobi, respectively. This pattern was mainly influenced by the low levels of human blood feeding detected in *Ae. aegypti* mosquitoes in Kisumu, and the low temperatures in Nairobi. Vector competence studies provided evidence that *Ae. aegypti* mosquitoes from all three cities were susceptible to infection with the East

African YFV genotype but unable to disseminate the virus. This was suggestive of a low risk of YFV transmission in all three cities. Two *Ae. aegypti* lineages, well supported by the maximum likelihood-tree, were observed in Kilifi, Kisumu and Nairobi. However, no unique pattern was observed in the clustering of the YFV-susceptible and non-susceptible specimens in the different lineages. It was therefore unlikely that the genetic differences within the *Ae. aegypti* population affect YFV susceptibility and hence yellow fever epidemic patterns in Kenya. Taken together, this study provides the most in-depth data on entomological risk factors relating to transmission of DENV/YFV and possible emergence of dengue and yellow fever in the major urban areas of Kenya. The improved understanding of the epidemiology of these diseases strongly suggests where to focus control efforts, especially vector control, as well as providing guidance for cost effective vaccination for yellow fever.

Keywords: Dengue and yellow fever viruses, risk assessment, vector surveillance, *Aedes aegypti*, *Aedes bromeliae*, *Stegomyia* indices, vector abundance, resting preference, feeding pattern, vector competence, vectorial capacity, BG-Sentinel traps, prokopack aspirators, genetic diversity, cytochrome oxidase subunit I, urbanization, Kenya.

Dedication

This thesis is dedicated to the memory of my late father (1949-2017). You encouraged me to reach greater heights and to be an independent person. I wished you had lived long enough to see me accomplish this. You will remain my hero for life. May your gentle soul rest in peace.

To my very precious daughter Princess Talia, all this would not have been possible without your unconditional love and prayers. You are a perfect example of the word understanding.

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Chapter 1

General Introduction, Rationale and Key Questions

Arboviruses (Arthropod-borne viruses) are globally distributed and are transmitted biologically among vertebrate hosts by hematophagous (blood-feeding) arthropod vectors such as mosquitoes, sandflies, biting midges and ticks (Weaver and Reisen, 2010). Except for dengue and chikungunya viruses, which have lost the requirement for enzootic amplification, arboviruses mainly circulate among wild animals, and cause disease after spill-over transmission to humans and/or domestic animals that are incidental or dead-end hosts (Gubler, 2002; Weaver and Reisen, 2010). They have a high propensity to cause encephalitis and/or viral hemorrhagic fever (VHF) syndromes and hence are a threat to public health (Sang and Dunster, 2001; LaBeaud et al., 2011). Of the more than 100 arboviruses known to cause disease in humans, most belong to members of the Flaviviridae, Bunyaviridae, and Togaviridae families (Gubler, 2002; LaBeaud et al., 2011). The impact of arboviruses on susceptible human hosts is increasing and this together with their geographic spread accounts for their emergence and re-emergence (Gubler, 2002). This is the case with dengue and yellow fever viruses, which represent the most important flaviviruses (Family: Flaviviridae, Genus: *Flavivirus*).

Dengue virus (DENV) represents the most important human arboviral pathogen. Dengue caused by DENV affects an estimated 50–100 million people annually with 40% of the world's population at risk (Gibbons and Vaughn, 2002; WHO, 2017a). The severe forms of the disease (dengue hemorrhagic fever and dengue shock syndrome) are responsible for as many as 15,000-25,000 deaths annually (Wuestewald, 2013). Yellow fever on the other hand affects 200,000 people yearly; with about 30,000 deaths, and a case fatality rate of 20-50% (WHO, 2014a; Wilder-Smith and Monath, 2016). An estimated 900 million people live in yellow fever endemic areas (WHO, 2014a). The yellow fever infection rate is higher in Africa with 90% of the total number of cases being reported from this part of the world (Tolle,

2009). About 15% of the severe yellow fever cases develop the characteristic jaundice symptoms (CDC, 2015).

Dengue virus comprises four related serotypes (DENV-1-4), that share a common transmission cycle but differ in their antigenicity (Gubler, 2002; WHO, 2017a). All four DENV serotypes are responsible for epidemics in Africa (Amarasinghe et al., 2011; Jaenisch et al., 2014), with three of these serotypes (DENV-1-3) causing acute fever in East Africa including Kenya (AFENET, 2013; Ellis et al., 2015; Konongoi et al., 2016). Also, DENV-2 is responsible for the highest number of epidemics in Africa (Sang, 2006; Amarasinghe et al., 2011).

For yellow fever virus (YFV), there exist 7 genotypes, two of which occur in South America, and five in Africa. The African genotypes are the East African genotype (Kenya, Uganda), Central/East African genotype (Sudan, Ethiopia, Central African Republic and Democratic Republic of Congo), West African genotype I (Nigeria, Cameroon, and Gabon), West African genotype II (Senegal, Guinea, Ivory Coast, and Ghana), and the Angolan genotype (Angola) (Chang et al., 1995; Stock et al., 2013). Nucleotide variability ranges from 25 to 30% among the different YFV genotypes, which together with existing genetic variability within vector species could affect the virulence of the YFV (Mutebi and Barrett, 2002; Stock et al., 2013). Although, five YFV genotypes are circulating in Africa, the 1992/93 outbreak in Kenya only witnessed the re-emergence of the East African YFV genotype, about 40 years after its last detection (Mutebi et al., 2001; Ellis and Barrett, 2008).

Dengue and yellow fever viruses are transmitted by *Aedes* (*Stegomyia*) mosquito species in Africa, and the *Haemagogus* or *Sabethes* mosquito species in South America (Mutebi et al., 2001; Rogers et al., 2006). The *Aedes* species include *Aedes aegypti, Aedes albopictus*, members of the *Ae. simpsoni* complex, *Aedes africanus, Aedes keniensis, Aedes metallicus Aedes tarsalis, Aedes furcifer/taylori* group and *Eretmapodite chrysogaster*, a non *Aedes* vector (Mutebi and Barrett, 2002; Ellis and Barrett, 2008). The immature forms of these vectors mostly develop in natural/artificial containers.

Aedes aegypti is responsible for urban transmission of both DENV/YFV (Ellis and Barrett, 2008). It is a highly anthropophilic, diurnal vector that is capable of biting multiple persons during a single gonotrophic cycle (Scott et al., 2000), thereby having the propensity for quickly spreading the viruses in dense urban settings (Rogers et al., 2006). The vector is most active approximately two hours after sunrise and several hours before sunset. While *Ae. aegypti* prefers to rest indoors in Asia and South America (Scott et al., 2000; Chadee, 2013; Dzul-Manzanilla et al., 2017), information on its resting habit in Africa is not available. *Aedes aegypti* exists in two forms*;* the domestic form *(Aedes aegypti aegypti)* and the forest form *(Aedes aegypti formosus*). The domestic form lives close to human settlements, predominantly in urban areas and can be found laying eggs in artificial containers (water storage containers, flower pots, discarded tyres etc) created by human activities (Gubler, 1998). The forest/wild subpopulation is zoophilic and breeds in rock holes, tree holes and fruit husks. The eggs of *Ae. aegypti* can survive desiccation for several months and will hatch into larvae when submerged in water, making it easy for the vector to be introduced into new areas, hence the (re-) emergence of dengue and yellow fever. About 2.5 billion people currently live within the range of the *Ae. aegypti,* thus there is a high risk of contracting either or both DENV or YFV (Gubler, 1998).

Aedes albopictus is the most invasive mosquito in the world, feeding aggressively on humans during the day (Benedict et al., 2007). It is an important secondary DENV vector**,** and breeds in tree holes especially in small, restricted, shaded bodies of water surrounded by vegetation. This mosquito species is ecologically flexible and this allows it to colonize many types of man-made sites and urban regions. Although it has not be documented in Kenya it is widely distributed in tropical and subtropical parts of the world and it is competent to develop YF as well (Mitchell et al., 1987).

Members of the *Ae. simpsoni* complex can feed on a number of hosts ranging from rodents to primates (Mukwaya, 1974), thus increasing the risk of disease transmisson. They feed outdoors in full sunlight all day but peak of activity is mid-afternoon. They prefer to rest among vegetation and can be found breeding in tree holes, leaf axils (pineapples, colocasias, certain varieties of bananas) and occasionally in man-made containers. This vector complex consists at least three species (*Aedes simpsoni s.s, Aedes bromeliae, Aedes lilii*) of which only *Ae. bromeliae* has been incriminated as a

YFV vector (Walter et al., 2014). *Aedes bromeliae* has also been described as the main YFV vector in East Africa (Ellis and Barrett, 2008). It is highly anthropohilic and breeds in containers in the domestic and peridomestic environments (WHO, 2014b), often serving as a bridge vector moving the YFV from one transmission cycle to the other.

Both DENV and YFV share a niche in the ecosystem being maintained in a cycle that involves humans and other non-human primates (especially for YFV), as well as *Aedes* vectors in the *Stegomyia* subgenus (Rogers et al., 2006; Ellis and Barrett, 2008; Monath and Vasconcelos, 2015). Different vectors are involved in distinct transmission settings such as wild/forest (*Aedes africanus*), semi-domestic (*Aedes bromeliae*) and domestic (*Ae. aegypti*); as such, the disease transmission cycles vary from sylvatic, to intermediary (for YFV), to urban (Monath, 2001; Rogers et al., 2006; Ellis and Barrett, 2008; WHO, 2014; Monath and Vasconcelos, 2015). Dengue and yellow fever viruses are known to have originated from sylvatic cycles that are maintained by non-human primates and forest-dwelling mosquitoes (Rogers et al., 2006; Vasilakis et al., 2011). Human-to-human transmission (urban) cycles, sustained by *Ae. aegypti* vectors, especially for DENV subsequently became established.

In the sylvatic cycle, YFV circulates between non-human primates and tree-dwelling/forest *Aedes* species mosquitoes such as *Ae. africanus*. When humans encroach into these forest habitats and are bitten by these infected mosquitoes, a spillover transmission occurs in the human population. In East Africa, yellow fever is known to be largely sylvatic involving a non-human primate-mosquito cycle with occasional spillover to human populations (Ellis and Barrett, 2008). The 1992-1993 YF outbreak in Kenya, was largely sylvatic, involving tree dwelling mosquitoes like *Ae. africanus* and *Ae. keniensis* (Reiter et al., 1998). The sylvatic cycle of yellow fever may also be maintained by vertical viral transmission (Fontenille et al., 1997). The sylvatic cycle of dengue in East Africa is not well understood although ancestral DENV are represented by forest strains that originated from primitive non-human primates in West Africa and Southeast Asia (Wang et al., 2000).

An intermediary transmission cycle occurs for YFV in rural areas of Africa. Here, peridomestic mosquito species (occurring within 10m of the house) such as *Ae.*

bromeliae, *Ae. furcifer* drive transmission between humans and non-human primates (Ellis and Barrett, 2008). These mosquito species are capable of biting both humans and monkeys, thus sustaining small-scale epidemics of yellow fever in rural human populations. This is the most common form of yellow fever seen in recent decades.

Once either the DENV/YFV is acquired by the highly anthropophilic and domestic vector *Ae. aegypti*, an urban transmission cycle can occur. In this case, humans are the main host and such transmissions spread rapidly, often involving a large population of susceptible human hosts (Monath, 2001; Rogers et al., 2006; Monath and Vasconcelos, 2015). The *Ae. aegypti* vector was widely controlled in the 1930s and this resulted in the urban form of the disease becoming uncommon. With the rise in urbanization, the vector has once again been reintroduced into areas, raising a new challenge of urban outbreaks. While recent outbreaks of dengue have been reported mainly in the urban areas of East Africa, urban yellow fever outbreaks are rather more common in West Africa (Ellis and Barrett, 2008). Urban outbreaks are feared the most by public health authorities as the disease may quickly spread in dense urban areas (Rogers et al., 2006).

Emerging and re-emerging infectious diseases have been highly linked to urbanization and globalisation, with DENV transmission being a classic example (Gubler, 1998, 2011; Weaver and Reisen, 2010). According to the UN-Wall chart, urbanization is growing rapidly and it is anticipated that by 2030, 60% of the world's population and 50% of Africa's population will live in urban areas (UN, 2003). However, expanding urbanization results not only in the extreme concentrations of susceptible human hosts frequently living under socioeconomic conditions conducive to vector population expansion, but also in the creation of urban "heat islands" that facilitate disease transmission (Weaver and Reisen, 2010). Recent yellow fever outbreaks in Angola and Democratic Republic of Congo, DRC have also demonstrated a change in the disease dynamics from the sylvatic transmission cycles to the urban cycles (WHO, 2017b). Urban epidemics can reach unprecedented scales and quickly become uncontrollable as 70% to 80% of the populations can be involved (Gubler and Trent, 1993). Also, international travel (movement of people) or globalization has changed substantially in the past 30 years and cities are now becoming important hubs for the introduction/transmission of infectious diseases such

as dengue and yellow fever into new areas (Gubler, 1998, 2011; Wilder-Smith and Monath, 2016). Global travel has been associated with the emergence of infectious diseases such as dengue, as they continue to emerge in areas of heavy tourism (Ostroff and Kozarsky, 1998).

Climatic factors (temperature, rainfall, humidity) also influence the emergence and re-emergence of infectious diseases (Patz et al., 1996) and dengue and yellow fever are among the most sensitive. The effect of climate change on disease epidemiology includes increases in vector abundance, increases in rate of feeding/reproduction, reduced pathogen incubation periods, and a shift in the geographic range of the vectors. Vector abundance often shows seasonal variation, a pattern, which is known to closely mirror dengue epidemics in Kenya (Lutomiah et al., 2016). Also, vector abundance has a positive relationship with temperature and rainfall (Barrera et al., 2011; Dhimal et al., 2015). In addition, increases in *Ae. aegypti* abundance or the abundance of other potential vectors will increase human-vector contact and hence increase the risk of disease transmission. Very limited information is currently available on the abundance and diversity of potential vectors of DENV/YFV that inhabit the major cities in Kenya.

However, for rapid progression of virus amplification to epidemic levels, competent vectors and susceptible host populations must converge repeatedly within a permissive environment (Weaver and Reisen, 2010). Therefore, in addition to urbanization, globalization and climatic factors, vector bionomics (ecology, feeding/rest patterns) as well as the vector's ability to successfully transmit the virus after exposure to an infectious dose will act in conjunction to influence the transmission potential of DENV/YFV.

The ecology of the vector (both immatures and adults) occurring in close association with humans impacts the risk of transmission of DENV/YFV, as with *Ae. aegypti*. While data on immature surveys can provide information on the risk levels (*Stegomyia* indices) of DENV/YFV transmission, the preferred vector breeding sites once identified can be targeted for vector control. Also exploiting the resting preferences of these vectors can provide baseline information, which can be used for control of adult mosquitoes during an emergency outbreak situation. *Aedes aegypti*

populations in dengue endemic regions have been associated with high human feeding (Scott et al., 2000, Harrington et al., 2001; Ponlawat and Harrington, 2005), demonstrating that human feeding is an important risk parameter in assessing transmission potential of arboviral diseases. However, studies on the feeding habits of *Ae. aegypti* populations present in the major Kenyan cities are presently lacking.

A vector's ability to successfully transmit a pathogen or vector competence is defined by the suite of factors that allows the vector to become infected after exposure to an infectious dose of the pathogen, and to transmit the pathogen once it encounters a susceptible host (Weaver and Reisen, 2010; Tabachnick, 2013). This mode of transmission often follows a series of processes which involve imbibing an infectious blood meal, infection of the vector's alimentary tract by the virus, dissemination of the virus to the haemocoel, virus replication in the salivary gland and eventually transmission to a susceptible host *via* infectious saliva during a blood meal (Weaver and Reisen, 2010; Tabachnick, 2013). The vector competence of a vector to a pathogen is affected by both extrinsic and intrinsic factors.

Extrinsic factors are influenced by climatic factors, with temperature being the most studied. Laboratory experiments have shown that increasing temperatures will generally increase viral replication in mosquitoes hence increasing their susceptibility and ability to transmit the virus (Watts et al., 1987). Intrinsic factors might be inherited traits that influence the host feeding pattern of a mosquito, the ability of the mosquito to pick up an infective viral dose in a blood meal and its subsequent transmission by bite or though the eggs (Tabachnick, 2013).

Whereas *Ae. aegypti aegypti* has been described as anthropophilic and a more competent DENV/YFV vector, *Ae. aegypti formosus* is zoophilic and considered to be a less competent vector (Tabachnick et al., 1985; Failloux et al., 2002; McBride et al., 2014). This behavioral difference in host feeding demonstrated by *Ae. aegypti* subspecies is genetically controlled (McBride et al., 2014). It varies within geographical areas, and may be associated with differential DENV/YFV transmission risks. Little is known about the host feeding pattern and the genetic variability that exists within the *Ae. aegypti* populations within the major urban areas of Kenya.

However should this variability affect the epidemiology of yellow fever, this may explain why it remains absent in urban areas of Kenya.

Once the vector ingests the pathogen in a blood meal, the pathogen must pass through the midgut infection barrier (MIB), the midgut escape barrier (MEB), the salivary gland infection barrier (SIB) and the salivary gland escape barrier (SEB) during the extrinsic incubation period. The vector competence of *Ae. aegypti* to dengue is controlled by two genes, one controlling the MIB and the other controlling the MEB (Bosio et al., 1998; Severson and Behura, 2016). It has been shown that *Ae. aegypti* mosquito populations vary in their ability to transmit DENV/YFV (Tabachnick et al., 1985; Dickson et al., 2014), and this can result in dengue and yellow fever outbreaks not being uniformly distributed within endemic areas.

Although, urban yellow fever outbreaks remain absent in Kenya, re-current dengue outbreaks are limited to the coastal areas of Kenya, predominantly in the city of Mombasa, despite the possibility of introduction into other urban areas. As part of a countrywide DENV/YFV risk assessment, this study focused on the major urban areas of Mombasa, Kisumu, and Nairobi, and assessed the potential of populations of *Aedes* (*Stegomyia*) vectors to sustain transmission of DENV/YFV. However, the different risk factors affecting DENV/YFV transmission present a challenge in the complete assessment of the risk of spread of dengue to other cities of Kenya or the emergence of an urban yellow fever outbreak. It is therefore important to examine the vector bionomics, the effect of environmental temperature on vector competence, and possible genetic differences influencing DENV/YFV transmission by *Ae. aegypti* mosquito populations in the major cities when assessing the risk associated with their emergence. Also, the combined interpretation of these risk factors can be determined from vectorial capacity equations, and used to estimate the risk of transmission of DENV/YFV, yet this is lacking in Kenya.

Relevance of this study

Dengue and yellow fever viruses represent the greatest health risk of arboviral emergence due to extensive tropical urbanization and the colonization of these expanding habitats by the highly anthropophilic *Aedes* mosquito vectors of the *Stegomyia* subgenus, and *Ae. aegypti* in particular (Weaver and Reisen, 2010). This is

underscored by recent outbreaks of dengue (Kenya, 2011-2017; Tanzania, 2013 among others) and yellow fever (Uganda, 2016), as well as sporadic yellow fever cases (Ethiopia, 2013, 2017) in East Africa (Ellis et al., 2015; Lutomiah et al., 2016; Mboera et al., 2016; Lilay et al., 2017; WHO, 2017b) demonstrating that the region is receptive to infection with either or both viruses.

Yellow fever remains a major public health challenge despite the availability of a safe and efficacious 17D vaccine. The yellow fever incidence in Africa shows that the vaccine coverage is inadequate and there is a real and present danger of a major epidemic in urban areas (Reiter et al., 1998, WHO, 2018). The re-emergence of yellow fever has been linked to the lapse in the immunization program in areas with past immunization records and it is therefore thought to be more of a policy and planning problem than a biological one (Calisher and Woodall, 2016). However, vector surveillance and risk assessment studies could identify risk areas and guide the public health authorities towards cost effective vaccination and targeted vector control. A new dengue vaccine (Dengvaxia) is available for use in highly endemic areas, although it is not yet licenced for use in many African countries including Kenya, hence the need for continuous vector surveillance and risk assessment studies (WHO, 2017c).

Both dengue and yellow fever are originally sylvatic diseases. However, while dengue epidemics in East Africa, including Kenya have mostly been reported from urban areas, urban yellow fever epidemics remain absent in this region despite the urban vector *Ae. aegypti* being widely distributed. The greatest threat in East Africa therefore remains the potential emergence of these viruses especially YFV from the sylvatic cycle, following proximal epizootic activity, and subsequent introduction into urban areas with dense populations of susceptible hosts and domestic vectors. Emergence of urban YFV could be mediated by the adaptation of sylvatic viruses to urban/domestic vectors like *Ae. aegypti* as has been previously suggested for DENV (Gubler, 2002). Although urban yellow fever epidemics are currently absent in Kenya, urban dengue epidemics are presently limited to the city of Mombasa and its environs (including Kilifi), with no reports of outbreaks in other urban areas such as Kisumu and Nairobi. The different risk parameters affecting DENV/YFV transmission are, however, complex, and investigating the factors underlying the

emergence and maintenance of these diseases in nature remains challenging. For these diseases to successfully emerge in an area, these different risk factors must align. The scenario in Kenya calls for a need to consolidate and compare the different risk factors affecting the transmission of DENV/YFV especially in the major cities/urban areas (population size > 400,000 people) of Kenya, which are known to be at greatest risk of their emergence.

Ultimately, this project will generate new information relating to the presence and/or distribution of DENV and YFV vectors in these cities, especially since very minimal data currently exist. It will also provide knowledge on the resting and feeding habits of the major *Aedes* (*Stegomyia* species) present as well as their DEN/YF virus transmission potential. This will help to improve the YFV vector distribution map, as well as information on the level of DEN and YF virus transmission risk in these urban areas.

Key research questions

The key research questions addressed in the present study include:

Chapter 2 - Assessment of risk of dengue and yellow fever virus transmission in three major Kenyan cities based on *Stegomyia* indices.

Key research questions:

Q1: Are the predominant *Aedes* (*Stegomyia*) species inhabiting the major urban areas of Kenya different?

Q2: Does the DENV/YFV transmission risk based on *Aedes (Stegomyia)* species infestation levels differ between Mombasa (dengue outbreak prone), Kisumu and Nairobi (no record of dengue)?

Q3: What are the most productive breeding sites for *Aedes (Stegomyia)* species that can be targeted for vector control?

Chapter 3 - Dengue and yellow fever virus vectors: seasonal abundance, diversity and resting preferences in three Kenyan cities.

Key research questions:

Q1: Does the adult mosquito abundance and diversity of potential dengue and yellow fever virus vectors vary by season as well as between the different major cities in Kenya?

Q2: Is there any difference in the *Ae. aegypti* resting preference (indoor vs outdoor) in the dengue endemic (Mombasa) and dengue free cities (Kisumu and Nairobi) in Kenya?

Chapter 4 - *Aedes aegypti* feeding preference and temperature shapes dengue virus transmission potential in three Kenyan cities.

Key research questions:

Q1: Does the *Ae. aegypti* human feeding pattern differ between dengue endemic (Mombasa) and non-endemic (Kisumu and Nairobi) cities in Kenya?

Q2: Is temperature an important factor in the DENV vector competence of *Ae. aegypti* mosquitoes in the endemic and non-endemic cities in Kenya?

Q3: Does the vectorial capacity of *Ae. aeypti* to transmit DENV differ between Mombasa, Kisumu and Nairobi?

Chapter 5 - Are urban *Aedes aegypti* mosquito populations from Kenya incompetent transmitters of the East African yellow virus genotype?

Key research questions:

Q1: Are urban *Ae. aegypti* populations in Kenya incompetent in sustaining yellow fever virus transmission?

Q2: Does the susceptibility of YFV relate to the genetic differences within *Ae. aegypti* populations?

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Chapter 2

Assessment of risk of dengue and yellow fever virus transmission in three major Kenyan cities based on *Stegomyia* indices

Sheila B. Agha[1,2*], David P. Tchouassi[1], Armanda D.S. Bastos[2], Rosemary Sang[1, 3]

[1]*International Centre of Insect Physiology and Ecology, Nairobi, Kenya*
[2]*Department of Zoology and Entomology, University of Pretoria, South Africa*
[3] *Arbovirus/Viral Hemorrhagic Fever Laboratory, Centre for Virus Research, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya*

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Abstract

Dengue (DEN) and yellow fever (YF) are re-emerging in East Africa, with contributing drivers to this trend being unplanned urbanization and increasingly adaptable anthropophilic *Aedes* (*Stegomyia*) vectors. Entomological risk assessment of these diseases remains scarce for much of East Africa and Kenya even in the dengue fever-prone urban coastal areas. Focusing on major cities of Kenya, we compared DEN and YF risk in Kilifi County (DEN-outbreak-prone), and Kisumu and Nairobi Counties (no documented DEN outbreaks). We surveyed water-holding containers for mosquito immature (larvae/pupae) indoors and outdoors from selected houses during the long-rains, short-rains and dry seasons (100 houses/season) in each County from October 2014-June 2016. House index (HI), Breteau index (BI) and Container index (CI) estimates based on *Aedes (Stegomyia)* immature infestations were compared by city and season. *Aedes aegypti* and *Aedes bromeliae* were the main *Stegomyia* species with significantly more positive houses outdoors (212) than indoors (88) (n = 900) (χ^2 = 60.52, P<0.0001). Overall, *Ae. aegypti* estimates of HI (17.3 vs 11.3) and BI (81.6 vs 87.7) were higher in Kilifi and Kisumu, respectively, than in Nairobi (HI, 0.3; BI,13). However, CI was highest in Kisumu (33.1), followed by Kilifi (15.1) then Nairobi (5.1). *Aedes bromeliae* indices were highest in Kilifi, followed by Kisumu, then Nairobi with HI (4.3, 0.3, 0); BI (21.3, 7, 0.7) and CI (3.3, 3.3, 0.3), at the respective sites. HI and BI for both species were highest in the long-rains, compared to the short-rains and dry seasons. We found strong positive correlations between the BI and CI, and BI and HI for *Ae. aegypti*, with the most productive container types being jerricans, drums, used/discarded containers and tyres. On the basis of established vector index thresholds, our findings suggest low-to-medium risk levels for urban YF and high DEN risk for Kilifi and Kisumu, whereas for Nairobi YF risk was low while DEN risk levels were low-to-medium. The study provides a baseline for future vector studies needed to further characterise the observed differential risk patterns by vector potential evaluation. Identified productive containers should be made the focus of community-based targeted vector control programs.

Author Summary

Despite the growing problem of dengue (DEN) and yellow fever (YF) evidenced from recent outbreaks in East Africa, risk assessment for their transmission and establishment through surveys of populations of the *Aedes* mosquito vectors, remain scarce. By estimating standard indices for the potential vectors, *Aedes aegypti* and *Aedes bromeliae* we partly could deduce the risk of transmission of these diseases in three major cities of Kenya, namely Kilifi (DEN-prone) and Kisumu and Nairobi (without any DEN outbreak reports). When compared to established threshold risk levels by WHO and PAHO, our findings suggest low-to-medium risk of urban YF, and high risk of DEN transmission for Kilifi and Kisumu but not Nairobi (low risk level for YF and low-to-medium risk for DEN). The observed seasonal risk patterns, higher *Aedes* infestation outdoors than indoors and productive container types (jerricans, drums, discarded containers and tyres), provide insights into the disease epidemiology and are valuable for targeted vector control, respectively.

2.1 Introduction

Dengue (DEN) and yellow fever (YF) are re-emerging diseases of public health importance caused by arboviral pathogens [1–4]. Both diseases share a common ecological niche including non-human primates as reservoir hosts and are vectored primarily by *Aedes* (*Stegomyia*) species [5]. Dengue fever is caused by one of the four serotypes of the dengue virus (DENV 1-4) with about 390 million infections reported worldwide each year, 16% of which are from Africa [6,7]. Additionally, an estimated 900 million people are living in YF endemic areas with about 90% of the global infections reported from Africa [8,9].

The rapid geographic spread of these diseases in recent times in Africa and especially in East Africa represents a worrying new trend with occurrence of major epidemics affecting urban human populations [10,11]. This is exemplified by recent DEN outbreaks in Somalia 2011, 2013 [12], Tanzania 2013, 2014 [4,13], Sudan 2010, 2015 [14,15] and various parts of Kenya 2011, 2013, 2015 [1,2]. An outbreak of YF was reported in Kenya in 1992-93 [16], in Sudan 2003, 2005, 2012 [17–19] and neighboring Uganda 2011, 2016 [20,21]. Despite the fact that the last YF outbreak in Kenya occurred over two decades ago, the country is still classified among countries with medium to high risk of YF transmission in Africa [22], and a number of YF cases have recently been imported from Angola where there was an ongoing outbreak [21]. There are currently no antiviral drugs available for either DEN or YF. However, there is a safe efficacious vaccine against YF, and a new, partially approved vaccine for DEN, for use only in geographical settings where epidemiological data indicate a high burden of the disease [23]. Unfortunately, the costs and availability of these vaccines have proved to be challenging for effective disease prevention. While the recent DEN and YF outbreaks in Africa have attracted renewed public health and research attention, effective monitoring and risk assessment for their occurrence remains limited.

Dengue virus (DENV) is known to be transmitted primarily by *Aedes furcifer* in Africa and *Ae. aegypti aegypti* in Asia and the Americas [5]. *Aedes aegypti aegypti* is highly anthropophilic and its larvae develop mostly in artificial containers in and around human habitations, compared to the more sylvatic *Ae. aegypti formosus*

subspecies which develop mostly in tree holes hence linking the emergence of DEN in tropical urban areas to *Ae. aegypti aegypti* [24,25]. Although the role of *Ae. aegypti* in the transmission of yellow fever virus (YFV) in East Africa is poorly understood, it plays an important role in YFV transmission in West Africa, driving human-to-human transmission and resulting in dreaded urban outbreaks [26,27]. Yellow fever outbreaks in East and Central Africa have so far been associated with *Ae. bromeliae,* a member of the *Ae. simpsoni* species complex [28–30]. *Aedes bromeliae* is a peri-domestic mosquito species capable of biting humans and monkeys, thereby driving small scale outbreaks in rural populations, with potential to move virus across species from primates to humans [5]. Other species such as *Ae. africanus* and *Ae. luteocephalus,* feed on forest monkeys and sustain the sylvatic cycle of YF [31]. Although *Ae. albopictus* a secondary DEN vector is not known to be present in Kenya, *Ae. aegypti* and *Ae. bromeliae* are present in the major cities [32], hence the need to assess the risk of arboviral disease emergence associated with these vectors.

Risk assessment through surveillance of abundance and distribution of *Aedes* mosquitoes, which are key players in transmission of the pathogens that cause these diseases is critical. This largely relies on estimation of traditional *Stegomyia* indices (House Index-HI, Container Index-CI and Breteau Index-BI) of immature mosquito populations in households [33–36]. Estimation of such indices may be of operational value and can facilitate the determination of local vector densities and measurement of the potential impact of container-specific vector control interventions such as systematically eliminating or treating larval habitats with chemicals [37]. Surprisingly, estimations of these indices as a means of assessing risk of DEN and YF in Kenya are scarce and/or exclusive to *Ae. aegypti* in outbreak situations [31]. Moreover, similar investigations on other *Stegomyia* species such as *Ae. bromeliae* are completely lacking, in spite of its' potential role in YFV transmission in Africa [5].

Unplanned urbanization remains an important risk factor that has contributed to the resurgence of these diseases by providing abundant larval habitats from water-retaining waste products and storage facilities in the presence of susceptible human populations [38–40]. A better epidemiologic understanding of entomological thresholds relating to risk can help to prevent a severe outbreak in urban settings.

Potential exists for emergence of these diseases, especially YF from proximal sylvan areas, and subsequent introduction into urban areas where dense susceptible populations and competent domestic vectors abound [41], as demonstrated by the recent YF outbreak in Angola and the Democratic Republic of Congo [11,21].

To assess the potential risk of urban transmission of these diseases in the three urban cities of Kenya, namely Kilifi (DEN-prone) and Kisumu and Nairobi (DEN-free) in the light of known differential outbreak reports of DEN, we hypothesized that 1) the predominant *Aedes* (*Stegomyia*) species inhabiting these cities differ. 2) the estimated HI, CI and BI, measures of the DENV/YFV transmission risk level by *Ae. aegypti* and *Ae. bromeliae* will be different for the three cities and will show seasonal variations. 3) the most productive container types (based on the number of immature mosquitoes following mosquito survey, rearing to adults, and identification) will vary between these three cities. Information of the productive container types can be used to guide targeted source reduction/control operations.

2.2 Methods

2.2.1 Study Area

The study was carried out on the outskirts of the major cities of Kenya; Nairobi and Kisumu (with no history of DEN outbreak) and Mombasa (DEN endemic and outbreak prone). While the phenomenon of DEN expansion is associated with urban human settlement, incidence of the disease in rural areas is also on the rise and is sometimes even higher than in urban and semi-urban areas/communities [40,42,43]. Therefore, our study targeted the cities, where we specifically selected sites in peri-urban suburbs around the main cities, Githogoro (Nairobi County), Kisumu (Kisumu County) and Rabai (suburbs within Kilifi County, at the outskirts of Mombasa city), mainly for logistical reasons, including ease of access to homesteads and households.

Githogoro is located about 13.1 km from the Central Business District (CBD) on the outskirts of Nairobi (01°17'S 36°48'E), the largest city and capital of Kenya (Fig 2.1). Nairobi has a total surface area of 696 km^2, a population of 3.1 million people [44], and is situated at an altitude of 1,661 m above sea level (asl). Githogoro is an urban

informal settlement with most of the houses made of iron sheeting and consisting of a single room. A few houses have more than one room and some yard space.

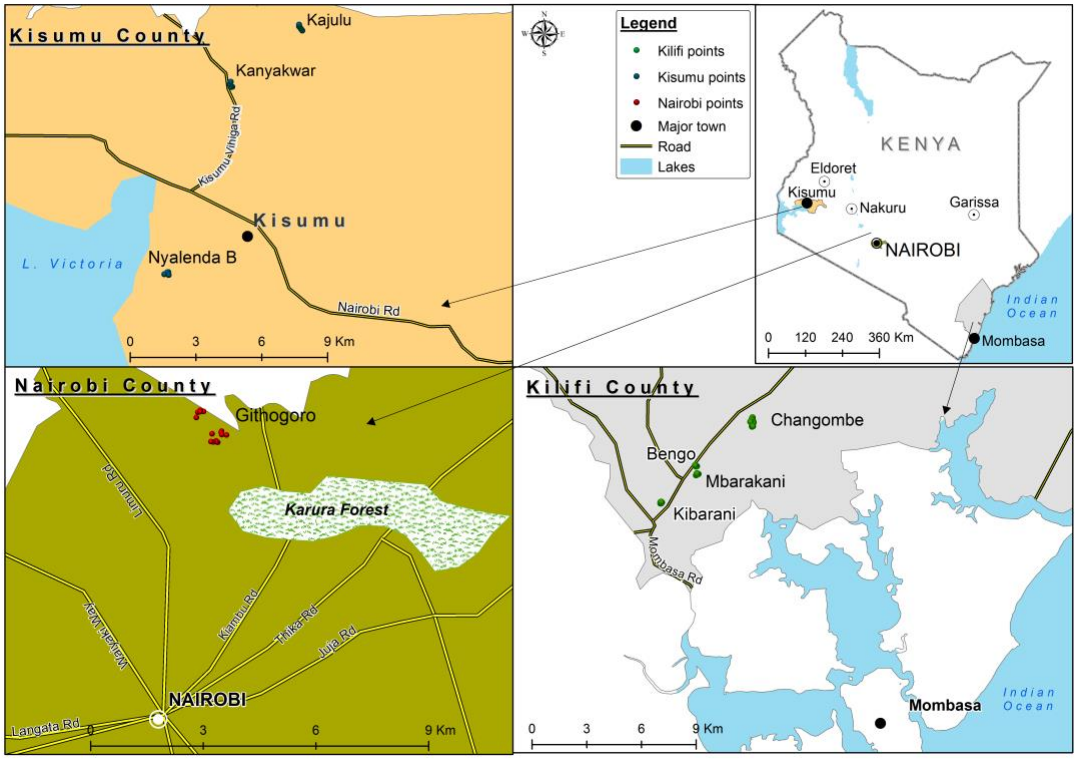

Figure 2.1. Map indicating the study sites within Kilifi, Kisumu, and Nairobi Counties of Kenya.

In Kisumu (00°03′S 34°45′E), the study sites included Nyalenda B, Kanyakwar and Kajulu located on the outskirts of Kisumu CBD at a distance of approximately 6.5 km, 5.8 km and 27.8 km, respectively. Kisumu is the third largest city in Kenya and the second most important city after Kampala in the greater Lake Victoria basin (Fig 1). It has a human population of >400,000 [44] and is situated at an altitude of 1,131 m asl. The houses in this area mostly have cemented walls and roofs made of iron sheeting. Water storage in containers is a common practice by the communities.

The study sites included Bengo, Changombe, Kibarani, and Mbarakani, in Rabai, which is located on the outskirts of Mombasa, though administratively it belongs to Kilifi County (Fig 1). Rabai is situated about 24.5km to the north-west of Mombasa CBD, the second largest city in Kenya, which is situated on an island (4°03'S 39°40'E). Mombasa has a total surface area of 294.7 km^2, a population of 1.2 million

people [44] and is situated at an attitude of 50 m asl. The houses in Rabai have walls that are either cemented, made of stones, or mud. The roofing system consists of iron sheeting or grass thatch. Water storage in containers is an equally common practice in these communities.

All three-study cities generally experience two rainy seasons, the long-rains season (April-June) and the short-rains season (October-December), interspersed by two dry seasons (January-March and July-September).

2.2.2 Study design

We conducted a cross-sectional survey of water holding containers situated both indoors and outdoors for presence of immature mosquito stages (larvae at all instars and pupae). The inspections and entomological surveys were conducted by a team of four trained personnel in houses that were selected at random for the initial survey. An interval of one house was applied during the first sampling and unique numbers assigned to each house for ease of identification in subsequent surveys during the next season. In cases where a house could not be sampled in subsequent surveys, either due to absence of the inhabitants or the owners declining entry, it was substituted for the next closest available house. Each survey was conducted over five consecutive days and 100 houses from the selected sites were targeted, within each of the three main urban areas (Nairobi, Kilifi, Kisumu). Repeat sampling of the same 100 houses / city was conducted for the dry season (July-September 2015 in Nairobi; January-March 2016 in Kilifi, and Kisumu) and for the long-rains (April-June 2015 in Kilifi, and Kisumu; April-June 2016 in Nairobi) and short-rains (October-December 2014 in Kilifi, and Kisumu, October-December 2015 in Nairobi) seasons. As such, there was a total of three sampling occasions (with 100 houses being sampled per study city and per season, corresponding to 900 sampling points), for the survey conducted from October 2014 to June 2016. Sampling in Nairobi was limited to Githogoro, whereas in Kilifi (Rabai) and Kisumu, operational surveys were conducted to reflect the proportionate size of each site in terms of the number of houses present. These sites were Bengo, Kibarani, Changombe and Mbarakani in Kilifi and Kajulu, Kanyakwar and Nyalenda B in Kisumu.

2.2.3 Survey of *Aedes* immatures

The survey of immature stages of *Aedes Stegomyia* mosquito species targeted artificial water-holding containers (indoors and outdoors) of any size and natural breeding sites (tree holes, banana axils, flower axils and colocasia) in peri-domestic areas of selected houses. Sampling was carried out using standardized sampling tools based on the type of water holding container encountered [45]. For small discarded containers (mostly found around the house, holding water which is not for household use), the water was emptied into a white tray and a plastic Pasteur pipette was used to collect the immatures. Jerrican (small plastic containers, 5-40L holding water for household use) surveys entailed pouring the water through a sieve into a bowl with a good contrast and collecting all immatures from the sieve with an aspirator. In large containers such as metal and plastic drums (50-210L containers used to store water for household use), the immatures were collected using ladles and aspirators when less than 20 were present or by emptying the water through a sieve when there were more than 20. Ladles, aspirators and pipettes were used to collect immatures from tyres as well as from tree holes and leaf axils. Flashlights were used where necessary. We captured information on each container sampled including: indoor or outdoor, natural or artificial, and the capacity of the container (>70L, 20L-70L, <20L). Immatures collected from containers were placed in whirlpaks (Nasco, FortAtkinson, WI) labeled with the pertinent information and transported to the field laboratory.

2.2.4 Rearing and identification of mosquitoes

Larval samples were placed in individual rearing trays for each container types. All pupae collected for the separate container types were transferred to individual adult cages. Larvae were fed fish food (Tetramin®) daily and the trays were inspected twice a day and pupae transferred to adult cages as well. This was done until all collected larvae/pupae had emerged to adults. During rearing, male and female *Aedes* mosquitoes were left together in a cage (small plastic buckets covered with fine netting materials and secured with rubber bands) and supplied with a 6% glucose solution on cotton wool. At the end of each sampling session, all adults were knocked down using triethylamine, placed in cryotubes and preserved in liquid nitrogen for transportation to the laboratory at the International Centre of Insect Physiology and Ecology in Nairobi. In the laboratory the resulting adult mosquitoes were morphologically identified using available taxonomic keys [46–48] and counted and

data on the species and number collected from the different container types were captured in Excel.

2.2.5 Data Analysis

A container was considered positive when at least one *Ae. aegypti* or *Ae. bromeliae* larva or pupa was found [45], and a house positive if at least one container type indoor was found infested with *Ae. aegypti* and/or *Ae. bromeliae* larvae. We estimated the classical *Stegomyia* indices: HI (percentage of houses infested with *Ae. aegypti* or *bromeliae* immatures), CI (percentage of water-holding containers infested with *Ae. aegypti* or *bromeliae* immatures), and BI [number of *Ae. aegypti* or *bromeliae* positive containers (indoor and outdoor) per 100 houses inspected].

We tested for significance of area/site and for seasonal effects in the patterns of observed indices (BI, HI, CI) using analysis of variance (ANOVA) followed by mean separation using the Tukey test (P = 0.05). Data for the different seasons were also pooled in each area to estimate the overall *Stegomyia* indices, and similarly compared for the different seasons and areas. Correlation analysis was performed to test for significant correlations between the indices- BI, HI, and CI.

The density of *Ae. aegypti* (total number of mosquitoes collected per total number of positive containers) indoors and outdoors was established and the difference compared within each area using a t-test.

The inspected containers were further categorized into 9 types based on similarity in certain features (e.g. size, natural or artificial, etc). The productivity of each of these container types was calculated per season and area as the percentage of the total number of immatures (larvae or pupae) determined by the adults reared from the container types (Productivity = 100 x (total number of immatures) / number of positive containers). We also applied ANOVA to test for significant differences in the proportion of positive containers (positivity) and compared the productivity among the container types after angular transformation. Container positivity for the different seasons was compared within an area using the Chi-Square test.

All analyses were carried out in R version 3.3.1 [49] at α=0.05 level of significance. Based on estimated indices we classified the areas/sites in terms of epidemic risk levels for YF or DEN as low, medium or high with reference to established epidemic thresholds [50,51]. HI values for *Ae. aegypti* and *Ae. bromeliae* were used to estimate risk of YFV transmission for the individual species with values of HI >35%, BI >50 and CI >20% considered as high risk of urban transmission of YFV; HI <4% BI <5 and CI <3% considered as unlikely or low risk of the disease transmission [50]. Similarly, the Pan American Health Organization (PAHO) has established threshold levels for dengue transmission based on HI for *Ae. aegypti* with low being an HI< 0.1%, medium an HI 0.1%–5% and high an HI> 5% [51].

Ethical statement

We sought permission from household heads through oral informed consent to allow water-holding containers in their residences to be surveyed. Household survey of mosquitoes was carried out with ethical approval from Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU) (Project Number SERU 2787).

2.3 Results

2.3.1 Mosquitoes collected

A total of 11,695 mosquitoes were reared from the larvae and pupae collected from water holding containers, both indoors and outdoors, from all sites and cities. These included *Ae. aegypti* (63.5%), *Ae. bromeliae* (2.9%), *Eretmapodite chrysogaster* (1.9%) and *Culex* spp. (31.53%). *Aedes metallicus*, other *Aedes* species (*Ae. tricholabis, Ae. durbanensis*) together with *Aedeomyia furfurea, Uranotaenia* spp, *Anopheles gambiae* s.l and *Toxorhynchites* spp. each comprised 0.1% or less of the total collection (Table 2.1). Focusing on our species of interest, a total of 7,424 *Ae. aegypti* were collected from all sites comprising 3,342 (45.0%) from Kilifi, 3,733 (50.3%) from Kisumu and 349 (4.7%) from Nairobi with an overall higher proportion (76%) being collected outdoors than indoors (24%). The *Ae. aegypti* densities

recorded indoors and outdoors were not significantly different in the DEN-outbreak prone county of Kilifi (n = 17.5 indoors, n=15.4 outdoors, P = 0.7). In contrast, counties of Kisumu (n = 8.3 indoors, n = 16.8 outdoors, P=0.036) and Nairobi (n = 0.7 indoors, n = 14.7 outdoors, P = 0.048) (with no documented records of DEN outbreaks) had significantly higher densities of *Ae. aegypti* outdoors compared to indoors (Fig 2.2).

Similarly, a total of 335 *Ae. bromeliae* were collected mainly outdoors (92%). The highest proportion was sampled in Kilifi (63%, n=211), followed by Kisumu (32.8%, n=110) and then Nairobi (4.2%, n=14) (Table 2.1).

Table 2.1. Mosquito composition collected indoors and outdoors in Kilifi, Kisumu, and Nairobi Counties, Kenya, October 2014 -June 2016.

	Kilifi		Kisumu		Nairobi		Total	
Mosquito species	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**	**Indoor**	**Outdoor**
Aedes aegypti	1441	1901	338	3395	2	347	1781	5643
Aedes bromeliae	24	187	3	107	0	14	27	308
Aedes metallicus	2	5	0	0	0	0	2	5
Other Aedes and Aedeomyia spp.	0	8	0	0	0	0	0	8
Eretmapodites chrysogater	2	206	0	0	0	10	2	216
Culex spp	561	801	44	1752	4	530	609	3083
Uranotaenia spp	0	0	0	1	0	0	0	1
Toxorhynchites brevipalpis	0	1	0	3	0	0	0	4
***Anopheles gambiae* s.l.**	0	0	0	5	0	1	0	6

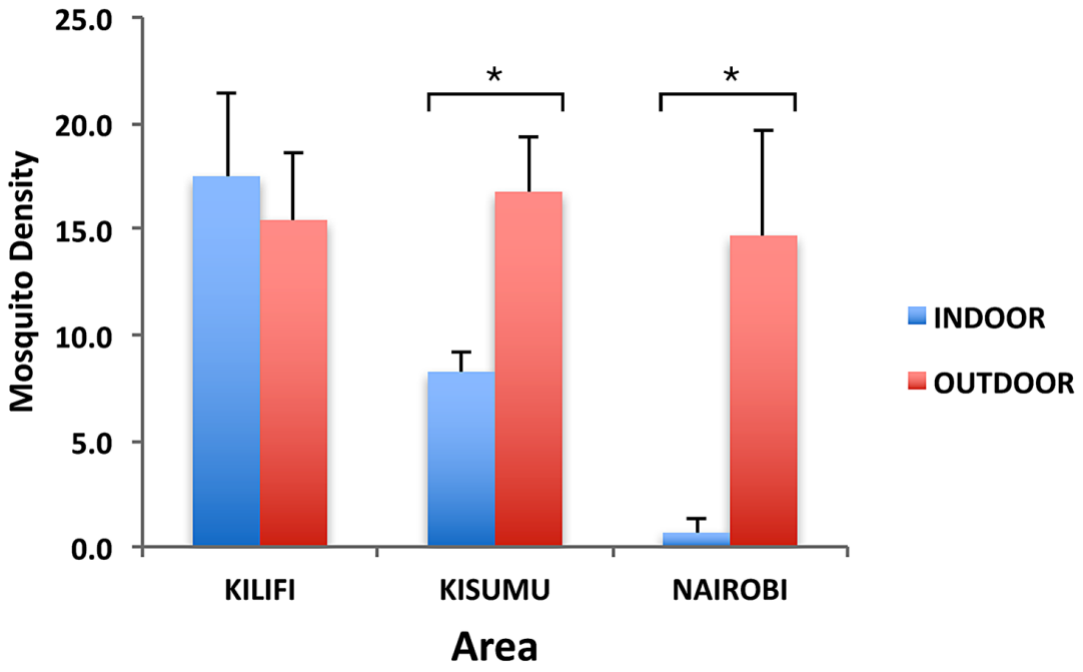

Figure 2.2. *Aedes aegypti* density, indoors and outdoors in Kilifi, Kisumu, and Nairobi Counties of Kenya. * Indicates significant differences between indoor and outdoor sampling, at P<0.05 in each of the three peri-urban areas sampled.

2.3.2 Dynamics of container productivity of *Aedes aegypti* and *Aedes bromeliae*

The rainy seasons recorded the highest proportions of *Ae. aegypti* in all three areas evaluated in this study. In Kilifi, long-rains constituted 1,648 (49.3%) of the total *Ae. aegypti* collected, followed by short-rains 1,172 (35.1%) with the lowest 522 (15.6%) observed during the dry season. An analogous pattern was found in Kisumu and Nairobi. In Kisumu, the long-rains, short-rains and dry season each accounted for 1,470 (39.4%), 1,441 (38.6%) and 822 (22.0%) of the total *Ae. aegypti* sampled. Surprisingly, collection of *Ae. aegypti* in Nairobi was highest during the short-rains 152 (43.6%), followed by the long-rains 143 (41%) and then the dry season at 54 (15.4%). However, the seasonal difference observed between long and short-rains in Nairobi was not statistically significant ($\chi^2 = 0.38$, P = 0.5).

Relative to *Ae. aegypti*, very low numbers of *Ae. bromeliae* were encountered from containers during our study. However, a seasonal pattern of abundance, with the highest proportion collected during one of the rainy seasons, was observed at all the areas. In Kilifi, *Ae. bromeliae* collected during the long-rains, short-rains and dry

seasons made up 52.9%, 45.1% and 1.9%, respectively, of the total collection. However, in Kisumu the highest proportion was recorded in the short-rains (70.9%), while the long-rains and dry seasons recorded 10% and 19.1%, respectively, of the total collection. In Nairobi, there was no record of *Ae. bromeliae* in the short-rains and dry seasons, and this mosquito species was only recorded in the long-rains. In terms of occurrence in container types, *Ae. aegypti* was mostly encountered in artificial containers such as jerricans, drums, tyres and other discarded containers at all the sites. However, to a lesser extent *Ae. aegypti* was found in natural container types such as tree holes and leaf axils in Kilifi and Kisumu (Table 2.2). Natural breeding sites like leaf axils were the most productive site for *Ae. bromeliae* at all the sites (Table 2.3). In fact, *Ae. bromeliae* was not found breeding in artificial containers in Nairobi, although to a minor extent it bred in artificial containers such as Jerricans and other discarded containers (Table 2.3) in Kilifi and Kisumu, mostly co-habiting with *Ae. aegypti.*

Table 2.2. Seasonal distribution of containers harboring *Aedes aegypti* immatures in Kilifi, Kisumu, and Nairobi Counties of Kenya.

	No. of positive containers /No. of containers surveyed								
	Kilifi			**Kisumu**			**Nairobi**		
Container Type	**Long-rains**	**Short-rains**	**Dry season**	**Long-rains**	**Short-rains**	**Dry season**	**Long-rains**	**Short-rains**	**Dry season**
Jerrican* /Jerrican, Plastic bottle	41/251	19/545	2/171	27/115	20/92	7/13	1/165	1/176	0/287
Tyre	20/26	9 /19	0	9/37	10/22	12/20	13/24	5/17	1/4
Drum❖ /Metal, Plastic	23/72	24/151	7/62	41/119	30/81	19/34	6/24	1/16	3/23
Basin /Basin, Bowl, Bucket	12/39	4/87	0/15	9/23	8/15	2/8	0/9	0/21	0/25
Natural breeding sites /Tree hole, leaf axils, flower pots	17/33	28/148	0	3/14	4/9	1/3	0/16	0/6	0/1
Animal drinking container	3/3	0/0	1/1	2/2	0	0	0/1	0/1	0/3
Pot /Clay pot, Aluminium pot	5/13	2/29	1/14	16/49	11/38	5/32	1/2	0	0
Tank✪ /Metal, Plastic	1/2	0/0	0/1	4/7	1/4	2/2	3/5	0/1	1/3
Discarded containers★	19/34	21/146	0/1	12/25	8/11	1/8	4/7	1/13	0/2
Others /Rock pools, stagnant water pools	0	0 /1	0	0/6	9/11	0	0	0	0
Total	**141 /473**	**107 /1126**	**11/165**	**123/397**	**101/283**	**49 /120**	**28 /253**	**8/251**	**5/348**

*5-40 liter capacity, ❖50-210 liter capacity, ✪>500 liter, ★Toilet parts, Coconut shells, Plastic and metal tins, Eating utensils, Plastic bags, Construction material.

Table 2.3. Distribution of *Aedes bromeliae* immature in different container types in Kilifi, Kisumu, and Nairobi Counties of Kenya.

	No. of positive containers /No. of containers surveyed		
Container Type	**Kilifi**	**Kisumu**	**Nairobi**
Natural breeding sites /Tree hole, leaf axils, flower pots	24 /133	11 /26	5 /23
Jerrican* /Jerrican, Plastic bottle	15 /967	1 /220	0 /628
Tyre	2 /45	8 /79	0 /45
Drum❖ /Metal, Plastic	7 /285	0 /234	0 /63
Basin /Basin, Bowl, Bucket	1 /141	0 /16	0 /55
Animal feeding container	3 /4	0 /2	0 /5
Pot /Clay pot, Aluminium pot	1 /56	2 /119	0 /2
Discarded container★	13 /181	3 /44	0 /22
Total	**66 /1812**	**25 /740**	**5 /843**

*5-40 liter capacity, ❖ 50-210 liter capacity, ★Toilet parts, Coconut shells, Plastic and metal tins, Eating utensils, Plastic bags, Construction material.

There was no significant difference in *Ae. aegypti* immature productivity by season or area. However, the contribution of container types to productivity of this species varied significantly (Df = 9, F = 6.41 P<0.0001). Significant differences were mostly observed between drums and animal drinking containers (P = 0.0008), drums and basins (P = 0.01), drums and natural breeding sites (P = 0.002), jerricans and animal drinking containers (P = 0.01), jerricans and natural breeding sites (P=0.02), tyres and animal drinking containers (P = 0.013) and between tyres and natural breeding sites (P = 0.022). Overall, in Kilifi, the most productive container types were jerricans (36.3%) in the long-rains, discarded containers (34.7%) in the short-rains, and drums (49.0%) in the dry season (Table 2.4). Similarly in Kisumu, the most productive container types were the jerricans (29.5%) in the long-rains, drums (24.5%) and discarded containers (24.1%) in the short-rains and drums in the dry (38.1%) season (Table 2.4). In Nairobi, drums (32.9%) were the most productive container types in the long-rains, tyres (84.9%) in the short-rains, and tanks (63.0%) in the dry season (Table 2.4).

Table 2.4. Productivity of containers harboring *Aedes aegypti* immature in Kilifi, Kisumu, and Nairobi Counties of Kenya.

	Immature Productivity (%)								
	Kilifi			Kisumu			Nairobi		
Container Type	**Long-rains (n)**	**Short-rains (n)**	**Dry-season (n)**	**Long-rains (n)**	**Short-rains (n)**	**Dry season (n)**	**Long-rains (n)**	**Short-rains (n)**	**Dry season (n)**
Jerrican* (Jerrican, Plastic bottle)	36.3 (599)	14.5 (170)	40.6 (212)	29.5 (433)	20.6 (297)	20.8 (171)	9.1 (13)	5.3 (8)	0
Tyre	1.2 (20)	18.7 (219)	0	7.3 (108	9.6 (138)	12.8 (105)	30.8 (44)	84.9 (129)	20.4 (11)
Drum❖ (Metal, Plastic)	18.3 (302)	24.6 (288)	49.0 (256)	23.5 (345)	24.5 (353)	38.1 (313)	32.9 (47)	0	16.7 (9)
Basin (Basin, Bowl, Bucket)	9.1 (150)	2.5 (29)	0	9.8 (144)	1.9 (28)	4 (33)	0	0	0
Natural breeding sites (Tree hole, leaf axils, flower pots)	5.9 (97)	3.8 (45)	0	3.5 (51)	0	0	0	0	0
Animal drinking container	3.8 (62)	0	5.4 (28)	0.3 (4)	0	0	0	0	0
Pot (Clay pot, Aluminium pot)	4.9 (80)	1.2 (14)	5.0 (26)	10.2 (150)	19.1 (275)	9.1 (75)	0	0	0
Tank✪ (Metal, Plastic)	0	0	0	0.5 (7)	0	10.3 (85)	13.3 (19)	0	63.0 (34)
Discarded containers★	20.5 (338)	34.7 (407)	0	15.5 (228)	24.1 (347)	4.9 (40)	14.0 (20)	9.9 (15)	0
Others (Rock pools, stagnant water pools)	0	0	0	0	0.2 (3)	0	0	0	0
Total	**100 (164)**	**100 (1172)**	**100 (522)**	**100 (1470)**	**100 (1441)**	**100 (822)**	**100 (143)**	**100 (152)**	**100 (54)**

*5-40 liter capacity, ❖50-210 liter capacity, ✪>500 liter, ★Toilet parts, Coconut shells, Plastic and metal tins, Eating utensils, Plastic bags, Construction material,

n= No. of *Aedes aegypti* reared out.

The most productive containers for *Ae. bromeliae* in Kilifi and Kisumu were discarded containers and natural breeding sites, while in Nairobi natural breeding sites were the most productive breeding sites.

Table 2.5. Productivity of *Aedes bromeliae* immature in different container types in Kilifi, Kisumu, and Nairobi Counties of Kenya.

	Immature Productivity (%)		
Container Type	**Kilifi (n)**	**Kisumu (n)**	**Nairobi (n)**
Natural breeding sites (Tree hole, leaf axils, flower pots)	34.1 (72)	27.0 (30)	100.0 (14)
Jerrican* (Jerrican, Plastic bottle)	17.1 (36)	2.7 (3)	0.0 (0)
Tyre	0.9 (2)	4.5 (5)	0.0 (0)
Drum❖ (Metal, Plastic)	1.9 (4)	0.0 (0)	0.0 (0)
Basin (Basin, Bowl, Bucket)	0.0 (0)	0.0 (0)	0.0 (0)
Animal feeding container	5.2 (11)	0.0 (1)	0.0 (0)
Pot (Clay pot, Aluminium pot)	2.4 (5)	0.9 (1)	0.0 (0)
Discarded container★	38.4 (81)	64.9 (72)	0.0 (0)
Total	**100 (211)**	**100 (111)**	**100 (14)**

*5-40 liter capacity, ❖ 50-210 liter capacity, ★Toilet parts, Coconut shells, Plastic and metal tins, Eating utensils, Plastic bags, Construction material,
n = No. of *Ae. bromeliae* reared out.

2.3.3 Positivity of the different container types

Based on the number of each container types surveyed and the number positive, we found significant differences in container positivity between the areas (Df = 2, F = 9.6, P = 0.0002) and seasons (Df = 2, F = 84.26, P = 0.018). Significant differences existed in the container type positivity between Kilifi and Kisumu [95% CI, (0.329, 26.392), P = 0.043], Kisumu and Nairobi [95% CI, (-37.214, -11.152), P<0.0001], but not between Kilifi and Nairobi. Generally, animal drinking containers and tyres were the most positive containers in Kilifi, tanks and discarded containers in Kisumu, and tyres and tanks in Nairobi. Similarly, container positivity was significantly different between the long-rains and dry seasons [95% CI, (2.393, 28.456), P = 0.016], long and short-rains [95% CI, (-27.122, -1.059), P = 0.03], but not between the short-rains and dry season. The proportion of positive containers was significantly different for all three seasons in Kilifi (χ^2 = 119.0, P<0.0001) and Nairobi (χ^2 = 31.7, P<0.0001) but not in Kisumu (χ^2 = 4.45, P< 0.1078). Tyres were the most positive containers

both in the long and short-rains in Kilifi while drums were the most positive containers in the dry season. In Kisumu, tanks constituted the most positive containers in the long-rains, basins in the short-rains and drums in the dry season. In Nairobi, discarded containers ranked as the highest positive containers in the long-rains, tyres in the short-rains and tanks in the dry season**.**

2.3.4 Larval indices and risk of dengue and yellow fever transmission

The overall *Ae. aegypti* CI was higher during the long-rains followed by dry season and then short-rains in Kilifi. In Kisumu, CI was higher in the dry season, followed by the long-rains and then short-rains, while in Nairobi, CI was higher in the long-rains followed by short-rains and then dry season (Fig 2.3A). The seasonal differences observed in all three cities were not significant (P = 0.14). However, the observed CI values were significantly different among the different cities (Df = 2, F = 16.69, P = 0.012), with differences recorded between Kilifi and Kisumu [95% CI, (0.483, 35.450), P = 0.046], Kisumu and Nairobi [95% CI, (-45.45, -10.48), P = 0.01], but not between Kilifi and Nairobi. CI was equally significantly different even at smaller scale among the sites (Df = 5, F=3.133, P = 0.037). Overall, CI was highest in Kanyarkwar (Kisumu) and lowest in Kibarani (Kilifi).

The overall *Ae. aegypti* HI was highest in the long-rains (24%, 15% and 0%), compared to the short-rains (20%, 12% and 0%) and dry season (8%, 7% and 1%) respectively in Kilifi, Kisumu, and Nairobi (Fig 2.3B). Our analysis showed that overall HI values varied significantly in the different cities (Df = 2, F = 11.24, P = 0.023) with among area differences recorded between Kilifi and Nairobi [95% CI, (-29.96, -4.04), P = 0.02], but not between Kilifi and Kisumu or Kisumu and Nairobi. Also, the overall HI was highest in Kanyarkwar (Kisumu) and lowest in Githogoro (Nairobi).

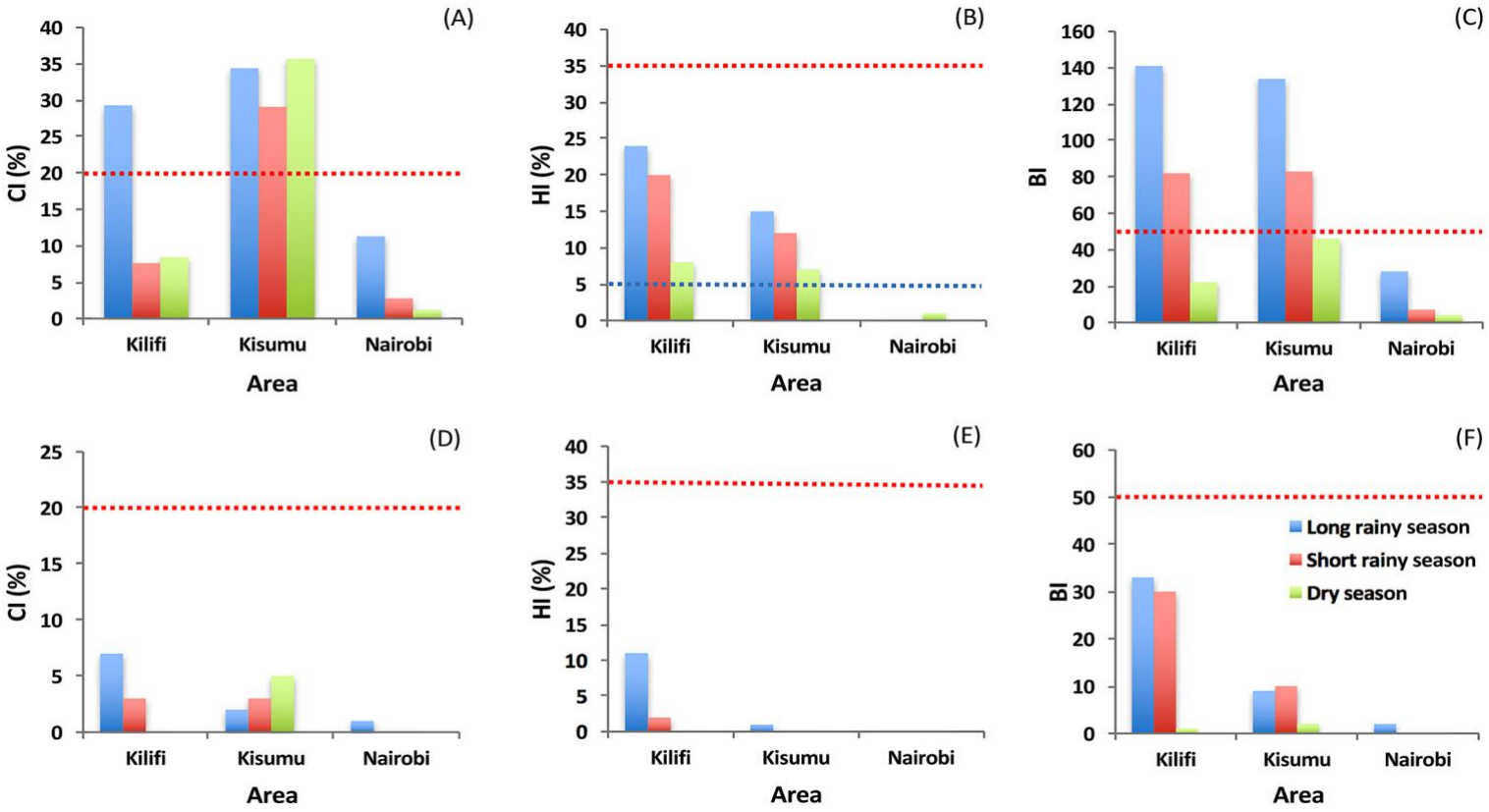

Figure 2.3. Seasonal risk levels of *Aedes aegypti* and *Aedes bromeliae* in Kilifi, Kisumu, and Nairobi Counties in Kenya. (A) Container Index (CI), (B) House Index (HI), (C) Breteau Index (BI) for *Aedes aegypti;* (D) Container Index (CI), (E) House Index (HI and (F) Breteau Index (BI) for *Aedes bromeliae.* Blue dashed line represents the DEN epidemic threshold level as defined by PAHO [51]. Red dashed line represents the YF epidemic threshold levels according to WHO [50].

Overall BI for *Ae. aegypti* varied significantly across the seasons (P = 0.044), with highest values observed in the long-rains (141, 134 and 28), compared to the short-rains (82, 83 and 7) and dry season (22, 46 and 7) seasons in Kilifi, Kisumu and Nairobi, respectively (Fig 2.3 C). Also, significant variation in the overall BI values was evident between areas (BI: Df = 2, F = 8.68, P = 0.035) and seasons (Df = 2, F = 7.52, P = 0.044). Among-area differences were observed between Kisumu and Nairobi [95% CI, (-145.66, -3.68), P = 0.043], but not between Kilifi and Kisumu or Kilifi and Nairobi. Likewise significant seasonal differences in BI values occurred between the long rains and dry seasons [95% CI, (6.01, 147.99), P = 0.0386], but not between the long and short-rains, or the short-rains and dry seasons in all three areas. Similarly, the overall BI was highest in Kanyarkwar (Kisumu) and lowest in Githogoro (Nairobi).

Based on HI values estimated for *Ae. aegypti* in reference to threshold levels for DEN transmission (low HI< 0.1%, medium HI 0.1%–5% and high HI> 5%) established by PAHO [51], both Kilifi and Kisumu were classified as being at high-risk for DEN transmission in all three seasons, while Nairobi was classified as being at low risk in both the long and short-rains and at medium risk in the dry season (Table 2.6). Even small-scale differences in DEN risk across sites among the major areas of Kilifi and Kisumu were evident, highest in Kanyakwar (Kisumu) and Mbarakani (Kilifi) (Table 2.6).

Similarly, with reference to the WHO threshold levels for urban YFV transmission (low HI<4%, Medium 4%-35% and high HI>35%), our risk level values for *Ae. aegypti*, show that Kilifi and Kisumu could be classified as being at medium-risk of an urban YF epidemic in all three seasons based on estimated HI values, and Nairobi at low risk in all three seasons (Table 2.7).

We found no significant difference in overall index values (CI, HI and BI) for *Ae. bromeliae* (Fig 2.3D, Fig 2.3E, Fig 2.3F*)*, among the three areas in the different seasons (P>0.05). However, based on the HI estimated for this species, compared to the established threshold levels for urban YFV transmission [50] and assuming that *Ae. bromeliae* could transmit YFV, only Kilifi could be classified as being at medium risk during the long-rains but at low risk in the short-rains and dry seasons. Both Kisumu and Nairobi can be classified as being at low risk levels of transmission in all three seasons (Table 2.8).

Table 2.6. Estimated dengue transmission risk levels in the long-rains, short-rains and dry seasons in Kilifi, Kisumu, and Nairobi Counties, Kenya.

		Long-rains				Short rains				Dry season				Overall Indices			
Area	**Site**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**
	Bengo	34.5	24	174	High	7.4	19.2	78.8	High	8.8	12	24	High	16.9	18.4	92.3	High
	Changombe	36.1	40	173.3	High	_	_	_	-	8.3	0	13.3	Low	22.2	20	93.3	High
Kilifi	Kibarani	3.6	0	20	Low	3.7	16.7	33.3	High	0	0	0	Low	2.4	5.6	17.8	High
	Mbarakani	33.8	30	125	High	7.7	22.9	105.7	High	14.8	10	40	High	18.8	21	90.2	High
	Overall	**29.3**	**24**	**141**	**High**	**7.6**	**20**	**82**	**High**	**8.4**	**8**	**22**	**High**	**15.1**	**17.3**	**81.7**	**High**
	Kajulu	22.2	0	80	High	13.9	5	55	Medium	16.3	10	35	High	17.5	5	56.7	High
	Kanyakwar	52.5	37.5	262.5	High	38.3	27.5	147.5	High	51.9	10	70	High	47.6	25	160	High
Kisumu	Nyalenda B	**11**	**0**	**32.5**	Low	25	0	32.5	Low	34.4	2.5	27.5	Medium	23.5	0.8	30.8	Medium
	Overall	**34.4**	**15**	**134**	**High**	**29.1**	**12**	**83**	**High**	**35.7**	**7**	**46**	**High**	**33.1**	**11.3**	**87.7**	**High**
	Githogoro	11.3	0	28	Low	2.8	0	7	Low	1.2	1	4	Medium	5.1	0.3	13	Medium
Nairobi	**Overall**	**11.3**	**0**	**28**	**Low**	**2.8**	**0**	**7**	**Low**	**1.2**	**1**	**4**	**Medium**	**5.1**	**0.3**	**13**	**Medium**

Risk levels estimated according to PAHO [51].

Table 2.7. Potential risk* of yellow fever virus transmission based on estimated *Aedes aegypti* indices in the long-rains, short-rains, and dry seasons in Kilifi, Kisumu, and Nairobi Counties, Kenya.

		Long-rains				Short-rains				Dry season				Overall Indices			
Area	**Site**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**
	Bengo	34.5	24	174	Medium	7.4	19.2	78.8	Medium	8.8	12	24	Medium	16.9	18.4	92.3	Medium
	Changombe	36.1	40	173.3	High	_	_	_	-	8.3	0	13.3	Low	22.2	20	93.3	Medium
Kilifi	Kibarani	3.6	0	20	Low	3.7	16.7	33.3	Medium	0	0	0	Low	2.4	5.6	17.8	Medium
	Mbarakani	33.8	30	125	Medium	7.7	22.9	105.7	Medium	14.8	10	40	Medium	18.8	21	90.2	Medium
	Overall	**29.3**	**24**	**141**	**Medium**	**7.6**	**20**	**82**	**Medium**	**8.4**	**8**	**22**	**Medium**	**15.1**	**17.3**	**81.7**	**Medium**
	Kajulu	22.2	0	80	Low	13.9	5	55	Medium	16.3	10	35	Medium	17.5	5	56.7	Medium
	Kanyakwar	52.5	37.5	262.5	High	38.3	27.5	147.5	Medium	51.9	10	70	Medium	47.6	25	160	Medium
Kisumu	Nyalenda B	**11**	**0**	**32.5**	Low	25	0	32.5	Low	34.4	2.5	27.5	Medium	23.5	0.8	30.8	Low
	Overall	**34.4**	**15**	**134**	**Medium**	**29.1**	**12**	**83**	**Medium**	**35.7**	**7**	**46**	**Medium**	**33.1**	**11.3**	**87.7**	**Medium**
	Githogoro	11.3	0	28	Low	2.8	0	7	Low	1.2	1	4	Low	5.1	0.3	13	Low
Nairobi	**Overall**	**11.3**	**0**	**28**	**Low**	**2.8**	**0**	**7**	**Low**	**1.2**	**1**	**4**	**Low**	**5.1**	**0.3**	**13**	**Low**

*The ability of this *Aedes aegypti* population to transmit YF in the region is unknown. It has never been implicated as a vector in East Africa but it is associated with urban YF transmission in West Africa [26,27]. Risk levels estimated according to WHO [50].

Table 2.8. Potential risk* of yellow fever virus transmission based on estimated *Aedes bromeliae* indices in the long-rains, short-rains, and dry seasons in Kilifi, Kisumu, and Nairobi Counties, Kenya.

		Long-rains				Short-rains				Dry season				Overall Indices			
	Site	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**	**CI (%)**	**HI (%)**	**BI**	**Risk level**
	Bengo	8	10	42	Medium	4	4	46	Low	1	0	2	Low	4.3	4.7	30	Low
	Changombe	14	33	67	Medium	-	-	-	-	0	0	0	Low	7	16.5	33.5	Medium
Kilifi	Kibarani	1	0	7	Low	0	0	0	Low	0	0	0	Low	0.3	0	2.3	Low
	Mbarakani	1	5	5	Medium	1	0	17	Low	0	0	0	Low	0.7	1.7	7.3	Low
	Overall	**7**	**11**	**33**	**Medium**	**3**	**2**	**30**	**Low**	**0**	**0**	**1**	**Low**	**3.3**	**4.3**	**21.3**	**Low**
	Kajulu	1	0	5	Low	6	0	25	Low	16	0	10	Low	7.7	0	13.3	Low
	Kanyakwar	4	3	20	Low	3	0	13	Low	0	0	0	Low	2.3	1	11	Low
Kisumu	Nyalenda b	0	0	0	Low	0	0	0	Low	0	0	0	Low	0	0	0	Low
	Overall	**2**	**1**	**9**	**Low**	**3**	**0**	**10**	**Low**	**5**	**0**	**2**	**Low**	**3.3**	**0.3**	**7**	**Low**
	Githogoro	1	0	2	Low	0	0	0	Low	0	0	0	Low	0.3	0	0.7	Low
Nairobi	**Overall**	1	0	2	**Low**	**0**	**0**	**0**	**Low**	**0**	**0**	**0**	**Low**	**0.3**	**0**	**0.7**	**Low**

*The ability of this *Aedes bromeliae* population to transmit YF in the coast is unknown. It has been associated with YF transmission in other regions [29,30]. Risk levels estimated according to WHO [50].

Equally strong positive correlations were recorded between the BI and HI ($R^2 = 0.887$, $P = 0.001$)) as well as the BI and CI ($R^2 = 0.721$, $P = 0.028$) (Table 2.9).

Table 2.9. Pearson correlations between the traditional *Stegomyia* indices in Kilifi, Kisumu, and Nairobi Counties, Kenya.

Stegomyia Indices	Container Index	House Index	Breteau Index
Container Index	1	0.498	0.721
	1	*0.172*	*0.028**
House Index	0.498	1	0.887
	0.172	*1*	*0.001**
Breteau Index	0.721	0.887	1
	*0.028**	*0.001**	*1*

* indicates significant correlations ($P < 0.05$); P-values are showed in italics.

2.4 Discussion

Aedes aegypti and *Ae. bromeliae* were the major *Stegomyia* species recorded at all sites/cities, justifying estimation of indices for the two species considering their potential roles in DENV and YFV transmission [26,27,29,30]. Our findings support the sympatric existence of both species in these growing urban ecologies in Kenya.

Although particular container types were more likely to be positive than others, it was noteworthy that these were not necessarily the most productive, suggesting that positivity did not always translate to productivity. *Aedes aegypti* in all three areas were mostly found breeding in jerricans, drums (which were particularly productive in all seasons), tyres, and discarded containers. This was equally observed in an earlier study in Mombasa city, during entomologic investigations of a recent DEN outbreak [2]. These containers could be targeted at the community level through awareness creation and public health education for the control of *Ae. aegypti* mosquitoes. In this way, the local inhabitants can help reduce *Ae. aegypti* larval sites by reducing these containers in and near their homes or by properly covering them to prevent gravid females from laying their eggs in them [37]. Observations from this study show that *Ae. aegypti* is also capable of developing in natural sites especially in the water holding axils of banana plants. *Aedes aegypti* breeding in banana and

colocasia plants have also been reported by Philbert and Ijumba (2013) in a study on the preferred breeding habitats of *Ae. aegypti* in Tanzania [52]. This adaptation should be monitored as it will take away any gains made from targeting control of breeding in artificial water holding containers. Immature stages of *Ae. bromeliae*, a species which is known to preferentially breed in phytotelmata, the water-holding axils of plants [53], were also found developing in artificial containers indoors and outdoors in this study. Its ability to develop in artificial containers both indoors and outdoors has also been reported in another study in coastal Kenya [54]. Both *Ae. aegypti* and *Ae. bromeliae* were also found co-developing in several artificial and natural breeding sites. Utilization of artificial breeding sites may be an indication that *Ae. bromeliae* is increasingly adapting to the urban environment, bringing it closer to human hosts and increasing the risk of transmission of a range of the arboviruses that cause human disease, including YFV.

Risk values for both *Ae. aegypti* and *Ae. bromeliae* were different not only between areas and seasons, but we found finer scale differences between the sites, suggesting spatio-temporal variation with non-uniform risk even within the same general ecology. Although water storage in containers is a common practice in these cities during the rainy and dry seasons, DEN outbreaks that have occurred in Mombasa have mostly been associated with the long and short-rains [2]. The estimated HI and BI for *Ae. aegypti* both showed the same seasonal pattern in all three areas. The strong correlations between the traditional *Stegomyia* indices observed in this study, clearly indicates that they are all important in determining risk of transmission. It will also be important to investigate how the *Stegomyia* indices correlate with the observed DEN cases, especially in the coastal site of Kilifi County.

Estimated risk values suggested that both Kilifi and Kisumu were at high risk of DEN transmission while Nairobi was at low risk. Based on our findings, risk of DEN in Kilifi is high especially during the long-rains (April-June) and short-rains (November-December). This correlates with reports of DEN outbreaks in coastal Kenya, with outbreak peaks during the long and short-rains in the 2013/2014 outbreaks [1,2]. High indices were also recorded in Mombasa city during this outbreak [2], with HI values comparable to that reported for Kilifi and Kisumu in our study. High indices have also been recorded in neighboring countries of Ethiopia [55] and Tanzania [56], which are

prone to DEN outbreaks. Low indices were recorded in Nairobi, and this may partially explain the absence of reports of epidemic DEN in this part of the country, in spite of people arriving with infection from endemic areas during outbreaks [57]. Surprisingly, this study recorded high DEN risk indices in Kisumu yet there has been no reported outbreak in the region. This finding suggests that the mere presence of high abundance of *Ae. aegypti* as observed in Kisumu, may not be sufficient in estimating the risk of DEN transmission and that other factors should be considered including susceptibility of the *Ae. aegypti* population to the DENV as well as their feeding behavior. All of these can affect vectorial capacity as has been demonstrated for *Ae. albopictus* [58].

We also observed significantly higher numbers of *Ae. aegypti* immatures outdoors compared to indoors in Kisumu and Nairobi. There is reason to believe that immatures will eventually emerge to adults posing biting risk to humans both indoors and outdoors in Kilifi compared to the outdoor risk in Kisumu and Nairobi, thereby leading to an increased risk of exposure to DEN transmission. This differential proximity of *Ae. aegypti* to human dwelling/activity may be a contributing factor to the differential epidemiology and outbreak pattern of DEN in the different cities. Earlier studies on the ecology of *Ae. aegypti* in the Kenyan coast suggested that the larvae of the domestic form *Ae. aegypti aegypti* develops indoors as opposed to the sylvatic form *Ae. aegypti formosus* which develops outdoors especially in forest tree holes and a polymorphic population which develops either indoors or outdoors in tree holes, steps cut into coconut palm trees, discarded tires, or tins [24]. Based on our observation, it is likely that the vector population in Kisumu and Nairobi is predominantly *Ae. aegypti formosus*, which has been described in other studies as a less efficient DEN vector when compared to *Ae. aegypti aegypti* [59,60]. A study to correlate the indoor vs outdoor larval habitats to possible genetic diversity among the species and susceptibility to DEN viruses is warranted.

Aside from the aforementioned biological factors which can impact occurrence of DEN outbreaks, temperature is by far the most important climatic variable that can modulate this pattern [61] and should also be considered. Generally, the different study areas have different average monthly temperatures, 22°C to 28°C in Nairobi, 28°C to 30°C in Kisumu and 27°C to 31°C in the coastal area of Kenya where DEN is

endemic. We are not sure how well the observed differences in the risk indices relate to the prevailing environmental temperature among the different areas. Higher temperatures have been shown to increase the ability of *Ae. aegypti* to transmit DENV by reducing the extrinsic incubation period [62–64]. However, it is important to note that the diurnal temperature fluctuations may be more important in modulating the transmission dynamics.

This study only inferred risk from infestation patterns of *Ae. aegypti.* How these risks relate to actual prevalence in the human population is deserving of further consideration. There is evidence to suggest that some silent DEN transmission goes unreported in Kisumu, as a serological survey carried out by Blaylock et al. (2011) in this part of the country reported DEN seroprevalence levels of 1.1 %. This value is similar to that reported by Morrill et al (1991) for DEN in the coastal area of Kenya during non-epidemic periods [65]. Dengue is known to manifest clinically like malaria and diagnostic tools for DEN detection are unavailable in most health centers in the East African region, including Kenya [57]. It is therefore very important to confirm undiagnosed malaria cases, as it is possible some of these cases may actually be DEN.

Generally, the risk of an urban YF epidemic occurring in Kenya based on vector abundance data observed in this study was classified as low to medium, with the risk due to *Ae. aegypti* being higher as compared to *Ae. bromeliae*. However, the role of *Ae. aegypti* in the transmission of YFV in East Africa has not been fully evaluated and in the documented outbreak that occurred in Kenya in 1992/93, it was observed that this was driven by sylvatic vectors mainly *Ae. africanus* and *Ae. keniensis* and that *Ae. aegypti* was not at all associated with the outbreak [31]. *Aedes bromeliae* has also been described as a YFV vector in this region, as it was the principal vector in the largest YF outbreak that occurred in Omo River in Ethiopia [29], as well as in outbreaks in Uganda [30]. *Aedes simpsoni* is a complex of at least three sister species of which only *Ae. bromeliae* has been implicated as a YFV vector [66]. To understand better the risk due to this species, it will be important to differentiate the sub-species occurring in these urban areas in parallel with vector competence status, which was outside the scope of this study.

In Kilifi and Kisumu the high abundance of *Ae. aegypti* especially in the rainy season is considered sufficient to allow YFV transmission in association with other YFV vectors species such as *Ae. bromeliae, Aedes metallicus* and *Er. chrysogaster* found at some of the sites. However, their ability to act as efficient YFV vectors in urban areas in Kenya needs to be evaluated as data on their vectorial capacity is completely lacking. It is important to note that high numbers of *Ae. bromeliae* were recorded in our study area in Kilifi, and that clarification of the role of this species in the transmission of endemic arboviruses, such as DENV and chikungunya virus is needed, as it may be acting as a potential secondary vector.

In conclusion, *Ae. aegypti* remains the only known DEN vector in Kenya with sufficient abundance in the major cities to sustain transmission. It is highly abundant and the risk values are indicative of high risk of DEN transmission in Kilifi and Kisumu. The key containers that are utilized by this species for oviposition are water storage containers that can be effectively targeted to reduce vector numbers and, consequently, the risk of virus transmission through community mobilization and public health education. The ovipositon site preference, indoor vs outdoor containers, between the study areas is suggestive of behavioral and/or genetic variation occurring in the different vector populations, calling for further studies. Overall, our findings provide a baseline for future studies to understand further the observed differential risk patterns especially with respect to the vectorial capacity of the different populations of *Ae. aegypti* and *Ae. bromeliae* for DENV and YFV transmission.

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Chapter 3

Dengue and yellow fever virus vectors: seasonal abundance, diversity and resting preferences in three Kenyan cities

Sheila B. Agha[1,2*], David P. Tchouassi[1], Armanda D.S. Bastos[2], Rosemary Sang[1, 3]

[1]International Centre of Insect Physiology and Ecology (icipe), P. O. Box 30772-00100, Nairobi, Kenya

[2] Department of Zoology and Entomology, University of Pretoria, Private Bag 20, Hatfield 0083, South Africa

[3]Arbovirus/Viral Hemorrhagic Fever Laboratory, Centre for Virus Research, Kenya Medical Research Institute (KEMRI), P.O Box 54840-00200, Nairobi, Kenya

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Abstract

Background

The transmission patterns of dengue (DENV) and yellow fever (YFV) viruses, especially in urban settings, are influenced by *Aedes* (*Stegomyia*) mosquito abundance and behavior. Despite recurrent dengue outbreaks on the Kenyan coast, these parameters remain poorly defined in this and areas of contrasting dengue endemicity in Kenya. In assessing the transmission risk of DENV/YFV, in three Kenyan cities, we determined adult abundance and resting habits of potential *Aedes Stegomyia* vectors in Kilifi (dengue-outbreak prone), and Nairobi and Kisumu (no dengue outbreaks reported). In addition, mosquito diversity, an important consideration for changing mosquito-borne disease dynamics, was compared.

Methods

Between October 2014 and June 2016, host-seeking adult mosquitoes were sampled for five consecutive days using CO_2-baited BG-Sentinel traps (12 traps daily) placed in vegetation around homesteads, across study sites in the three major cities. Also, indoor and outdoor resting mosquitoes were sampled using Prokopack aspirators. Three samplings were conducted during the long-rains, short-rains and dry season for each city. Inter-city and seasonal variation in mosquito abundance and diversity was evaluated using general linear models while mosquito-resting preference (indoors versus outdoors) was compared using Chi-squared test.

Results

Aedes aegypti, which comprised 60% (n = 7,772) of the total 12,937 host-seeking mosquitoes collected, had comparable numbers in Kisumu (45.2%, n = 3,513) and Kilifi (37.7%, n = 2,932), both being significantly higher than Nairobi (17.1%, n = 1,327). *Aedes aegypti* abundance was significantly lower in the short-rains and dry season relative to the long-rains ($P<0.0001$). *Aedes bromeliae,* which occurred in low numbers, did not differ significantly between seasons or cities. Mosquito diversity was highest during the long-rains and in Nairobi. Only 10% (n = 43) of the 450 houses aspirated were found positive for resting *Ae. aegypti*, with overall low captures in all areas. *Aedes aegypti* densities were comparable indoors/outdoors in Kilifi; but with higher densities outdoors than indoors in Kisumu and Nairobi.

Conclusions

The presence and abundance of *Ae. aegypti* near human habitations and dwellings especially in Kilifi/Kisumu, is suggestive of increased DENV transmission risk due to higher prospects of human vector contact. Despite low abundance of *Ae. bromeliae* suggestive of low YFV transmission risk, its proximity to human habitation as well as the observed diversity of potential YFV vectors should be of public health concern and monitored closely for targeted control. The largely outdoor resting behavior for *Ae. aegypti* provides insights for targeted adult vector control especially during emergency outbreak situations.

Key words. *Aedes aegypti, Aedes bromeliae,* vector abundance, mosquito diversity, resting preference, urbanization, Kenya, Dengue and Yellow fever risk.

3.1 Introduction

Global epidemics of dengue and yellow fever are on the rise in most tropical and subtropical regions, with geographic expansion and increasing frequency of outbreaks being reported especially in Africa [1–4]. Dengue virus (DENV) is the most rapidly spreading arbovirus in the world, with over 390 million global infections reported yearly [5,6]. Also, yellow fever virus (YFV), which has a mortality rate of 20-50%, rivaling that of Ebola virus, is among arboviral diseases of major public health concern [4].

Since the first dengue outbreak in Kenya in 1982, which occurred in Kilifi and Malindi, subsequent outbreaks have mostly been limited to the Kenyan coast, especially in the urban city of Mombasa [7–9] and recently also affecting the Kenya-Somali border area [10]. This expansion in the geographical range of dengue outbreaks is of concern, as it highlights the potential for further spread. Urban yellow fever outbreaks are on the rise, as recently reported in Angola (Luanda) and Democratic Republic of Congo (Kinshasa), with cases imported into China and Kenya [4,11,12]. Although the last yellow fever outbreak in Kenya occurred in 1992-93 [13], the disease is still considered a public health threat. This could be driven in part by the potential for spread through national/international travel [4,14] as well as the widespread presence of peri-domestic vectors, *Aedes aegypti* and *Aedes bromeliae* [15].

Yellow fever has continued to re-emerge in the last decade despite the availability of a safe and efficacious vaccine. Although, a new dengue vaccine for use in emergency situations in highly endemic countries is currently available [16], the vaccine has not been licensed for use in many endemic countries, including Kenya. Current efforts for controlling dengue in Kenya therefore rely on reducing man-vector contacts as well as continuous suppression of the vector *Ae. aegypti* by targeting the immature stages. Based on previous studies carried out in Kenya, the most productive containers types for *Aedes* immature were determined for targeted vector control, and the associated *Stegomyia* risk indices were established for assessing risk of DENV/YFV transmission [9,17]. However, studies focusing on adult mosquito populations are known to be more informative in estimating risk of transmission of these diseases

[18]. Also, emergency interventions targeting adults remain crucial during outbreaks, the effectiveness of which depends on a good understanding of the adult abundance and resting behavior.

Aedes aegypti aegypti and *Ae. aegypti formosus (*hereafter referred to as *Ae. aegypti)* are genetically diverse forms of *Ae. aegypti,* with the former being highly domesticated and often found in close association with humans, especially in urban settings, as opposed to the more zoophilic genetic form *Ae. aegypti formosus* [19]. As reported in large areas of Asia and South America, vectorial capacity of *Ae. aegypti* is influenced to a large extent not only by its extremely high human feeding tendency, but importantly, abundance and indoor resting habits [1,20], which serve to enhance man-vector contact and maximize disease transmission. Surprisingly, knowledge of these attributes remains poorly defined in major dengue foci of Africa, especially in Kenya.

As part of an epidemiological assessment of risk of dengue and yellow fever outbreaks in Kenya, it was hypothesized that 1) the abundance and diversity of potential DENV/YFV vectors differs between the dengue-endemic (Kilifi County) and dengue-free (Kisumu and Nairobi County) cities in Kenya 2) the domestic and peri-domestic resting habits of potential DENV/YFV vectors differ in these cities. Data on the adult abundance and diversity can guide on the level of risk of transmission within each city, while identification of resting habits can be useful in vector control programs, providing baseline information on the different adult mosquito control strategies that can be implemented in case of an epidemic.

3.2 Materials and Methods

3.2.1 Study site

The study was carried out in three of the largest cities in Kenya, which despite all being major trade and travel hubs, differ with respect to dengue status with Kilifi being endemic to dengue, whereas there are no reports of dengue from, Kisumu and Nairobi (Fig 3.1). The capital Nairobi (01°17'S 36°48'E) is the largest city of Kenya and is located at an altitude of 1,661 m above sea level (asl). Average monthly

temperature ranges from 22°C to 28°C. Kilifi County, situated at an altitude of 50m asl, occurs within the Coastal Region, with Mombasa (4°03'S 39°40'E), the second largest city in Kenya. With an average monthly temperature range of 27°C to 31°C, the Coastal Region has been endemic for dengue since 1982, and has experienced recent, as well as recurrent outbreaks in the last decade. Kisumu (00°03′S 34°45′E), the third largest city in Kenya is second only to Kampala in importance, within the Lake Victoria Region. It is situated at an altitude of 1,131 m asl and has an average monthly temperature range of 28°C to 30°C.

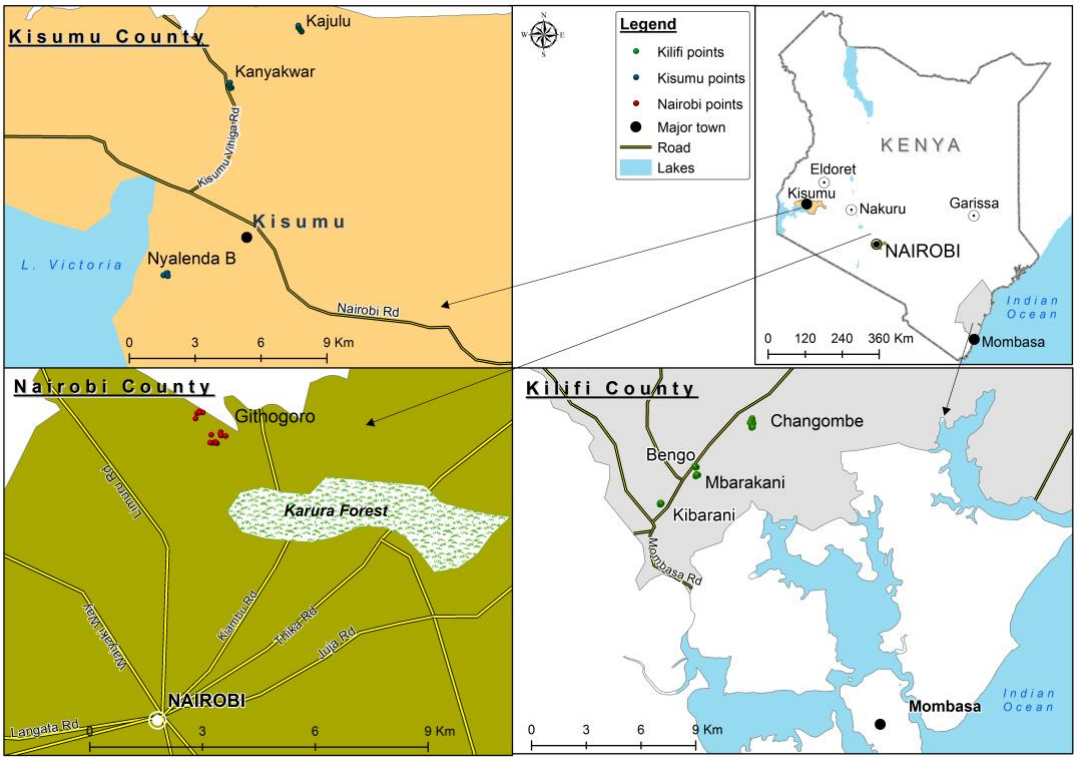

Figure 3.1. Map showing the location of the study sites within Kilifi, Kisumu and Nairobi Counties of Kenya.

Traps were set in four sublocations within Kilifi County in Rabai including Bengo, Changombe, Kibarani, and Mbarakani. Similarly, trapping in Kisumu (Kisumu County), covered the three sublocations- Kajulu, Kanyakwar, and Nyalenda B. In Nairobi (Nairobi County), all traps were set in Githogoro. Sampling at the sublocations, conducted to ensure the widest possible coverage within each city, balanced against logistical considerations, such as ease of accessibility to homesteads, particularly within Nairobi.

3.2.2 Study design

Sampling was conducted in the long-rains (April-June), short-rains (October-December) and dry season (January-March or July-September), from 2014 to 2016. The seasons were primarily defined by the amount of rainfall. We obtained average rainfall data two weeks prior to mosquito sampling from the Kenya Meteorological Department which during the long-rains was 12.4, 10.8 and 8.3 mm; short-rains 5.5, 4, and 7.3 mm and the dry season 0, 0.3 and 0 mm in Kilifi, Kisumu and Nairobi, respectively.

Adult host-seeking mosquitoes were collected using BG-Sentinel traps, baited with CO_2 supplied in the form of dry ice and placed outdoors in the vegetation around human habitations. The CO_2 was dispensed by placing ~0.5kg in a thermos Igloo (2L) per trap and suspended slightly above the trap entry. Traps (12 daily) were set up at 7 am in the morning and retrieved at 6pm in the evening on the same day, for 5 consecutive days, in each season in each city, translating to a total of 540 BG-Sentinel traps being set (180 per city and 60 per season).

Collection of resting adult mosquitoes was performed using Prokopack aspirators targeting *Aedes* resting mosquitoes indoors (sitting room, bedroom, and kitchen) and outdoors (on nearby vegetation and the walls outside the house). Houses in each city were purposively selected to include houses with a common design and most importantly availability of surrounding vegetation. Each sampling season targeted a total of 50 houses per city; so, a total of 450 houses (50 per season and 150 per city) were sampled in all three cities. Sampling was done in the long-rains, short-rains and dry season for 5 consecutive days. Collection was done between 11:00am to 3:00pm daily by a team of three people (one indoor and 2 outdoor) lasting 20 min per house.
Trapped mosquitoes were taken to a temporary field site laboratory in each city and immobilized using triethylamine (TAE), placed in cryovials and immediately preserved in liquid nitrogen for transportation to the laboratory at the International Centre of Insect Physiology and Ecology, Nairobi for identification. Morphological identification was done using available taxonomic keys [21–23]. Data on the collection date, species, season and city of collection was captured in Excel. Some mosquitoes that could not be identified to species level because some of the

morphological features were damaged or lost were classified to genus level as *Aedes* spp, *Mansonia* spp, and *Culex* spp.

3.2.3 Data analysis

Mosquitoes collected during each of the 5-day trappings per season (i.e., BG-Sentinel collections) from the different sites within each city were pooled and counted. We analyzed the total mosquito abundance and specific-species abundance (*Ae. aegypti* and *Ae. bromeliae*) using General linear models (GLMs) with seasons and cities as predictors. As a measure of mosquito community structure, we estimated the Shannon diversity index (hereafter referred to as diversity) for each city per trapping season using the vegan package in R version 3.1.1 (R development Core Team). We explored seasonal and city influence on mosquito diversity by applying GLMs after log-transformation to normalize the data. Best-fit models (normal or poisson or quasipoisson or negative binomial generalized linear models) were selected based on model residuals for species richness, diversity, total abundance and species-specific abundance. Data normality was confirmed by performing Shapiro–Wilk tests on model residuals of mosquito diversity. Kilifi was taken as the reference city, and the dry season as the reference season.

For resting mosquito collections, we limited our comparisons to *Ae. aegypti* only. Resting *Ae. aegypti* collected for the different seasons in each city were pooled, and broadly classified as indoors (sitting room, bedroom and kitchen) and outdoors (walls around the house and vegetation). The mosquito resting density estimated as the total number of resting *Ae. aegypti* by number of collectors was compared, indoors *versus* outdoors, for each city using the Chi-squared test. The proportion of houses positive indoors or outdoors was also compared using the Chi-squared test. All data were analyzed in R version 3.3.1 [24] at α=0.05 level of significance.

3.3 Results

3.3.1 Mosquito abundance and composition

A total of 12,937 mosquitoes representing 6 genera and 25 species was captured throughout the survey from the three cities using the BG-Sentinel traps (Table 3.1).

Aedes aegypti was the most dominant DENV/YFV vector represented across all the cities and seasons except for Nairobi where *Aedes tricholabis* dominated collections during the long-rains. Kilifi however had a wider *Aedes* species representation (9 spp). *Mansonia* species was primarily encountered in Kisumu especially in the long-rains and dry seasons. Collections of *Culex* species were generally low; dominated by *Culex pipiens* and *Culex univittatus* in Kisumu during the long-rains, and wider species representation (10 species) in Nairobi. *Culex rubinotus* was limited to Kilifi, *Culex poicilipes* to Kisumu and *Culex zombaensis* to Nairobi although in low numbers. *Toxorhynchites brevipalpis* was also recorded in Kilifi and Kisumu, *Eretmapodites chrysogaster* in Kilifi and Nairobi, while *Anopheles* species were recorded in all three areas during the long-rains, although in low numbers.

Total mosquito abundance was significantly higher in Kisumu than Kilifi (Estimate = 0.593±0.29, t = 2.08, P = 0.043). However, total mosquito abundance did not differ between Kisumu and Nairobi (Estimate = 0.30±0.27, t = 1.12, P = 0.27) or Kilifi and Nairobi (Estimate = 0.293±0.30, t = 0.97, P = 0.34) (Table 3.2). Overall abundance during the long-rains was significantly higher than the short-rain (Estimate = 2.316±0.38, t = 5.459, P < 0.0001) and dry season (Estimate = 2.119±0.39, t = 5.46, P < 0.0001), but collections between the short-rains and dry season did not differ significantly (Estimate = -0.198±0.51, t = 0.39, P = 0.7) (Table 3.2).

Aedes aegypti accounted for 60% (n = 7,772) of the total host-seeking mosquitoes collected, with Kilifi yielding 37.7% (n = 2,932), Kisumu 45.2% (n = 3,513), and Nairobi 17.1% (n = 1,327) (Table 3.1). While *Ae. aegypti* abundance in Kilifi and Kisumu were comparable (Estimate = 0.321±0.241, t = 1.33, P = 0.19), when each was compared to Nairobi, a two-fold and three-fold increase in *Ae. aegypti* abundance was observed in Kilifi (Estimate = -0.653±0.32, t= -2.06, P = 0.045), and Kisumu (Estimate = 0.97±0.31, t = 3.17, P = 0.027), respectively (Table 3.2). While *Ae. aegypti* abundance varied significantly between the long- and short-rains (Estimate = 2.004±0.31, t = 6.50, P < 0.0001), and the long-rains and dry season (Estimate = 2.109±0.378, t = 5.59, P < 0.0001), the numbers recorded in the short-rains and dry season were not significantly different (Estimate = 0.104±0.46, t = 0.23, P = 0.82) (Table 3.2).

Table 3.1. Seasonal adult mosquito abundance in Kilifi, Kisumu, and Nairobi between October 2014 and June 2016 using CO_2-baited BG-Sentinel traps.

	Kilifi			Kisumu			Nairobi		
Mosquito species	**Long-rains**	**Short-rains**	**Dry season**	**Long-rains**	**Short-rains**	**Dry season**	**Long-rains**	**Short-rains**	**Dry season**
**Aedes aegypti*	2235	581	113	2577	414	522	1071	180	76
#Aedes bromeliae	6	5	0	0	5	0	13	0	0
#Aedes metallicus	1	3	0	10	0	0	0	0	0
#Aedes tarsalis	3	0	0	0	0	0	0	0	0
Aedes dentatus	0	1	0	0	0	0	0	0	0
Aedes mcintoshi	2	0	0	50	*1*	16	101	1	1
Aedes tricholabis	57	19	0	1	3	0	2295	18	6
Aedes hirsutus	23	3	0	0	1	0	0	0	0
Aedes longipalpis	33	0	0	0	0	0	0	0	0
Aedes spp.	109	1	0	19	0	0	0	39	6
#Eretmapodites chrysogaster	5	6	0	0	0	0	2	0	0
Mansonia africana	2	0	0	789	39	185	0	0	0
Mansonia uniformis	0	0	0	224	7	220	0	0	0
Mansonia spp	0	0	0	37	0	0	0	0	0
Culex pipiens	55	4	2	126	1	4	44	5	48
Culex annuloris	0	2	0	3	0	0	33	2	14
Culex univittatus	2	1	0	140	4	0	12	0	3
Cx vansomereni	3	0	0	0	0	0	21	0	0
Culex rubinotus	0	1	7	0	0	0	0	0	0
Culex zombaensis	0	0	0	0	0	0	54	0	10
Culex tigripes	0	0	0	1	0	2	3	0	0
Culex poicilipes	0	0	0	10	0	0	0	0	0
Culex ethiopicus	0	0	0	7	1	0	0	0	3
Culex bitaeniorhynchus	0	0	0	2	0	0	0	3	0
Culex spp	7	2	0	23	0	0	0	2	2
Toxorhynchites brevipalpis	3	0	0	0	1	0	0	0	0
Anopheles gambiae s.l	2	0	0	1	0	0	2	0	0
Anopheles coustani	0	0	0	1	0	0	0	0	0
Total	**2548**	**629**	**122**	**4021**	**477**	**949**	**3651**	**250**	**169**

* = Major vector of DENV and urban YFV, # = Potential YFV vectors

Table 3.2. Total mosquito abundance and diversity in the long-rains, short-rains and dry season in Kilifi, Kisumu and Nairobi. Analyses are quasipoisson generalized linear model (Abundance df = 2, 47), normal linear models (Shannon diversity df = 2, 47).

	Total abundance			***Aedes agypti* abundance**			***Aedes bromeliae* abundance**			**Shannon Diversity Index**		
City	Estimate ±se	t-value	*P*-value	Estimate ±se	t-value	*P*-value	Estimate ±se	t-value	*P*-value	Estimate ±se	t-value	*P*-value
Kisumu	0.593±0.285	2.083	0.043*	0.321±0.241	1.33	0.19	-0.469±0.803	-0.584	0.562	0.063±0.035	1.821	0.075
Nairobi	0.293±0.303	0.969	0.337	-0.653±0.317	-2.058	0.045*	0.487±0.615	0.792	0.432	0.186±0.035	5.36	<0.0001***
Long-rains	2.119±0.388	5.459	<0.0001***	2.109±0.378	5.585	<0.0001***	18.497±2128.62	0.009	0.993	0.086±0.037	2.303	0.026*
Short-rains	-0.198±0.509	0.388	0.700	0.104±0.455	0.229	0.82	17.497±2128.62	0.008	0.993	-0.009±0.035	-0.262	0.794

*P < 0.05, ***P < 0.001.

Kilifi was considered as the reference city and the dry season as the reference season in the analyses.

Aedes bromeliae was the second most dominant *Stegomyia* species recorded in all three cities comprising 0.23% (n = 29) of the total mosquitoes collected, of which 37.9% (n = 11) occurred in Kilifi, 17.2% (n = 5) in Kisumu, and 44.8% (n = 13) in Nairobi. *Aedes bromeliae* abundance however, did not vary by city or season (Table 2). Other potential vectors of YFV recorded include *Aedes metallicus, Aedes tarsalis,* and *Erytmapodites chrysogaster* although in very low numbers, each representing less than 0.1% of the total mosquitoes collected (Table 3.1). *Aedes metallicus, Ae. tarsalis*, and *Er. chrysogaster* were all recorded in Kilifi, with no record of *Er. chrysogaster* and *Ae, metallicus* in Kisumu and Nairobi, respectively. The mosquito species composition encountered throughout the sampling periods in the different cities is shown in Table 3.1.

3.3.2 Mosquito species richness/diversity

Of the total 25 species observed in all three areas, 10 belonged to the *Culex* genus and nine to the *Aedes* genus (Table 3.1). Mosquito species richness varied by city and season, being comparable in Kisumu and Nairobi (Estimate = 0.133±1.03, t = 0.13, P = 0.90), but significantly higher when each was compared to Kilifi (Nairobi-Kilifi Estimate = 2.168±0.96, t = 2.26, P = 0.03, and Kisumu-Kilifi Estimate = 2.301±0.96, t = 2.40, P = 0.02). Mosquito species richness was significantly higher in the long-rains compared to the short-rains (Estimate = 5.77±0.96, t = 6.02, P < 0.0001) and dry season (Estimate = 6.87±1.03, t = 6.68, P < 0.0001), but not between the short-rains and dry season (Estimate = 1.098±0.96, t = 1.15, P = 0.26).

Species richness varied from 6 to 20 in Kilifi, 6 to 22 in Kisumu, and 10 to 18 species in Nairobi from the dry to rainy seasons. Also, the overall mosquito diversity varied by city and season. Mean mosquito diversity was two- fold higher in Nairobi (n = 1.03) compared to Kisumu (n = 0.60, Estimate = -0.123±0.037, t = -3.30, P = 0.002), and three-fold higher in Nairobi compared to Kilifi (n = 0.31, Estimate = 0.186±0.035, t = 5.36, P < 0.0001) (Table 3.2, Fig 3.2). Mosquito diversity was, however, not significantly different between Kilifi and Kisumu (Estimate = 0.063±0.035, t =1.821, P = 0.075) (Table 3.2). We found significantly higher mosquito diversity in the long- *versus* short-rains (Estimate = 0.095±0.035, t = 2.73, P = 0.009), then between the long-rains and dry season (Estimate = 0.086±0.037, t = 2.303, P = 0.026), but no difference between the short-rains and dry season (Estimate

= 0.009±0.035, t = 0.26, P = 0.79) (Table 3.2). Mosquito diversity ranged from 0.04 to 1.9, with the lowest value recorded in Kisumu during the dry season and highest in Nairobi in the dry season.

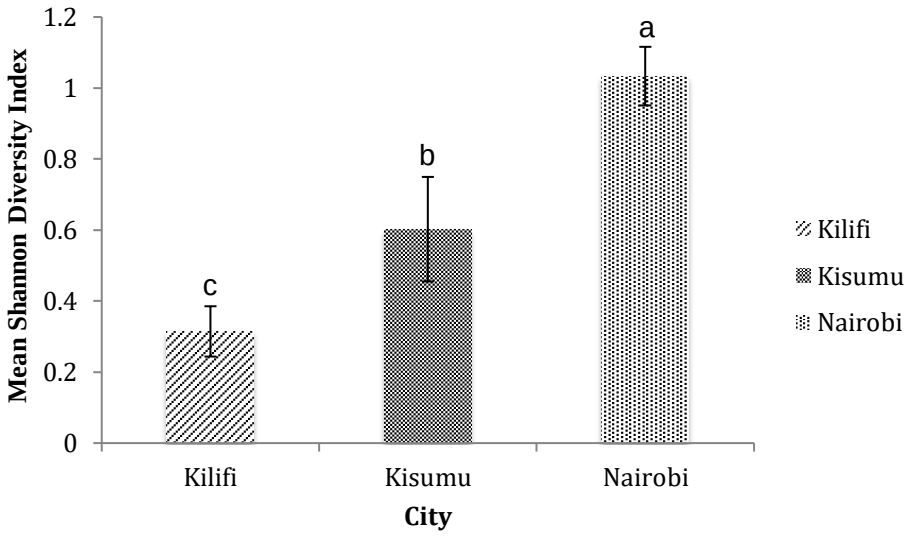

Figure 3.2. Mean Shannon diversity index for mosquitoes collected using BG-Sentinel traps in Kilifi, Kisumu, and Nairobi in Kenya. means followed by different letters are significantly different at α=0.05**.**

3.3.3 Resting preferences

Of the total 450 houses (150 per city, 50 per season) inspected from all three cities, 10% (n = 44) were positive for either male or female *Ae. aegypti*. Of these positive houses, 27.3% (n = 12) were from Kilifi, 52.3% (n = 23) from Kisumu and 20.4% (n = 9) from Nairobi. A total of 73 *Ae. aegypti* only were aspirated from all three cities, 44% (n = 32) females and 56% (n = 41) males both indoors and outdoors. This translated to a resting density of 0 to 5 mosquitoes indoors and 6 to 21 mosquitoes outdoors for the different cities (Table 3). Apart from one *Ae. bromeliae,* which was collected outdoors in Kilifi, *Ae. aegypti* was the only other *Stegomyia* species sampled in the resting collection. While there was no significant difference in the number of *Ae. aegypti* found resting indoors and outdoors in Kilifi ($\chi^2 < 0.0001$, Df = 1, P = 1.0), significantly higher numbers were found resting outdoors compared to indoors in Kisumu ($\chi^2 = 28.17$, Df = 1, P < 0.0001). In Nairobi, resting was exclusively outdoors. Also, in Kilifi, the catches of females ($\chi^2 = 0$, Df = 1, P = 1.0) either indoors or outdoors, like that of males ($\chi^2 = 1$, Df = 1, P = 0.32), did not differ significantly.

Table 3.3. Indoor and outdoor resting densities of *Aedes aegypti* mosquitoes collected in Kilifi, Kisumu and Nairobi using Prokopack aspirators from October 2014-June 2016.

			Female		Male		Total[a]	
Area	Location	Positive houses[b]	No. Collected	Resting density[c]	No. Collected	Resting density[c]	No. Collected	Resting density[c]
	Indoor	3.3 (5, 0.01-0.08[d])	5	5	0	0	5	5
Kilifi	Outdoor	5.3 (8, 0.03-0.11[d])	7	4	4	2	11	6
	Indoor	1.3 (2, 0.002-0.05[d])	1	1	1	1	2	2
Kisumu	Outdoor	14.0 (21, 0.09-0.21[d])	14	7	28	14	42	21
	Indoor	0.0 (0, 0.0-0.03[d])	0	0	0	0	0	0
Nairobi	Outdoor	6.0 (9, 0.03-0.11[d])	5	3	8	4	13	7

[a] Total males and females *Ae. aegypti* collected.
[b] % Positive houses (No. of positive houses, 95% Confidence interval).
[c] Resting density = No. collected / No. of collectors.
[d] P<0.0001
Indoors = Sitting room, bedroom and kitchen.
Outdoor = Nearby vegetation and outside walls.

However, in Kisumu significantly more females (χ^2 = 6.25, Df = 1, P = 0.012) and males (χ^2 = 19.2, Df = 1, P 0.0001) were captured outdoors than indoors. Overall, in Kilifi the total number of females aspirated was significantly higher than the number of males (χ^2 = 6.55, Df=1, P = 0.011), while in Kisumu (χ^2 = 3.13, Df = 1, P = 0.077), and Nairobi (χ^2 = 0, Df = 1, P = 1), there was no significant difference.

3.4 Discussion

Aedes aegypti the known DENV vector in Kenya [9], was generally found to be the most abundant mosquito species. Abundance was highest during the long-rains relative to the short-rains and dry season, and comparably higher in Kilifi and Kisumu, versus Nairobi. We found very low occurrence of *Ae. bromeliae*, a species which did not vary by city or season. Furthermore, variation in mosquito diversity was evident, being highest in Nairobi and during the long-rains (Table 3.2). Variation in abundance and diversity, both important epidemiologic risk parameters, may impact differentially on transmission risk of DENV/YFV across seasons and cities. More outdoor resting was observed for *Ae. aegypti*, suggesting the existence of an exophilic population especially in Kisumu and Nairobi. In Kilifi, resting data is suggestive of a more endophilic population of *Ae. aegypti*.

The *Ae. aegypti* abundance pattern was strongly correlated with seasonal rainfall, with higher abundance during the long-rains compared to the other periods in all three cities (Table 3.1). In fact, 5 and 8 times more *Ae. aegypti* were captured during the long-rains compared to short-rains and dry seasons, respectively (Table 3.1). This is expected as abundance of mosquitoes including *Ae. aegypti* is generally associated with rainfall [25]. Previous findings have found rainfall as an important driver of *Ae. aegypti* populations and dengue incidence [25], which corroborates the occurrence of dengue outbreaks in Kenya and East Africa during periods of heavy rainfall [9,26]. Taken together, our findings suggest higher risk of DENV transmission in Kilifi/Kisumu than Nairobi, particularly during the long-rains. Nonetheless, the persistence of *Ae. aegypti* throughout the short-rains and dry season suggests that disease transmission could potentially persist through the year, albeit at lower level, due to continued presence of the vector.

Factors relating to availability of breeding sites, temperature or altitudinal differences may have influenced the abundance patterns of *Ae. aegypti* across the cities [25,27]. Being a typical container breeder, we previously found an increase in the number of breeding sites in Kilifi and Kisumu in the long-rains, compared to Nairobi [17], which is located at a higher altitude (1,661 m asl), and has lower average monthly temperatures (22°C to 28°C) compared to Kilifi (50 m asl, 27°C to 31°C) and Kisumu (1,131 m asl, 28°C to 30°C). This may partly explain the low *Ae. aegypti* abundance found in Nairobi, as significant reductions in *Ae. aegypti* abundance with an increase in altitude have previously been reported [27]. The same study identified temperatures to be a positive risk factor for *Ae. aegypti* abundance, with vector abundance increasing with an increase in temperature [27]. Autochthonous cases of dengue can be facilitated by local *Aedes* vectors. However, despite Nairobi being a major hub in East Africa, there has been no outbreak of dengue reported. A possible contributing factor to this pattern could be low *Aedes* abundance, as was observed in our study, among other factors. The high vector abundance in Kilifi and Kisumu corroborates with their increased breeding sites, especially during the long-rains. This high *Ae. aegypti* abundance in Kilifi may explain the dengue epidemics reported in this region [7–10]. Also, the high abundance in Kisumu may explain the recent reports of sporadic cases of dengue (Sang, unpublished data) although outbreaks have not been reported. The high abundance of *Ae. aegypti* in Kisumu and its potential role in dengue epidemics is therefore one deserving of further consideration.

Aedes bromeliae, Ae. metallicus, Ae. tarsalis and *Er. chrysogaseter* are sylvatic vectors mostly found inhabiting discarded plastic containers as well as natural containers (treeholes, rock pools and discarded coconut shells) [17]. These YFV vectors have been implicated in yellow fever outbreaks in East and Central Africa [28–31]. Trap captures for these species were generally low and it is not certain if this could be related to potential sampling bias of the sampling tool employed- the BG-Sentinel trap. This trap whilst designed to target *Ae. aegypti* [32,33], has been shown to effectively collect other disease vectors including *Anopheles* species and even sandflies [34,35]. As such it appears unlikely that this trapping tool employed may have affected the overall mosquito diversity and abundance, particularly as we also baited the traps with CO_2 which is thus far known to be the most potent attractant to mosquito species [36,37]. However, given that in our previous study [17], we

encountered high numbers of *Ae. bromeliae* and *Er. chrysogaster* immatures, the low numbers of adults recorded in this study suggest that adults may be poorly attracted to the BG-Sentinel trap. This indicates that developing better sampling tools for targeting adults of these species is an important consideration. In addition, the introduction of sylvatic YFV vectors into urban areas, as was observed in this study, is of public health concern. The YFV could adapt to these vectors given their ability to act as potential enzootic vectors. Their ability to transmit the YFV therefore warrants further assessment.

Although, species richness was comparable in Nairobi and Kisumu, Nairobi had the highest overall diversity of mosquitoes (Fig. 3.2). The species observed in Kilifi, especially *Ae. bromeliae, Ae. metallicus*, *Ae. tarsalis,* and *Er. chrysogaster* which in addition to *Ae. aegypti,* are known vectors for YFV, should not be ignored. These species may be of epidemiological value, in the light of pathogen adaptation to multiple vectors as observed for chikungunya virus in Senegal [38,39]. Their role in sustaining an outbreak of dengue and chikungunya therefore needs to be assessed, as these vectors especially *Ae. bromeliae* could serve as bridge vectors [40], moving the virus from the sylvatic to the urban transmission cycle. While mosquito diversity was higher during the rainy seasons compared to the dry season in Kilifi and Kisumu, the observed pattern was different in Nairobi where higher mosquito diversity was recorded in the dry season. The diversity pattern in Nairobi may have been influenced by the *Culex* collections, which were fairly represented in Nairobi with only sparse occurrence in Kilifi/Kisumu, especially during the dry season. Although *Culex* species are not important vectors in the epidemiology of DENV/YFV, they play an important role in the transmission of other arboviruses, such as West Nile virus [41].

Despite extensive sampling effort, we found very low numbers of resting *Ae. aegypti* mosquitoes, both indoors/outdoors. The sampling regime and effort is in line with techniques employed elsewhere [42,43]. The low numbers of *Ae. aegypti* resting indoors is in stark contrast to the largely indoor habit known for this species in Asia and South America [42,44,45]. This suggests a difference in the resting patterns of *Ae. aegypti* found in Kenya and corroborates findings from previous studies reporting generally low numbers of resting *Ae. aegypti* in this region [9,46]. The largely outdoor resting habit of this species concurs with its breeding pattern being mainly

outdoors [9,17,46]. However, the low outdoors numbers recorded here suggest other resting sites apart from vegetation that require further elucidation. Although, most of the outdoor resting was observed on vegetation, this study did not investigate in detail the preferred plant types, as this was not within the scope of the study. Overall, the knowledge of these resting patterns can be exploited in emergency operations targeting adults to break transmission in an outbreak situation. The proportion of adults found resting indoor/outdoor varied between Kilifi and the other cities; for Nairobi and Kisumu it was largely outdoors. This finding indicates possible indoor/outdoor temperature differences in these study areas as well as population differences in resting habits among these cities, which is worth exploring.

Conclusion

Aedes aegypti was the most dominant mosquito species recorded and its occurrence varied by city and season. The abundance pattern suggests that the risk of DENV transmission is elevated during the long-rains and in Kilifi/Kisumu compared to Nairobi assuming that the vector population is efficient in transmitting the virus. The low abundance of *Ae. bromeliae* recorded is suggestive of a low risk of YFV transmission in all three cities. The overall mosquito abundance pattern neither correlated with species richness nor diversity. In addition to vector abundance, the feeding habit and vector competence are factors that can differentially drive the emergence of dengue and yellow fever in an area. Therefore to fully understand the risk associated with the transmission of DENV/YFV in these cities of Kenya, these factors need to be assessed. The resting pattern for *Ae. aegypti* is suggestive of a more endophilic population in Kilifi and an exophilic population in Kisumu and Nairobi. This knowledge on the resting behavior can be exploited in adult mosquito control to break transmission during emergency outbreak situations. Continuous vector surveillance should however be routinely performed for early detection of changing vector dynamics that may precipitate an outbreak of dengue/yellow fever.

Declarations

Abbreviations

DENV, dengue virus; YFV, yellow fever virus; TEA, triethylamine; asl, above sea levels; GLM, general linear models.

Ethics approval and consent to participate

We sought permission from household heads through oral informed consent to allow their residences to be surveyed for resting mosquitoes. Household survey of mosquitoes was carried out with ethical approval from Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU) (Project Number SERU 2787).

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Chapter 4

Aedes aegypti feeding pattern and temperature shapes dengue virus transmission potential in three Kenyan cities

Sheila B. Agha[1,2], David P. Tchouassi[1], Armanda D.S. Bastos[2], Rosemary Sang[1, 3]

[1]*International Center of Insect Physiology and Ecology (icipe), P. O. Box 30772-00100, Nairobi, Kenya*

[2] *Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0083, South Africa*

[3]*Arbovirus/Viral Hemorrhagic Fever Laboratory, Center for Virus Research, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya*

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Abstract

The burden and frequency of dengue epidemics continue to increase globally, including Kenya. Despite urbanization being a risk factor for dengue virus transmission (DENV) across Kenya, the underlying reasons for recurrent dengue epidemics in Mombasa city (coastal Kenya) whilst other major cities such as Kisumu and Nairobi remain less affected, is not clear. The differential dengue occurrence may be related to variation in *Aedes aegypti* vector population (blood feeding pattern, vector competence) and ambient temperature. In infection studies, no difference in dengue virus serotype -2 (DENV-2) transmission was found, between the vector populations. However, enhanced transmission at higher temperatures was evident. Interestingly, blood meal analyses revealed that field-collected *Ae. aegypti* from coastal Kenya (Kilifi, outskirts of Mombasa) exhibited higher human feeding than those from Kisumu. Estimated vectorial capacity or potential of DENV transmission to humans in Mombasa was about 9-fold higher than in Kisumu and 14-fold higher than in Nairobi at the selected temperatures. Despite comparable transmission rates observed for *Ae. aegypti* across the cities, the higher human feeding and temperatures substantially elevated the potential for DENV transmission in Mombasa than in Kisumu and Nairobi respectively. In conclusion, recurrent outbreaks of dengue in Mombasa and not in Kisumu and Nairobi, in spite of heavy population movements among them is largely influenced by the mosquitoes feeding pattern and environmental temperature.

4.1 Introduction

Dengue virus (DENV) is a global public health threat with epidemics mostly reported in urban and semi urban areas [1,2]. The virus comprises of four related dengue virus serotypes (DENV-1-4), belonging to the genus *Flavivirus* (Family: *Flaviviridae*) [3]. The most recent epidemics in Africa have predominantly been reported in East African countries, with DENV-2 responsible for the highest number of cases [4,5]. Dengue epidemics have been linked to urbanization, globalization, climate change and the broad distributional range of the *Ae. aegypti* vector [6–9]. Amidst the increasing urbanization, recurrent dengue outbreaks in Kenya remain mostly limited to the coastal areas, especially in and around the city of Mombasa [10–12]. There has been no confirmation of any locally acquired dengue cases in the other major cities despite serological studies demonstrating circulating DENV antibodies [13–15]. Determination of the underlying reasons for the observed differential outbreak patterns in Kenyan cities is important in improving our understanding of the major disease drivers and disease risk in all major cities.

Mombasa is Kenya's second largest city and is located at the coastal part of the country. Apart from being a major tourist site and hosting a major international airport, it is also an important port city. Other major cities include Nairobi, Kenya's largest city, and Kisumu, the third largest city in Kenya, which is located on the shores of Lake Victoria, and is an important transportation route within East Africa. All three cities are characterized by either the presence of a national/international airport or a seaport, or both, thus serving as local, regional, and international transport hubs. These cities serve as main gate ways to East Africa, and due to the ease of interconnectivity, we would expect periodic generation of dengue epidemics resulting from either importation of infectious cases or infected vectors into these cities [8] from within and outside the country. This, however, has not been the case in Kenya, as epidemics remain limited to the city of Mombasa [10,12]. The possible drivers responsible for this differential outbreak pattern therefore remain unexplained.

Nevertheless, temperatures and vector factors, among others, could be possible contributing risk factors to this outbreak pattern in Kenya, yet limited information is currently available to support this contention. All three cities have different ambient temperatures and temperature has been reported to influence DENV transmission [16,17]. Entomological risk factors may also relate to variation in the vector abundance, genetics, vector competence, and host

feeding preferences. Previous studies in Kenya have examined risk of transmission drawing from vector presence/abundance data mainly [12,18]. These risk parameters, whilst valuable, are rarely studied in tandem with other aspects influencing transmission, yet their combined effects may be critical to fully understanding their complex interrelationship in defining risk of DENV transmission. Also, the DENV transmission potential (vectorial capacity) can be evaluated and used to predict the risk of dengue outbreak in these and other cities [19,20]. While vectorial capacity estimates take into account all the different factors affecting disease transmission by a single mosquito species [19,20], studies on vectorial capacity in relation to dengue risk measures is lacking in Kenya.

To unravel the urban dengue epidemic patterns in Kenya's three major cities of Mombasa (outskirts in Kilifi), Kisumu and Nairobi (no dengue reported thus far), it was hypothesized that 1) The *Ae. aegypti* population in Mombasa is more anthropophilic compared to that of Kisumu and Nairobi. 2) The ability of *Ae. aegypti* to transmit DENV (vector competence) differs between populations from these cities and is influenced by temperature. To understand how the changes in these parameters might translate to disease dynamics, the vectorial capacity of *Ae. aegypti* for DENV across the three major Kenyan cities was estimated.

4.2 Materials and methods

4.2.1 *Aedes aegypti* host blood meal analysis

Blood meal analysis was performed on wild-caught blood fed samples collected using BG-Sentinel traps (Biogents) and Prokopack aspirators from the selected sites in Mombasa, Kisumu and Nairobi described in detail elsewhere (Agha et al. unpublished data). The abdomen of individual blood fed mosquitoes were cut using scalpels that were sterilized with 70% ethanol between specimens to prevent cross contamination of samples. Genomic DNA was extracted from whole blood contained in each mosquito abdomen using DNeasy blood and tissue Kit (Qiagen, GmbH-Hilden, Germany) according to the manufacturer's instructions. The DNA amplification targeted the 12S mitochondrial rRNA gene, a target that has been widely used for mammalian blood meal identification [21]. The 12S3F (5'-GGGATTAGATACCCCACTATGC-3') and 12S5R (5'-TGCTTACCATGTTACGACTT-3') primers amplify a 500 bp fragment of the 12S gene. Genomic amplification reactions

were performed in a final reaction volume of 10µl, containing 5.0µl of 10x Mytag HS mix, 2µl of water, 0.5µl (0.5µM) of each of the forward and reverse primers, and 2µl (1-10ng) of the genomic DNA template. The cycling conditions were 95°C for 3mins, followed by 95°C for 20 secs, 56°C for 30 sec, 72°C for 30 sec for 35 cycles, and a final extension at 72°C for 5 mins.

4.2.2 Nucleotide sequencing and analysis

Amplicons were run on a 1.5% agarose gel against a 1KB DNA ladder (Thermo Fisher Scientific, USA). The amplicons were individually purified using ExoSap PCR purification kit (USB Corp., Cleveland, OH), according to supplier recommendations. Blood fed samples showing double bands were purified from the gel using a gel purification kit (Bioline, London, UK). Unidirectional sequencing using the forward primer was outsourced from a commercial company (Macrogen, Seoul, Republic of Korea) and sequences were cleaned and analyzed using MEGA v 5 software [22]. The host blood meal was identified after a blast nucleotide searches against the Genbank database (www.ncbi.nlm.nih.gov/blast).

4.2.3 Vector competence of *Aedes aegypti* mosquitoes to dengue virus

4.2.4 Mosquito colonies

Immature *Ae. aegypti* mosquitoes were collected from artificial water holding containers in and around houses between October and November 2016 from sites in three major urban areas; Mombasa (site in outskirts), Kisumu, and Nairobi. Mombasa has an average monthly temperature of 27-31°C, Kisumu 28-30°C, and Nairobi 22-28°C. In Mombasa, we specifically selected sites from the outskirt (Kilifi-Rabai) based on previous history of DENV circulation [11]. In Kisumu and Nairobi (with no DENV outbreak reports) selection of sites was driven mainly by logistical constraints, such as access to housing. The study sites were Kanyakwar and Kajulu in Kisumu and Githogoro in Nairobi.

Samples were transported to the BSL-2 insectary at the International Centre of Insect Physiology and Ecology (*icipe*), Duduville campus, Nairobi and they were reared at 28°C and a 12:12 (L:D) photoperiod to adults as previously described [23]. The resulting F2 generation mosquitoes from all three urban areas were used in the vector competence study. This study focused on *Ae. aegypti* because it is a primary vector for DENV, with recent

isolations of the virus confirming its role in disease transmission in the coastal region of Kenya [12].

4.2.5 Dengue virus strain and assay

The DENV-2 strain used in this study was isolated from a patient (Sample number: 008/01/2012) during the 2012 outbreak in Mandera, Kenya. The virus had been previously passaged twice on C6/36 cells, and was available as a cryo-preserved stock in the BSL-2 laboratory at *icipe*. Before use, the virus was passaged once on Vero cells to generate sufficient quantities for use in the vector competence study. Briefly Vero cells (Green African Monkey cell line, ATTC® CCL-81) were grown in a T-25 cell culture flask using growth media as previously described [23]. Once the Vero cells were ~80% confluent, the cells were inoculated with 200ul of the virus. The flask containing cells were incubated for 1hr in a 5% CO_2 incubator set at 37°C for virus adsorption, after which the cells were maintained with maintenance media (Minimum essential media (MEM) supplemented with 2% FBS). The cells were observed for peak viral levels and the supernatant was harvested and used, without freezing, in an infectious blood meal to expose mosquitoes.

4.2.6 *Aedes aegypti* infection

Vector competence experiments were performed in a BSL-2 laboratory at *icipe* using a hemotek membrane feeder (Discovery Workshops, Accrington, the United Kingdom) covered with a mouse skin and maintained at 37°C. Pre-starved (12hrs before feeding) mosquitoes aged 4-9 days from all three areas were exposed to about 2ml (per well of the feeder) of an infectious blood meal consisting of 1:2 parts of freshly harvested DENV-2 and defibrinated sheep blood (Central Veterinary Laboratories Kabete, Kenya). After 1hr of feeding, unfed mosquitoes were removed from each of the experimental cages. The fully blood fed mosquitoes from each area were divided into three new groups (cages), which were incubated at 22°C, 28°C and 31°C representing either the minimum/maximum average monthly temperatures for each urban area. A proportion of the mosquitoes were sampled on days 7, 14 and 21 (for the different temperatures and areas) and tested for infection, dissemination and transmission of the DENV-2. The sample sizes for each day/city are given in Table S3. To quantify the virus to which the mosquitoes were exposed, 100µl of the pre- and post-feeding blood/virus mixtures were collected before and after the experiment, and added separately to 900µl of homogenization media (MEM supplemented with 15% FBS).

These mixtures were frozen immediately at -80°C until thawed for virus quantification by plaque assay.

4.2.7 Infection, dissemination and transmission of dengue virus-2 by *Aedes aegypti*

For the different urban areas and selected temperatures, individual mosquitoes were assayed for virus infection, dissemination and transmission (by the capillary tube method) as previously described [23]. With slight modifications, the individual mosquito body and legs were homogenized in 500ul of homogenization media, and the capillary tube contents were emptied into a mirocentrifuge tube containing 150ul of homogenization media. All legs, body, and saliva samples were stored at -80°C until assayed for the presence of the virus by cytopathic effect (CPE) or plaque assay.

4.2.8 Cytopathic effect and plaque assays

The body samples were removed from the freezer and thawed on wet ice. Samples were homogenized using a minibeadbeater (BioSpec Products Inc, Bartlesville, OK 74005 USA) aided by a copper bead (BB-caliber airgun shot) and tested for the virus by CPE assay. Briefly, samples were centrifuged (Eppendorf centrifuge 5417R) at 4°C at a speed of 12,000rpm for 10mins to clarify the homogenate. Vero cells that had been freshly grown in a 24-well cell culture plate were inoculated with 50µl of the individual mosquito sample, one sample per well. Plates were incubated for 1hr with agitation to allow for virus adsorption. The cells were then maintained by adding 1ml of maintenance media into each well. Plates were incubated for up to 12 days and the wells were observed daily for CPE. The supernatant of all wells showing CPE were harvested and frozen for retesting by plaque assay. In addition, approximately 25% of the negative samples were randomly selected for a blind passage by CPE assay.

For the plaque assay, serial 10-fold dilutions of the frozen supernatants were prepared and inoculated on freshly grown Vero cells in a 12-well cell culture plate. Plates were incubated for 1hr to allow for virus adsorption, after which cells were maintained by adding 2ml of maintenance media (2.5% methylcellulose mixed with 2X MEM) per well. After 9 days of incubation at 37°C (5% CO_2), the media was gently poured off from each well and the cells were fixed with 10% formalin for 2 hrs. The cells were stained with 0.5% crystal violet overnight and the plaques were observed using a light box. Similarly, serial 10-fold dilutions

of the pre- and post-feeding blood/virus mixtures were prepared and the virus was quantified by plaque assay as described above.

All the legs of mosquitoes with positive bodies and the saliva samples of mosquitoes with positive legs were tested by CPE assay followed by plaque assay as described above.

4.2.9 Vectorial capacity estimation

Aedes aegypti is the major vector for DENV in Kenya [12]. To quantify the potential for DENV transmission risk to humans, we estimated its vectorial capacity. The vectorial capacity is indicative of the basic reproductive rate of a vector-borne pathogen and is estimated using the equation:

$$\mathbf{VC} = \frac{\mathbf{ma^2p^Nb}}{\mathbf{-ln\,(p)}} \qquad [20]$$

where m = the vector density in relation to the host, a = is the human biting rate, p = the probability of daily survival, N = the extrinsic incubation period (EIP), b = the vector competence.

Our vectorial capacity estimates were limited to the long rainy season because this period corresponds with highest adult *Ae. aegypti* mosquito abundance, highest risk of DENV transmission and the highest number of dengue epidemics in the coastal part of Kenya [12,18]. Therefore, we only considered abundance data for host-seeking female *Ae. aegypti* mosquitoes as well as blood fed mosquitoes collected during the long rainy season in all three areas (Agha et al. unpublished data).

For mosquito density (m), trap abundance data for female *Ae. aegypti* mosquitoes reported in a previous study was used (Table 4.1).

Also the biting rate (a), was estimated based on data from blood meal analysis and the estimated feeding frequencies of *Ae. aegypti* (a = host preference index x feeding frequency) [20]. The host preference index was considered to be the proportion of human blood-fed *Ae. aegypti* mosquitoes estimated from this study, while the feeding frequency was the total number of female *Ae. aegypti* mosquitoes collected per trap per day (m) (Table 4.1).

We assumed a value of 0.8 for *Ae. aegypti* survival rates (p) as previously reported in a mark-release field experiment at the Kenyan coast of Rabai-Kilifi [26]. We assumed a constant survival rate for all three areas and for all three temperatures in this study as *Ae. aegypti* mosquitoes have been shown to tolerate a wide range of temperatures [27].

N and b were derived from our vector competence experiment. N was considered to be the time in days for the virus to reach the salivary gland after virus exposure (Table S1). b the vector competence was estimated for each *Ae. aegypti* population based on the transmission rate (proportion of exposed mosquitoes with virus present in the saliva) (Table 4.2).

4.2.10 Statistical Analyses

Viral infection was ascertained by confirming the presence of the virus in the mosquito's body, while viral dissemination was ascertained by confirming the presence of the virus in both the mosquito's body and legs [23]. If the virus was present in the body but not the legs, the mosquito was considered to have a non-disseminated infection limited to the midgut. Similarly, transmission was ascertained by confirming the presence of infectious virus in the mosquito's saliva.

The overall dissemination and transmission rates at each temperature were compared for the different areas using Fisher's Exact test. The analysis was performed in R version 3.3.1 [28] at α=0.05 level of significance.

Ethical statement

Scientific and ethical approval was obtained from Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU) (Project Number SERU 2787). The animal use component was reviewed and approved (approval number KEMRI/ACUC/ 03.03.14) by the KEMRI Animal Use and Care committee (KEMRI ACUC). The KEMRI ACUC adheres to national guidelines on the care and use of animals in research and education in Kenya enforced by National Commission for Science, Technology and Innovation (NACOSTI). The Institute has a foreign assurance identification number F16-00211 (A5879-01) from the Office of Laboratory Animal Welfare (OLAW) under the Public

Health Service and commits to the International Guiding Principles for Biomedical Research Involving Animals.

4.3 Results

4.3.1 *Aedes aegypti* abundance and host blood meal preferences

The number of female *Ae. aegypti* captured in each of the three locations during the long rainy season, April-June, are shown in Table 4.1.

Table 4.1. Density of host seeking female *Aedes aegypti* mosquitoes collected in Mombasa, Kisumu and Nairobi during the long rainy season (April-June).

	Mombasa	**Kisumu**	**Nairobi**
Total No. of *Ae. aegypti* collected	2235	2577	1071
No. of females	995	1266	534
Vector Density	16.58	21.1	8.9

We examined the host blood meal sources in a total of 76 *Ae. aegypti* mosquitoes from Mombasa (n=37), Kisumu (n=20) and Nairobi (n=19), identifying 13 different hosts blood meal sources (Fig 4.1). Human feeding was observed for *Ae. aegypti* mosquitoes collected from all three areas, with human feeding of 35%, 5% and 16% in Mombasa, Kisumu and Nairobi respectively.

Also, most of the *Ae. aegypti* mosquitoes that fed on humans were collected during the long-rains (April-June) in all three areas (Table 4.2). Multiple blood feeding was observed for two *Ae. aegypti* mosquitoes collected from Mombasa. One had fed on a cow and a tortoise, while the other had fed on two different species of tortoises.

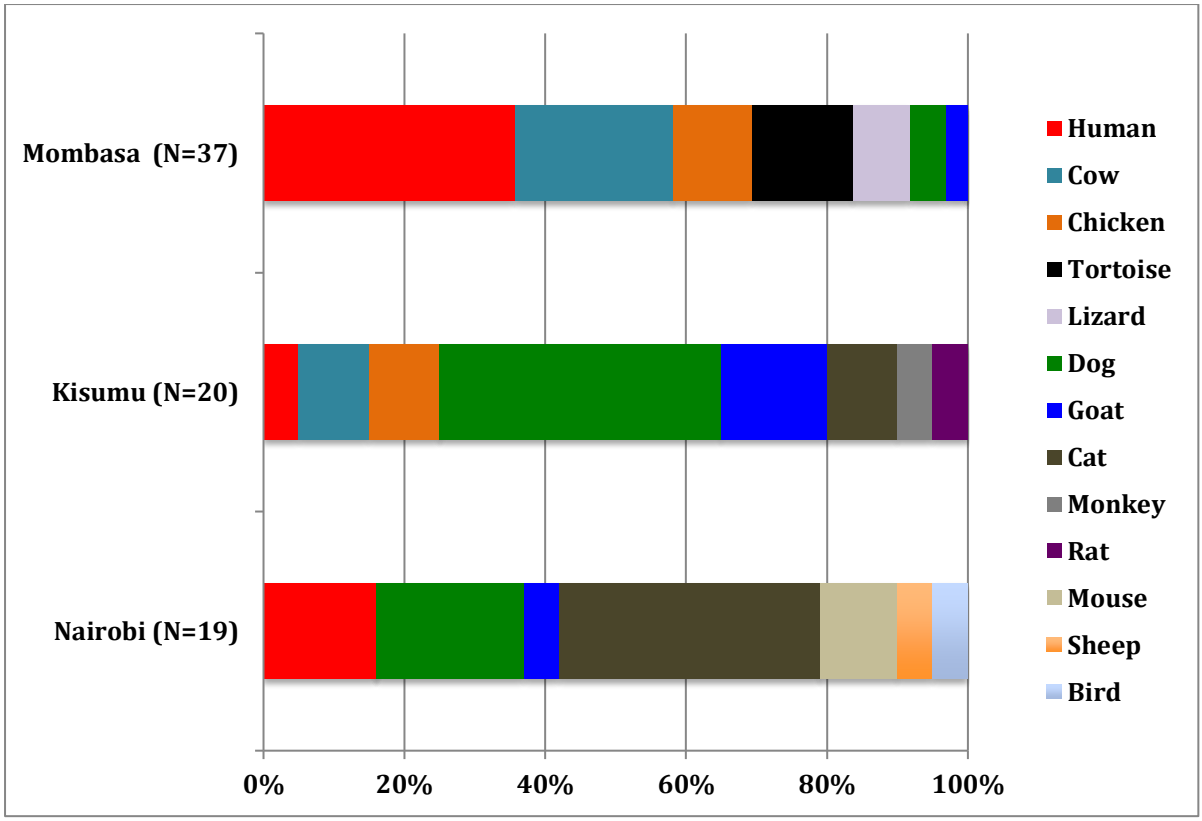

Figure 4.1. Host blood meal sources for *Aedes aegypti* mosquitoes collected from Mombasa, Kisumu and Nairobi from October 2014 to June 2016.

Table 4.2. Human blood index of *Aedes aegypti* mosquitoes collected during the long rainy season in Mombasa, Kisumu and Nairobi.

	Total collected	No. fed on human	Human blood index
Mombasa	30	9	0.30
Kisumu	14	1	0.07
Nairobi	16	3	0.19

Table 4.3. Vector competence of *Aedes aegypti* to dengue virus-2 at selected temperatures.

Origin	Infection rate[a]	Dissem rate[b]	Dessem(I) rate[c]	Trans rate[d]	Trans(I) rate[e]	Trans(D) rate[f]
			Mosquitoes held at 22°C			
Mombasa	26 (14/53)	4 (2/53)	14 (2/14)	2 (1/53)	7 (1/14)	50 (1/2)
Kisumu	42 (19/45)	0 (0/45)	0 (0/19)	0 (0/45)	0 (0/19)	n.a.
Nairobi	12 (5/45)	0 (0/42)	0 (0/5)	0 (0/42)	0 (0/5)	n.a.
			Mosquitoes held at 28°C			
Mombasa	44 (19/43)[g]	14 (6/43)	32 (6/19)	3 (1/40)	5 (1/19)	17 (1/6)
Kisumu	28 (23/83)	14 (12/83)	52 (12/23)	1 (1/83)	4 (1/23)	8 (1/12
Nairobi	25 (14/56)	13 (7/56)	50 (7/14)	0 (0/56)	0 (0/14)	0 (0/7)
			Mosquitoes held at 31°C			
Mombasa	25 (15/61)	10 (6/61)	40 (6/15)	3 (2/61)	13 (2/15)	33 (2/6)
Kisumu	44 (28/63)[h]	17 (11/63)	39 (11/28)	7 (4/61)	14 (4/28)	36 (4/11)
Nairobi	24 (14/59)[h]	19 (11/59)	79 (11/14)	5 (3/57)	21 (3/14)	27 (3/11)

n.a., not appicable
[a]Infection rate: Percent infected (No. infected/No. tested)
[b]Dissemination rate: Percent with a disseminated infection (No. disseminated/No. tested)
[c]Dissemination(I) rate: Percent of infected with a disseminated infection (No. disseminated/No. infected)
[d]Transmission rate: Percent with virus in their saliva (No. transmitting/No. tested)
[e]Transmission(I) rate: Percent of infected mosquitoes with virus in their saliva (No. transmitting/No. infected)
[f]Transmission(D) rate: Percent of disseminated mosquitoes with virus in their saliva (No. transmitting/No. disseminated)
[g] Includes three mosquitoes not tested for transmission.
[h] Includes two mosquitoes not tested for transmission.

4.3.2 *Aedes aegypti* susceptibility to dengue virus

A total of 505 mosquitoes were exposed to a DENV-2 infectious blood meal with average titers of $10^{7.1}$ before and $10^{6.9}$ after feeding for the different experiments. At each of the three

temperatures tested, there were no significant differences in infection, dissemination, or transmission rates in *Ae. aegypti* from Mombasa, Kisumu, or Nairobi (Table 4.3).

However, with the exception of two mosquitoes from Mombasa, none of the mosquitoes held at 22°C developed a disseminated infection, and only one of these (1/140 for all those tested at 22°C) transmitted virus. Although there was no significant difference in dissemination or transmission rates between mosquitoes from each of the three areas, both dissemination and transmission rates increased with an increase in holding temperature in mosquitoes from all three areas (Fig 4.2). For mosquitoes held at 22, 28, or 31°C, dissemination rates were 2/140 (1%), 25/182 (14%), and 27/183 (15%), respectively. The dissemination rates for mosquitoes held at either 28 or 31°C were significantly higher than the dissemination rate for mosquitoes held at 22°C (Fisher's exact test, $p<0.0001$). Similarly, for mosquitoes held at 22, 28, or 31°C, the transmission rates were 1/140 (0.7%), 2/179 (1.1%) and 9/179 (5.0%), respectively. Again, mosquitoes held at 31°C had a significantly higher (Fisher's exact test, $p = 0.048$) transmission rate than those held at 22°C.

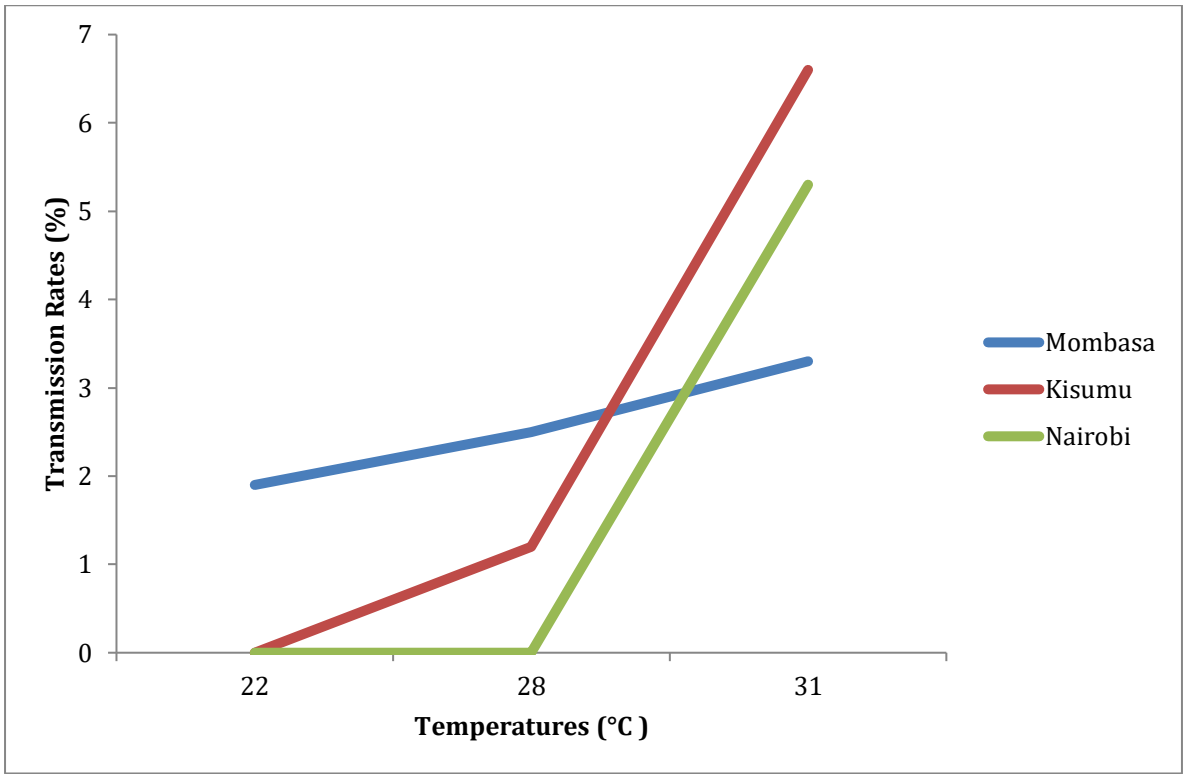

Figure 4.2. Virus transmission rates by *Aedes aegypti* mosquitoes from Mombasa, Kisumu and Nairobi, exposed to dengue virus-2 & incubated at selected temperatures.

Table 4.4. Estimated Vectorial Capacity values for Mombasa, Kisumu and Nairobi at selected temperatures.

Parameter	Definition	Calculation	Temperature	Mombasa	Kisumu	Nairobi
m	Mosquito density	# of mosquitoes/ trap/day		16.6	21.1	8.9
a	Human biting rate	(Human blood index) × # of mosquitoes/ trap/day		5.0	1.5	1.8
p	Daily survival			0.8	0.8	0.8
n	Extrinsic incubation period		22°C	14	14	14
			28°C	14	14	14
			31°C	7	7	7
b	vector competence		22°C	0.7	0.7	0.7
			28°C	1.1	1.1	1.1
			31°C	5.0	5.0	5.0
VC	Vectorial capacity		22°C	63	7	4
			28°C	100	11	7
			31°C	2158	241	148

At 22°C, no viral dissemination was observed in all three populations at 7 days post exposure, but by day 14, both viral dissemination and transmission were observed in mosquitoes from Mombasa, but not in those from Kisumu or Nairobi. At 28°C, while no virus transmission was observed in Nairobi, virus transmission was observed in Mombasa and Kisumu at the earliest of 14 days. Also at 31°C virus transmission was observed by day 7 (Table S1).

4.3.3 Vectorial capacity of *Aedes aegypti*

We calculated the vectorial capacity of *Ae. aegypti* for DENV by combining our data on human blood index (Table 4.2) and vector competence at selected temperatures (Table 4.3). Because there were no statistically significant differences between any of the three populations at any of the temperatures, we combined the vector competence data at each temperature to get a better estimate of vector competence in order to calculate the vectorial capacity for the mosquitoes from each of the cities. We used a fixed value of 0.8 for the *Ae. aegypti* survival rate [26] and estimated *Ae. aegypti* density in these cities (Table 4.1).

Based on these parameters, the overall estimated vectorial capacity value in Mombasa was about 9-fold and 14-fold higher than Kisumu and Nairobi respectively, at all three temperatures. Also, the vectorial capacity values increased with an increase in temperature, ranging from 4.0 at 22°C in Nairobi to 2158 at 31°C in Mombasa (Table 4.4). While at temperatures of 22°C and 28°C the estimated vectorial capacity values were comparably low, a 22-34 fold increase was observed when the vectorial capacity was estimated at 31°C in all three areas.

4.4 Discussion

Our study indicates the greater prevalence of dengue in Mombasa than in Kisumu is likely due to difference in *Ae. aegypti* feeding preference rather than differences in vector competence. However, lower environmental temperature appears to be the principal reason for the lack of dengue outbreaks in Nairobi. The risk of DENV transmission, as observed in this study, was generally high in Mombasa and low in Kisumu and Nairobi. These results correspond to the recurrent epidemics reported in Mombasa, while Kisumu and Nairobi remain less affected.

The *Ae. aegypti* population from Mombasa appears to be more anthropophilic compared to the population in Kisumu (Fig 4.1, Table 4.2). Increased human feeding has been associated with increased risk of DENV transmission due to increase human-vector contact [29]. This high anthropophily result thus corroborates with the dengue epidemics reported in Mombasa and the coastal area of Kenya at large [10–12]. High human feeding has also been reported in dengue endemic areas of Asia [30,31]. The low human feeding/biting rates observed for the *Ae. aegypti* population in Kisumu is indicative of a poor vector population, thus a likely reason why the area is less affected by dengue. Also, we observed that the *Ae. aegypti* population in Nairobi were about 3-times more anthropophilic than the ones from Kisumu. Thus, the absence of dengue in Nairobi may not be due to the *Ae. aegypti* population being less anthropophilic, but rather due to low temperatures and low *Ae. aegypti* abundance as was reported in Chapter 3. To fully understand the *Ae. aegypti* feeding habits, further studies should consider using larger sample sizes.

Identification of multiple blood feeding in the *Ae*. aegypti mosquito population in Mombasa is an important finding in understanding DENV epidemiology. Multiple host blood feeding increases the probability of *Ae. aegypti* acquiring or transmitting the DENV, a feeding behavior which is known to vary geographically as well as under different climatic conditions and has also been associated with differential dengue epidemic patterns [31,32]. Apart from humans, *Ae. aegypti* mosquitoes also feed on domestic animals such as dogs, cats, goats, sheep and this has been previously reported [30,32]. Although bovine has been reported to be an avoidance host by *Ae. aegypti* [30], we found a 22% preference for this animal species in Mombasa (Fig 4.1). This can be exploited in dengue control by diverting *Ae. aegypti* feeding from humans to insecticide-treated cows, as has been suggested for *Anopheles* mosquitoes in malaria control [33].

Although mosquito host blood feeding pattern in mosquitoes is said to be dependent on host availability [34], previous studies have reported that *Ae. aegypti* will preferentially feed on humans even when other domestic animals are present [29–32]. Thus, the *Ae. aegypti* blood feeding pattern observed in our study areas may not only be dependent on host availability but also on host preference which is largely genetically controlled [35]. The observed feeding patterns are therefore suggestive of

genetically distinct *Ae. aegypti* populations in these study areas, with a more anthropophilic population (*Ae. aegypti aegypti*) in Mombasa and Nairobi and a more zoophilic population (*Ae. aegypti formosus)* in Kisumu. This finding is supported by the results of the phylogenetic analyses in Chapter 5 (Table 5.3). As a limitation, data on the density of the different host types in the study areas were not available, and future studies should consider this aspect of blood feeding. Thus, our blood meal preference values should only be considered as an estimate.

Dengue virus transmission was observed in mosquitoes originating from all three areas and increased with an increase in temperature (Fig 4.2). Previous studies have demonstrated a positive relationship between increase in temperature and increasing DENV transmission [17]. The temperatures in Kisumu range between 28-30°C and are similar to those in Mombasa, 27-31°C, therefore, it is unlikely that temperature is responsible for the lower DENV transmission in Kisumu. Because the mosquito population from Kisumu was competent to transmit DENV in the laboratory, the absence of epidemics in Kisumu may therefore be linked to the low human feeding preference observed for this *Ae. aegypti* population. However, in Nairobi, where temperatures are only 22-28°C, it is likely that the lack of dengue epidemics in this part of Kenya is due to the reduced vector competence at these lower temperatures.

The estimated vectorial capacity values increased with increasing temperatures, with Mombasa recording the highest values at all three temperatures compared to Kisumu and Nairobi (Table 4.4). The high vectorial capacity observed in Mombasa was greatly influenced by the high human feeding preference of the *Ae. aegypti* mosquitoes in addition to high vector density. Although, this study observed DENV transmission in the mosquitoes from Kisumu, vectorial capacity estimates were quite low suggesting that the potential for *Ae. aegypti* to sustain a DENV transmission in Kisumu was about 9 times less likely than in Mombasa. The vectorial capacity estimate in Kisumu was greatly reduced by the low human feeding potential of the *Ae. aegypti* mosquitoes, despite their high density in this city (Table 4.1). Poor human blood index is known to greatly reduce vectorial capacity [34]. It is therefore worth noting that the DENV transmission potential in Kisumu could increase over time should the more anthropophilic *Ae. aegypti* population increase.

The vectorial capacity estimate in Nairobi was quite low, with the highest value estimated at an experimental temperature of 31°C. However, 31°C is an unusually high temperature for Nairobi. Also, considering the low *Ae. aegypti* density in Nairobi (Table 4.1), DENV transmission is unlikely in this part of the country, as was previously suggested on the basis of *Stegomyia* risk indices [18]. Therefore, with climate change and increasing potential for disease transmission [36,37], the risk of DENV transmission in Nairobi would increase over time should temperatures in this area increase [20].

Because there are currently no suitable animal models to estimate DENV transmission [38], the capillary tube method was used, although it is known to underestimate virus transmission [39]. The transmission rates observed should therefore be considered as an underestimate of the actual rates and should be discussed with caution. However, an increase in human feeding has a more significant effect on vectorial capacity compared to a corresponding increase in vector competence [20,31]. Therefore, our vectorial capacity estimate would not be significantly affected should the observed transmission rates increase. Although there is also circulation of other dengue serotypes (DENV-1 and -3) in Kenya [35,40], our study only focused on DENV-2. However, even if the Kisumu and Nairobi *Ae. aegypti* populations are more competent in transmitting DENV-1 and DENV-3, this should not increase the DENV transmission potential in these cities as the major factors affecting DENV transmission in these areas have been identified to be low human feeding rates and temperature respectively.

In conclusion, the risk of DENV transmission is high in Mombasa and low in Kisumu and Nairobi. Risk factors such as host feeding preference, environmental temperature, mosquito population density, EIP, and vector competence, when interpreted individually, are insufficient in assessing risk of transmission of DENV. An assessment of a combination of all risk factors is therefore more informative and highly recommended. Continued assessment of risk of DENV transmission and vector surveillance are highly recommended and must be performed routinely as a means of early warning.

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Supplementary Table S1. Infection, dissemination and transmission rates of *Aedes aegypti* mosquitoes from Mombasa, Kisumu and Nairobi days post exposure to dengue virus-2 at selected temperatures.

Days of incubation		Rate (No. Positive /No. Tested)								
		22°C			28°C			31°C		
		Mombasa	Kisumu	Nairobi	Mombasa	Kisumu	Nairobi	Mombasa	Kisumu	Nairobi
7	Infection	8.7 (2/23)	33.3 (4/12)	3.8 (1/26)	50 (9/18)*	27.6 (8/29)	25.0 (5/20)	29.2 (7/24)	52.2 (12/23)	22.7 (5/22)
	Dissemination	0.0 (0/23)	0.0 (0/12)	0.0 (0/26)	11.1 (2/18)	0.0 (0/29)	5.0 (1/20)	0.0 (0/24)	13.0 (3/23)	13.6 (3/22)
	Dissem/Infected	0.0 (0/2)	0.0 (0/4)	0.0 (0/1)	22.2 (2/9)★	0.0 (0/8)	20.0 (1/5)	0.0 (0/7)	25.0 (3/12)★	60.0 (3/5)★
	Transmission	0.0 (0/23)	0.0 (0/12)	0.0 (0/26)	0 (0/15)	0.0 (0/29)	0.0 (0/20)	0.0 (0/24)	4.8 (1/21)	0.0 (0/20)
	Trans/Infected	0.0 (0/2)	0.0 (0/4)	0.0 (0/1)	0 (0/6)	0.0 (0/8)	0.0 (0/5)	0.0 (0/7)	10.0 (1/10)	0.0 (0/3)
	Trans/Dissem	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	0 (0/2)	0.0 (0/0)	0.0 (0/1)	0.0 (0/0)	100 (1/1)	0.0 (0/1)
14	Infection	40.9 (9/22)	38.9 (7/18)	60.0 (3/5)	28.6 (4/14)	23.1 (6/26)	11.1 (2/18)	30.4 (7/23)	57.1 (12/21)	35.0 (7/20)
	Dissemination	9.1 (2/22)	0.0 (0/18)	0.0 (0/5)	7.1 (1/14)	11.5 (3/26)	5.6 (1/18)	21.7 (5/23)	19.0 (4/21)	30.0 (6/20)
	Dissem/Infected	22.2 (2/9)	0.0 (0/7)	0.0 (0/3)	25.0 (1/4)	50.0 (3/6)	50.0 (1/2)	71.5 (5/7)	33.3 (4/12)	85.7 (6/7)
	Transmission	4.5 (1/22)	0.0 (0/18)	0.0 (0/5)	7.1 (1/14)	0.0 (0/26)	0.0 (0/18)	8.7 (2/23)	4.8 (1/21)	15.0 (3/20)
	Trans/Infected	11.1 (1/19)	0.0 (0/7)	0.0 (0/3)	25.0 (1/4)	0.0 (0/6)	0.0 (0/2)	28.6 (2/7)	8.3 (1/12)	42.9 (3/7)
	Trans/Dissem	50.0 (1/2)	0.0 (0/0)	0.0 (0/0)	100.0 (1/1)	0.0 (0/3)	0.0 (0/1)	40.0 (2/5)	25.0 (1/4)	50.0 (3/6)
21	Infection	37.5 (3/8)	43.3 (8/15)	9.1 (1/11)	54.5 (6/11)	32 (9/28)	38.9 (7/18)	7.1 (1/14)	21.1 (4/19)	11.8 (2/17)
	Dissemination	0.0 (0/8)	0.0 (0/15)	0.0 (0/11)	27.3 (3/11)	32.1 (9/28)	27.8 (5/18)	7.1 (1/14)	21.1 (4/19)	11.8 (2/17)
	Dissem/Infected	0.0 (0/3)	0.0 (0/8)	0.0 (0/1)	50.0 (3/6)	100.0 (9/9)	71.4 (5/7)	100.1 (1/1)	100.0 (4/4)	100.0 (2/2)
	Transmission	0.0 (0/8)	0.0 (0/15)	0.0 (0/11)	0.0 (0/11)	3.6 (1/28)	0.0 (0/18)	0.0 (0/14)	10.5 (2/19)	0.0 (0/17)
	Trans/Infected	0.0 (0/3)	0.0 (0/8)	0.0 (0/1)	0.0 (0/6)	11.1 (1/9)	0.0 (0/7)	0.0 (0/1)	50.0 (2/4)	0.0 (0/2)
	Trans/Dissem	0.0 (0/0)	0.0 (0/0)	0.0 (0/0)	0.0 (0/3)	11.1(1/9)	0.0 (0/5)	0.0 (0/1)	50.0 (2/4)	0.0 (0/2)

Dissem = Dissemination, Trans = Transmission, * Includes one mosquito not tested for disseminaation and transmission, ★ Includes two mosquitoes not tested for transmission

Chapter 5

Are urban *Aedes aegypti* mosquito populations from Kenya incompetent transmitters of the East African yellow virus genotype?

Sheila B. Agha[1,2*], David P. Tchouassi[1], Armanda D.S. Bastos[2], Rosemary Sang[1,3]

[1]International Centre of Insect Physiology and Ecology (icipe), P. O. Box 30772-00100, Nairobi, Kenya

[2] Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0083, South Africa

[3]Arbovirus/Viral Hemorrhagic Fever Laboratory, Centre for Virus Research, Kenya Medical Research Institute (KEMRI), Nairobi, Kenya

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Abstract

Background

Amidst the rise in urbanization and intensifying international travel, the risk of urban yellow fever (YF) outbreaks and importation are on the rise. Whereas *Aedes bromeliae* has been described as the major YFV vector in the east African region, the role of *Ae. aegypti* remains largely unknown. In this study, the vector competence of *Ae. aegypti* populations from three major urban areas in Kenya; Kilifi (outskirts of Mombasa), Kisumu and Nairobi was assessed. As vector genetics is known to influence a species' ability to transmit a virus, further analysis was performed on susceptible and non-susceptible *Ae. aegypti* specimens from each population for possible genetic differentiation.

Methodology and principal findings

A total of 632 *Ae. aegypti* mosquitoes were orally exposed to a $10^{5.8\text{-}6.2}$ PFU/ml of an east African YFV genotype strain (BC 7914). Fully engorged mosquitoes were incubated at 28^{o}C for 21 days and assayed for viral susceptibility at 7, 14 and 21 days post-exposure. Individual mosquito body and legs were assessed by cell culture and plaque assays, to ascertain viral infection and dissemination, respectively. Overall, 22% (n=136) of the exposed *Ae. aegypti* mosquitoes were susceptible, with infection rates being comparable for the Kilifi (23%) and Kisumu (26%) populations (χ^2=0.35, DF=1, P=0.56), but significantly lower for Nairobi (11%) ($\chi^2 \geq 8.27$, DF=1, $P \leq 0.004$). However, no viral dissemination was observed in the three populations of *Ae. aegypti* within the 21 days of incubation, suggestive of either a long viral extrinsic incubation period or a possible barrier to YFV dissemination. Phylogenetic analyses revealed the presence of two sympatric *Ae. aegypti CO1* gene lineages in all three sampling sites. YFV susceptibility did not differ between the two lineages.

Conclusion

Urban *Ae. aegypti* mosquito populations sampled from three Kenyan cities, were found to be susceptible to YFV, but failed to disseminate the virus within 21 days of exposure, indicating they are refractory to virus transmission. Also, YFV susceptibility did not differ between the two *CO1 Ae. aegypti* lineages. Based on these results, the risk of *Ae. aegypti* sustaining urban YFV transmission is considered to be low in Kenya.

5.1 Introduction

Yellow fever (YF) has re-emerged in the recent decades as one of the major public health challenges, attracting renewed health and research attention. Although historically absent from Asia [1], the disease is currently endemic in 33 African and 11 South American countries [2,3]. With a high case fatality rate of 20-50%, YF epidemics have often resulted in deaths despite the availability of a safe and efficacious vaccine. The recent outbreak in Angola was of great concern as the number of cases spread in the city of Luanda and to other regions, then to neighboring Congo. Through international travels cases were subsequently imported into other countries including China and Kenya, putting a largely naïve and unvaccinated population in Asia and other countries at risk [4–6].

In the east African region, YF epidemics have been reported in Ethiopia (1960-1962), Kenya (1992-1993), Sudan (2003, 2005) and Uganda (2010) [7,8]. This region remains endemic for the yellow fever virus (YFV) evident by the recently reported cases in Uganda and Ethiopia [5]. YFV has three transmission cycles in Africa (urban, intermediate or rural and the sylvatic cycles) with each cycle involving a specific set of hosts and vectors [1,9–11]. The YF epidemics in East Africa have mainly been sylvatic, involving sylvatic and peri-domestic mosquito species such as *Aedes africanus* and *Aedes bromeliae* (a member of the *Aedes simpsoni* complex), while urban YF epidemics involving *Aedes aegypti* have been reported in West African countries [12–14]. Recent YF outbreaks in Angola (Luanda) and Democratic Republic of Congo (Kinshasa) have however, clearly demonstrated a changing disease dynamic for these regions, from sylvatic to urban transmission cycles [5].

Urban migration in Africa, characterized by movement of non-immune rural populations to already densely populated cities often leads to poor housing/living conditions that encourage mosquitoes to thrive [15], posing a ready recipe for a massive urban epidemic. The greatest threat of YF in East Africa is the potential emergence of the virus from the sylvatic cycle following proximal epizootic activities and the subsequent introduction into urban areas where dense populations of susceptible hosts and competent local vectors exist, as has been previously suggested for dengue [16,17]. Urban epidemics could be initiated by a single viremic human

arriving in an area where domestic and peri-domestic vectors are abundant and competent [8], a situation that could have played out in 2016 in Kenya if the two cases imported from Angola during the outbreak had arrived in a viremic state. The widely distributed *Ae. aegypti* and *Ae. bromeliae* mosquito species are known vectors of YFV. While *Ae. bromeliae* has been described as the major YFV vector in East Africa [7,18], the role of *Ae. aegypti* in sustaining a YFV transmission remains largely unknown.

Urban YF epidemics are highly linked to the *Ae. aegypti* vector, yet studies on the ability of *Ae. aegypti* in sustaining a YFV transmission remains lacking especially in East Africa. In the previous and only outbreak to occur in Kenya, it was found that *Ae. africanus* and *Ae. keniensis* were the key vectors and that although *Ae. bromeliae* may have played a role in the earlier stages of the outbreak, *Ae. aegypti* was not implicated (Reiter et al 1998). A previous study reported up to five times higher YFV dissemination rates among *Ae. bromeliae* compared to *Ae. aegypti* from selected rural and peri-urban sites in Kenya [19]. As data from urban localities are presently lacking, there is a need to evaluate the potential of the vector populations in the urban areas of Kenya. *Aedes aegypti* in East Africa is known to exist in two genetically distinct forms, the domestic form *Ae. aegypti aegypti*, and the forest form *Ae. aegypti formosus* [20–22]. Both forms exist in sympatry at the Kenyan coast but information on the *Ae. aegypti* population structure in the other urban areas of Kenya is lacking. It is also not clear whether existing genetic differences within *Ae. aegypti* affects susceptibility to the YFV in this region [11].

With the aforesaid knowledge gaps, it was hypothesized that 1) *Ae. aegypti* populations from urban areas of Kenya (Kilifi (outskirts of Mombasa), Kisumu and Nairobi), are incompetent in transmitting the east African YFV genotype, 2) the genetic differences existing within the *Ae. aegypti* population do not influence YFV transmission potential.

5.2 Materials and Methods

5.2.1. Mosquito collection and rearing

Populations of *Ae. aegypti* mosquitoes were collected from selected sites in three urban areas of Kenya; Kilifi (outskirts of Mombasa), Kisumu and Nairobi. In Kilifi, samples were collected from Bengo and Mbarakani in Rabai. In Kisumu samples were collected from Kanyarkwar and Kajulu, while in Nairobi, samples were collected from Githogoro. Immature *Ae. aegypti* were collected from water holding containers indoors and outdoors from houses. Samples were transported to the enhanced BSL-2 insectary at KEMRI set at 28^{o}C and 12:12 (L:D) photoperiod for rearing. The larvae were fed on tetramine fish food (Tetramine) and emerging adults for each area were put in separate cages. Adults were fed on 6% glucose solution supplied on cotton wool and morphologically identified to confirm species as *Ae. aegypti*. Female adults *Ae. aegypti* were blood fed using anesthetized laboratory mice (Kenya Medical Research Institute, Animal House) to stimulate egg development and the resulting F1 adults were used for the vector competence study.

5.2.2 Yellow fever virus amplification

The east African genotype of the YFV (BC 7914) [23] isolated from a patient during the 1992/93 YF outbreak in Kenya was used in this study. The virus was passaged once in sucking mice brain, prior to passage on Vero cells (Green African Monkey cell line, ATTC® CCL-81) grown in cell culture media consisting of Minimum Essential Medium (MEM) (Sigma-Aldrich, St. Louis, MO) as previously described (Agha et al., 2017). Viral amplification was achieved by inoculating 600 µl of the virus suspension on freshly grown Vero cells in a T-75 cell culture flask (Corning Incorporated, USA). Following a 1-hour incubation (in a 5% CO_2 incubator set at 37^{o}C) with intermittent rocking to allow for adsorption, the virus-infected cells were maintained in 20 ml maintenance media (MEM supplemented with 2% FBS). The cells were incubated and observed daily, and once 80% cytopathic effect (CPE) was observed the contents of the flask were frozen down at -80^{o}C. The contents of the flask were again gently thawed on wet ice and centrifuged (Eppendorf centrifuge 5417R) at 1500 rpm for 5 mins at 4^{o}C. The supernatant was aliquoted into 1.5 ml cryotubes and stored at -80^{o}C until required for vector competence studies.

5.2.3 Mosquito susceptibility to the yellow fever virus

An infectious blood meal was prepared by adding two parts of defibrinated sheep blood (Central Veterinary Laboratories Kabete, Kenya) to one part of YFV of titers $10^{7.5}$ PFU/ml. Using a hemotek membrane feeder (Discovery Workshops, Accrington, the United Kingdom) with the wells covered with mouse skin as membrane, 2 ml of the infectious blood was introduced into each well and the feeding system was maintained at 37ºC. Female *Ae. aegypti* mosquitoes, 5-12 days old and pre-starved for 12hrs, were exposed to feed for one hour. Before and after mosquito exposure, 100 µl of the infectious blood was added to 900 µl of homogenization media (MEM, supplemented with 15% FBS) to determine the virus titer before and after feeding. The blood/media mixtures were immediately stored at -80^{0}C until virus quantification by plaque assay. To get a good sample size representative of each area, the experiments were repeated three times.

5.2.4 Infection and dissemination assays with the yellow fever virus

All the unfed mosquitoes were aspirated out from the cages and the visibly blood fed mosquitoes incubated at 28ºC for up to 21 days. Mosquitoes were maintained on 6% glucose and on day 7, 14 and 21, a proportion of the mosquitoes were picked out and frozen at -80ºC until virus detection by CPE and plaque assays. Before testing for the presence of YFV, the legs of individual mosquitoes were separated from the body and the legs and body samples were stored in individual microcentrifuge tubes. To determine virus infection, the body sample was homogenized separately in 500 µl of homogenization media, while to determine virus dissemination the legs, five legs (with the sixth being preserved for molecular species identification) were homogenized in 400 µl of homogenization media.

5.2.5 CPE and Plaque assays to test for the yellow fever virus

With the aid of a copper bead (BB-caliber airgun shot) mosquito body samples were homogenized singly using a minibeadbeater (BioSpec Products Inc, Bartlesville, OK 74005 USA) and centrifuged at 12,000 rpm for 10 min at 4ºC (Eppendorf centrifuge 5417R) to clarify the solution. After centrifuging, 50µl of each sample was inoculated in a single well of a 24 well-plate of freshly grown Vero cells, and the cells overlaid with 1ml of maintenance media (per well). The cells were incubated and observed daily for up to 12 days. The supernatant of wells showing CPE were harvested and

frozen down at -80^{0}C. Leg samples for mosquitoes with positive bodies were also tested by CPE assay. For confirmation, 25% of the negative samples (body and legs) were retested using a CPE assay.

The supernatant of samples, which tested positive by CPE assay, were retested for YFV by plaque assay. This was done by preparing and inoculating 10-fold serial dilutions (up to 10^{-3}) of the supernatant on 95% confluent freshly grown Vero cells in a 12-well plate. The cells were overlaid with maintenance media (2.5% methylcellulose mixed with 2X MEM) and incubated at 37^{o}C for 9 days. On day 9, the maintenance media was gently poured off and the cells fixed using 10% formalin for 2 hours. The cells were stained with 0.5% crystal violet solution overnight after which, the plates were washed under running tap water and the plaques observed with the aid of a light box. Similarly, 10-fold serial dilutions of the virus/blood mixture were prepared and tested by plaque assay to quantify the virus following the same protocol above.

5.2.6 Yellow fever virus transmission assay

To more efficiently assess transmission, some of the *Ae. aegypti* mosquitoes which did not take any blood meal were inoculated with a YFV suspension to produce mosquitoes with disseminated infection [24]. The suspension was prepared by adding 100µl of YFV (containing $10^{7.5}$PFU/ml) to 900µl of homogenization media. Each mosquito was inoculated with 0.3µl of YFV suspension. After 10 days of incubation at 28^{o}C, these mosquitoes were placed in plastic cups (covered with a fine netting material and secured with rubber bands) and immobilized by placing them in a -20^{o}C freezer for 40 seconds. Virus transmission using the capillary tube method was assessed for the individual mosquitoes as previously described [25]. The saliva content was emptied into a microcentrifuge tube containing 150µl homogenization media, while the legs and body samples were homogenized in 500 µl of homogenization media. The samples were stored at -80^{o}C until tested for YFV by CPE and Plaque assays. These groups of mosquitoes were however not used to calculate the infection and dissemination rates, as inoculation will bypass the midgut infection and escape barriers.

5.2.7 Molecular phylogenetic analysis

Genomic DNA was extracted from the individual legs of YFV infected and non-infected *Ae. aegypti* mosquitoes from each of the three populations to determine the subspecies. DNA extraction was performed using DNeasy blood and tissue Kit (Qiagen, GmbH-Hilden, Germany) per the manufacturer's instructions. DNA amplification targeted the cytochrome c oxidase subunit 1 (*CO1*) barcoding region using *CO1* FOR (5'-TGTAATTGTAACAGCTCATGCA-3') and REV (5'-AATGATCATAGAAGGGCTGGAC-3') primers for *Ae. aegypti* species identification [26]. DNA amplification was performed in a 25µl mixture containing 12.5µl of Mytag HS mix, 5.5µl of water, 1µl of each of the forward and reverse primers and 5µl (1-10ng) of the genomic DNA template. The cycling conditions were 95^{o}C for 3 mins, followed by 95^{o}C for 20 seconds, 48^{o}C for 30 seconds, 72^{o}C for 40 seconds for 35 cycles, and a final extension at 72^{o}C for 7 minutes. Amplicons were run on a 1.5% agarose gel against a 1kb DNA ladder (Thermo Fisher Scientific). Amplicons of the expected size (860 bp) were individually purified using ExoSap PCR purification kit (Thermo Fisher Scientific), according to recommendations by the manufacturer. Unidirectional sequencing using the forward primer was outsourced to a commercial firm (Macrogen, Seoul, Republic of Korea) and sequences were, viewed and edited in Chromas, prior to phylogenetic analysis using MEGA v 5 software [27]. Homologous sequences in the Genbank database were identified through BlastN searches and aligned using ClustalW in MEGA, together. Reference *CO1* sequences for domestic *Ae. aegypti* (Genbank Accession No. MF194022 and No. AF390098) and *Ae. aegypti formosus* (Genbank Accession No. AY056597) were included. The best-fit model of sequence evolution identified under the BIC, was used to infer a Maximum Likelihood (ML) tree in Mega5. Nodal support for the different clades in a phylogenetic tree was assessed through 1000 bootstrap replications.

5.2.8 Data analysis

Mosquito infection was ascertained by confirming the presence of the virus in the body. Mosquitoes with a positive body but negative legs were considered to have a non-disseminated infection limited to the mid gut. If both the body and legs were positive, the mosquito was considered to have a disseminated infection [29]. For the inoculated samples, positive saliva indicated virus transmission. The infection and dissemination rates of *Ae. aegypti* from different areas were compared using the Chi-

squared test. Also, in each area the proportion of YFV infected and non-infected *Ae. aegypti* mosquitoes within each lineage was compared. All analyses were performed in R version 3.3.1 [30] at α=0.05 level of significance.

Ethical statement

Scientific and ethical approval was obtained from Kenya Medical Research Institute Scientific and Ethics Review Unit (KEMRI-SERU) (Project Number SERU 2787). The animal use component was reviewed and approved (approval number KEMRI/ACUC/ 03.03.14) by the KEMRI Animal Use and Care committee (KEMRI ACUC). The KEMRI ACUC adheres to national guidelines on the care and use of animals in research and education in Kenya enforced by National Commission for Science, Technology and Innovation (NACOSTI). The Institute has a foreign assurance identification number F16-00211 (A5879-01) from the Office of Laboratory Animal Welfare (OLAW) under the Public Health Service and commits to the International Guiding Principles for Biomedical Research Involving Animals.

5.3 Results

***Aedes aegypti* susceptibility to oral infections with the yellow fever virus**

The YFV titers of the blood meals to which mosquitoes were exposed were $10^{6.2}$ PFU/ml before feeding, and $10^{5.8}$ PFU/ml after feeding. The titers for the different experiments were constant as the same frozen virus stock was used. A total of 632 mosquitoes distributed as follows: Kilifi (n = 209), Kisumu (n = 263) and Nairobi (n = 160), were exposed. Irrespective of the area and the number of days post exposure, 22% (n = 136) of the exposed mosquitoes were infected with the YFV (Table 5.1). Although no significant difference in the overall infection rates was observed between Kilifi and Kisumu (χ^2 = 0.35, DF = 1, P = 0.56), infection rates were significantly lower when Nairobi was compared to Kilifi (χ^2 = 8.27, DF = 1, P = 0.004), and to Kisumu (χ^2 = 12.77, DF = 1, P=0.0004) (Fig 5.1).

In Kilifi, a total of 78, 63 and 68 mosquitoes were tested on days 7, 14 and 21, respectively. Although the rates were higher at day 14 (30%) (Table 5.1), this did not

differ significantly from the other days post exposure (χ^2 = 4.49, DF = 2, P = 0.10). In Kisumu, 92, 87 and 84 mosquitoes were sampled on days 7, 14 and 21, respectively, with the infection rates being higher on day 21 (29%) (Table 5.1) although the difference was not significant (χ^2 = 0.87, DF = 2, P = 0.65). Also in Nairobi, 70, 56 and 34 mosquitoes were sampled on days 7, 14 and 21. The infection rate was higher at day 7, but the difference was not statistically significant (χ^2 = 1.60, DF = 2, P = 0.45) (Table 5.1).

The leg samples of the 136 mosquitoes with infected bodies, from Kilifi (n = 49), Kisumu (n = 69) and Nairobi (n = 18) all tested negative for YFV dissemination (Table 5.1).

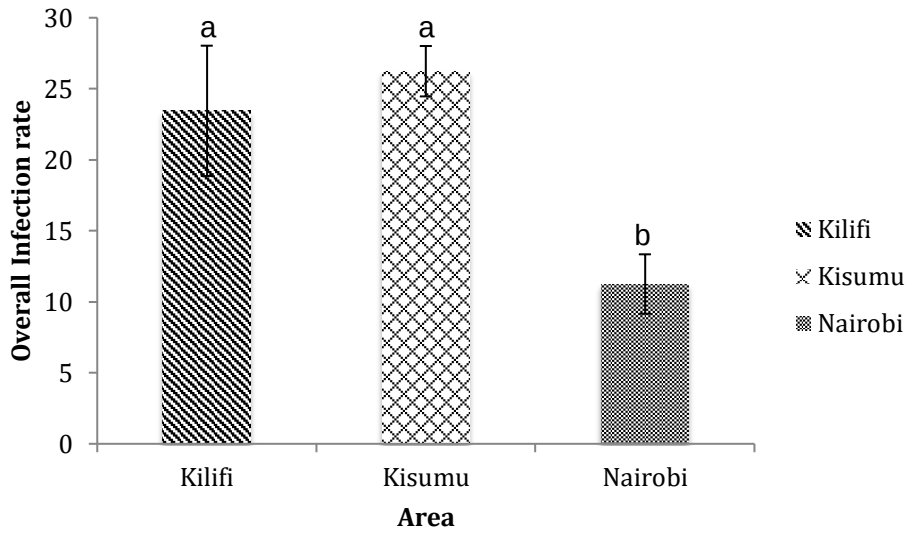

Figure 5.1. Overall oral infection rates for *Aedes aegypti* mosquitoes from Kilifi, Kisumu and Nairobi exposed to the yellow fever virus. Bars followed by same letter are not significantly different at α=0.05 level of significance.

Yellow fever virus susceptibility among intrathoracic inoculated *Aedes aegypti* mosquitoes

High mortality (>75%) was observed among YFV inoculated mosquitoes with only 11 surviving out of the total 45 inoculated. After 10 days post inoculation, the bodies of all the 11 surviving mosquitoes tested positive. However, only a few legs tested positive and all saliva samples tested negative for the YFV (Table 5.2).

Table 5.1. Susceptibility of *Aedes aegypti* mosquitoes from Kilifi, Kisumu and Nairobi to oral infection with yellow fever virus

	Infection rates (No. Infected/No. Tested) by days post exposure to YFV				**Dissemination rates (No. Disseminated/No. Tested) by days post-exposure to YFV**			
Area	7	14	21	Total	7	14	21	Total
Kilifi	26.0 (20/78)	30.0 (19/63)	15.0 (10/68)	23.0 (49/209)	0.0 (0/20)	0.0 (0/19)	0.0 (0/10)	0.0 (0/49)
Kisumu	23.0 (21/92)	28.0 (24/87)	29.0 (24/84)	26.0 (69/263)	0.0 (0/21)	0.0 (0/24)	0.0 (0/24)	0.0 (0/69)
Nairobi	14.0 (10/70)	7.0 (4/56)	12.0 (4/34)	11.0 (18/160)	0.0 (0/10)	0.0 (0/4)	0.0 (0/34)	0.0 (0/18)
Total	21.0 (51/240)	23.0 (47/206)	20.0 (38/186)	22.0 (136/632)	0.0 (0/51)	0.0 (0/47)	0.0 (0/38)	0.0 (0/136)

Table 5.2. Susceptibility of *Aedes aegypti* mosquitoes inoculated intrathoracically with yellow fever virus.

	Percentage (No. positive/No. tested)		
Area	**Body**	**Legs**	**Saliva**
Nairobi	100.0 (7/7)	29.0 (2/7)	0.0 (0/7)
Kisumu	100.0 (2/2)	0.0 (0/2)	0.0 (0/3)
Kilifi	100.0 (2/3)	33.0 (1/3)	0.0 (0/3)

Genetic diversity of yellow fever virus-susceptible and non-susceptible *Aedes aegypti* specimens

A selection of 94 *Ae. aegypti* mosquitoes exposed to YFV were analyzed, 31 from Kilifi (16 infected and 15 non-infected), 32 from Kisumu (16 infected and 16 non-infected) and 31 Nairobi (15 infected and 16 non-infected). Phylogenetic analyses of the samples recovered two lineages in Kilifi (Fig 5.2), Kisumu (Fig 5.3) and Nairobi (Fig 5.4), which were well supported by the ML tree. Also, two discrete monophyletic lineages were recovered from one of the lineages in Kilifi (lineage 1) and Kisumu (lineage 2) (Fig 5.2, 5.3). Samples in lineage 1 clustered closely with domestic *Ae. aegypti* (Genbank Accession No. MF194022 and No. AF390098), while samples in lineage 2 clustered with *Ae. aegypti formosus* strain (Genbank Accession No. AY056597).

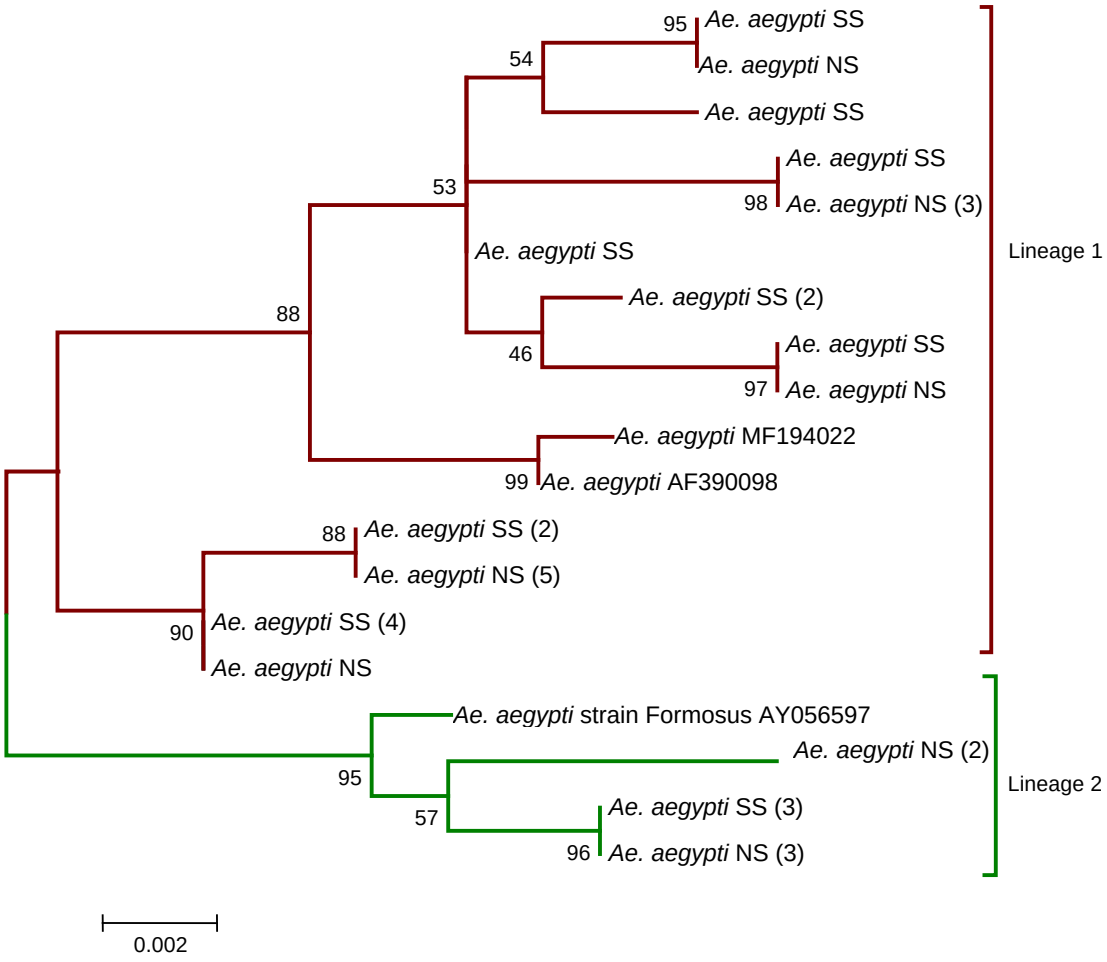

Figure 5.2. Maximum likelihood tree with a Tamura 3-parameter model, Gamma distributed (G) for *CO1* barcode region (860 bp) of yellow fever susceptible (SS) and non-susceptible (NS) *Ae. aegypti* samples from Kilifi, Kenya. The numbers above branches indicate a bootstrap value for 1000 replicates.

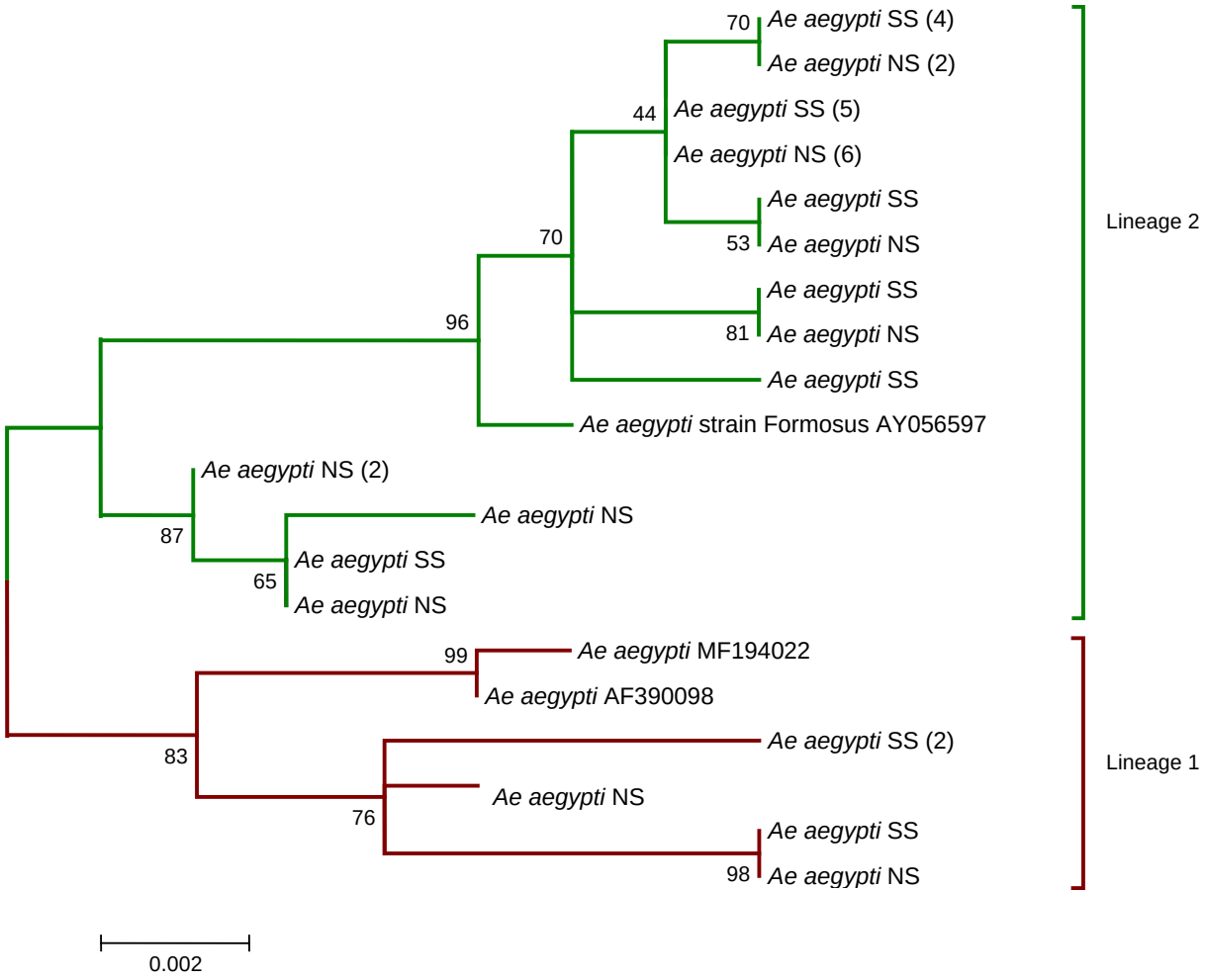

Figure 5.3. Maximum likelihood tree with a Tamura 3-parameter model, uniform rates for *CO1* barcode region (860 bp) of yellow fever susceptible (SS) and non-susceptible (NS) *Ae. aegypti* samples from Kisumu, Kenya. The numbers above branches indicate a bootstrap value for 1000 replicates.

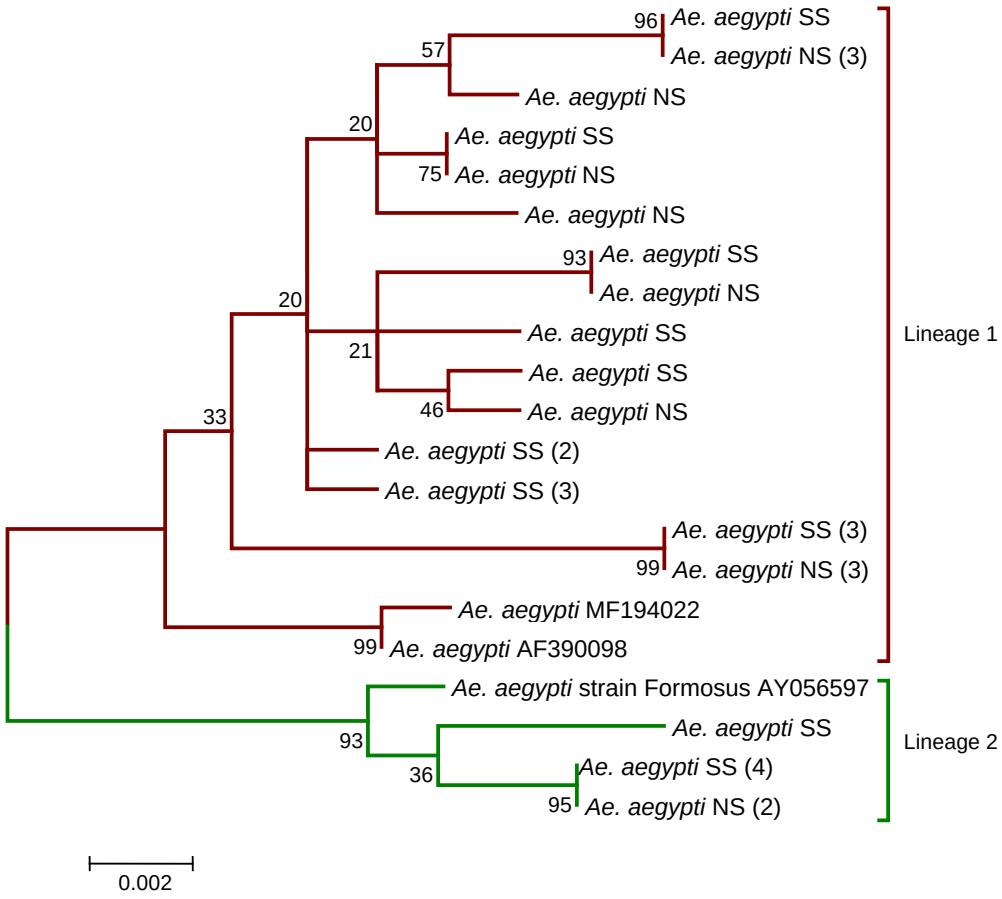

Figure 5.4. Maximum likelihood tree with a Tamura 3-parameter model, Gamma distributed (G) for *CO1* barcode region (860 bp) of yellow fever susceptible (SS) and non-susceptible (NS) *Ae. aegypti* samples from Nairobi, Kenya. The numbers above branches indicate a bootstrap value for 1000 replicates.

In Kilifi, lineage 1 comprised 71.9% of the total *Ae. aegypti* mosquitoes analysed, while lineage 2 comprised 25.8%. Similarly, in Nairobi lineage 1 made up 77.4% while lineage 2 made up 22.6% of the population analysed. An analogous pattern was observed in Kisumu where lineage 1 constituted 15.6% and lineage 2 84.4% of the *Ae. aegypti* mosquitoes analysed (Table 5.3). Viral susceptibility however did not vary by lineage, as both the YFV susceptible and non-susceptible *Ae. aegypti* mosquitoes were fairly represented across the different lineages in all three areas (Table 5.4).

Table 5.3. *Aedes aegypti* mosquito lineages in Kilifi, Kisumu and Nairobi.

	Percentage (proportion tested)	
	Lineage 1	**Lineage 2**
Kilifi	71.9 (23/32)	25.8 (8/31)
Kisumu	15.6 (5/32)	84.4 (27/32)
Nairobi	77.4 (24/31)	22.6 (7/31)

Table 5.4. Yellow fever virus susceptible and non-susceptible *Ae. aegypti* mosquitoes from Kilifi, Kisumu and Nairobi across the different lineages.

	Percentage (proportion tested)			
	Lineage 1		**Lineage 2**	
	SS	NS	SS	NS
Kilifi	40.6 (13/32)	31.3 (10/32)	37.5 (3/8)	62.5 (5/8)
Kisumu	60.0 (3/5)	40.0 (2/5)	48.1 (13/27)	51.9 (14/27)
Nairobi	41.7 (10/24	58.3 (14/24	71.4 (5/7)	28.6 (2/7)

Positive = YFV susceptible mosquitoes (SS)

Negative = YFV non-susceptible mosquitoes (NS**)**

Discussion

Although, *Ae. aegypti* from all three urban areas were susceptible to the YFV as early as 7 days, interestingly, they failed to disseminate the virus within 21 days post viral exposure. This is suggestive that urban *Ae. aegypti* populations in Kenya may not be competent East African YFV genotype vectors. Also, the molecular phylogeny revealed the sympatric occurrence of both subspecies of *Ae. aegypti* in all three urban areas. However, none of the lineages varied in their susceptibility to the YFV (Table 5.4). It is therefore highly unlikely that their sympatric existence would impact YF epidemic patterns in this region.

The overall infection rates for *Ae. aegypti* as reported in this study (22%) (Table 5.1) are comparable to the 21% reported in a previous study [19]. Infection with YFV has also been reported for *Ae. aegypti* mosquitoes from Kenya, parts of Australia and Asia, although urban epidemics of YFV have never been reported [31,32]. However, infection rates of 26% were reported for an incompetent urban population of *Ae. aegypti* mosquitoes which reportedly sustained a YF epidemic [13]. The competence of a vector is therefore not only determined by the efficiency of viral infection, but by its ability to efficiently disseminate and transmit the virus.

Although this study did not record any YFV dissemination, previous studies with *Ae. aegypti* mosquito populations from Kenya (using YFV isolated from Sudan) and South Africa (using YFV isolated from Kenya) recorded viral dissemination rates of 8% and 7.7% respectively [19,33]. Both studies had used head tissues to estimate viral dissemination as opposed to using leg tissues as was the case in this study. However, it has been reported that the tissue analysed for dissemination (head or legs) should not affect the dissemination rates [34]. More importantly, to draw conclusions about vector competence for a specific geographical locality, it is important to use a virus and a vector population that are geographically proximate as suggested by Dickson et al. [34]. This was the approach taken in this study in which a virus isolated from Kenya was used to infect mosquito populations derived from Kenya. However, it is worth mentioning that Ellis et al. [19] also reported failure of the Nairobi mosquitoes to disseminate the YFV, a finding that concurs with the results reported here. However, if the virus cannot be disseminated to all body tissues including the

legs, then viral transmission in saliva might not be possible. However, the risk of an urban YFV outbreak cannot be completely rolled out in Kenya, as arboviruses are known to persist at low rates only to later result in a widespread amplification facilitated by some change in single or multiple factors [35]. This was the case in La Réunion, where a Chikungunya epidemic emergence and was sustained by *Aedes albopictus* after its fitness was increased as a result of mutations in the chikungunya virus [36].

A previous study with the chikungunya virus also reported that mosquitoes with disseminated infection had titers at least a log higher than mosquitoes without a disseminated infection [37]. Therefore, a study quantifying the YF viral load in the body (viral infection) of *Ae. aegypti* mosquitoes from these urban areas is highly recommended. It may be that the mosquitoes are susceptible to infections with the YFV, but the body titers may be too low to allow for virus dissemination to all body tissues. Should this be the case, it implies that *Ae. aegypti* vector populations in Kenya are incompetent in transmitting YFV relative to chikungunya, which it efficiently transmits [37].

The viral titer and the state of the virus (frozen or fresh) are factors that can also influence the mosquito's susceptibility to the virus. Viral titers used in this study ($10^{5.8\text{-}6.2}$) are comparable to those reported for natural YFV infections (10^5-10^6) in patients [9]. A previous study had reported that *Ae. aegypti* mosquitoes are more susceptible to freshly grown YFV compared to frozen virus [38]. This hypothesis was tested on South African *Ae. aegypti* mosquitoes and there was no difference in viral susceptibility between mosquitoes fed on freshly grown or frozen YFV [33]. Comparable infection rates have also been reported for *Ae. aegypti* mosquitoes populations from Kenya using frozen (21%) [19] as well as freshly grown (29%) [31] YFV. Therefore, the low infection rates and failure to disseminate the YFV by *Ae. aegypti* as observed in this study is unlikely attributable to low viral titers or the use of frozen virus.

Failure to observe viral dissemination in all three populations of *Ae. aegypti* mosquitoes up to 21 days post exposure to the YFV is suggestive of a possibly long extrinsic incubation period (EIP). Because EIP is critically affected by mosquito's

survival [39], long EIP can negatively affect the ability of urban *Ae. aegypti* mosquito to transmit the YFV in Kenya, should they be unable to survive long enough to go through the EIP. Future vector competence studies should consider the EIP and test mosquitoes for viral dissemination after 21 days of virus exposure. Also, higher temperatures are known to greatly reduce the EIP of a virus hence reducing the number of days the virus will take to reach the salivary glands [40]. Therefore, performing the experiment at a higher temperature may be worth considering, since the average monthly temperature in Kilifi (27^{o}C to 31^{o}C) and Kisumu (28^{o}C to 30^{o}C) often go above 28^{o}C (the temperature used in this study).

Susceptibility of a mosquito vector to an arbovirus is influenced by both genetic and non-genetic factors [41,42]. Reporting viral infection in this study confirms that the midgut infection barrier (MIB) may not be the major barrier to YFV transmission in *Ae. aegypti* mosquitoes in Kenya. On the other hand, failure to report viral dissemination provides supporting evidence of a possible midgut escape barrier (MEB), which may be genetic or otherwise. Also, the *Ae. aegypti* mosquitoes that were inoculated had a 100% infection rate but viral dissemination was not observed in every single mosquito (Table 5.3). This was surprising as inoculation is supposed to bypass the barriers to viral infection and dissemination. This observation is strongly suggestive of a non-genetic barrier to YFV dissemination such as the mosquito's digestive and immune processes [41,42]. Further studies to identify possible genetic and non-genetic factors affecting YFV dissemination in urban *Ae. aegypti* mosquitoes from Kenya is therefore warranted.

The molecular phylogeny revealed the sympatric existence of two *Ae. aegypti* lineages in the major urban areas of Kenya (Fig. 5.2, 5.3 and 5.4). A previous study had also reported the existence of behaviorally distinct populations of *Ae. aegypti* in Kilifi [22]. This study therefore provides supporting evidence that the behaviorally distinct populations observed by Trpis and Hausermann [22], may also be genetically different. Based on a previous study, YFV susceptibility varies within the *Ae. aegypti* strains, with *Ae. aegypti aegypti* being more susceptible compared to *Ae. aegypti formosus* [31]. However, in this study, a significant increase in viral susceptibility could not be clearly linked to any *Ae. aegypti* lineage (table 5.4) suggesting that susceptibility may not be genetically determined for *Ae aegypti* mosquito populations

in Kenya, and the genetic variation within the vector may also not play a role in the epidemiology of YF in Kenya [11].

Conclusions

There currently exists two *Ae. aegypti* mosquito lineages in the three major cities of Kenya assessed in this study. All two lineages were susceptible to YFV infection but none was able to disseminate the virus within 21 days post exposure. The urban *Ae. aegypti* populations in Kenya is incompetent in transmitting the East African YFV geneotype, and the genetic variability existing within this mosquito species does not influence the epidemiology of yellow fever. Vector competence remains an important component of risk assessment and continuous vector surveillance and control actions are highly recommended and should be performed routinely.

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Chapter 6

General Conclusions and Recommendations

In the absence of a licensed vaccine against dengue, targeting the main vector, *Ae. aegypti*, has been the means to control the disease. Control programs are mainly geared towards source reduction and elimination of breeding sites for immature mosquitoes, as well as fumigation and residual spraying targeting resting adult populations. These efforts have impacted on vector populations with varying degrees of success in the Americas (Gubler, 2004). However, despite recurrent outbreaks of dengue at the Kenyan coast, large-scale vector control initiatives/programs are scarce. And even if present, cost-effective means to implementing such measures rely on a good understanding of the biology of potential vectors in these areas, which so far remains poorly characterised in Kenya. Entomological risk assessment studies can therefore guide vector control efforts in endemic areas, by providing useful information as to where control should be focused.

The purpose of this study was to assess the risk of DENV/YFV transmission in relation to urbanization focusing on three of the largest cities in Kenya; Mombasa, Kisumu and Nairobi. The study identified the major vectors inhabiting these urban areas, including their abundance and diversity. Also, for surveillance and control purposes, aspects of the vector ecology (immature breeding sites and resting preference), especially for *Ae. aegypti*, the most abundant species, were characterized. The host feeding preference for *Ae. aegypti* was characterized by analyzing sequences of the ribosomal 12S gene. The ability of *Ae. aegypti* to transmit the DENV/YFV at selected temperatures of 22°C, 28°C and 31°C, representing the minimum/maximum average monthly temperatures in these cities was also investigated. In addition, the genetic composition of YFV susceptible and non-susceptible *Ae. aegypti* mosquitoes in these cities was characterized by sequencing the mitochondrial cytochrome oxidase subunit 1 (*CO1*) gene, in order to gain a deeper insight as to whether *Ae. aegypti* population genetics could shape the epidemiology of yellow fever. Sampling for this study was done in the cities of Mombasa (Kilifi, dengue endemic) and in Kisumu and Nairobi (with no reported dengue thus far). All three areas have no record of yellow

fever. To achieve these goals, we formulated research questions (listed in chapter 1), which are addressed in detail in the four research chapters (chapter 2-5). Combining both field surveillance and laboratory experiments, data generated from this study were analyzed and results used to address the different research questions.

In chapter 2, the risk of DENV/YFV transmission in these three cities based on *Stegomyia* indices was assessed. Results from immature container surveys performed in and around houses revealed that *Ae. aegypti* and *Ae. bromeliae* were the major *Stegomyia* species present in all three cities. This justified estimation of *Stegomyia* indices for the two species considering their potential roles in DENV and YFV transmission (Smithburn and Haddow, 1946; Sérié et al., 1968; Germain et al., 1980 Nasidi et al., 1989; Ellis and Barrett, 2008; Lutomiah et al., 2016). While the population of *Ae. bromeliae* was considered low, it was concluded that *Ae. aegypti* remains the only known DENV vector in Kenya with sufficient abundance in the major cities to sustain a DENV transmission. Also, on the basis of established vector index thresholds, estimated risk values were indicative of high risk of DENV transmission in Kilifi and Kisumu, and low-to-medium risk in Nairobi. Although low numbers of *Ae. bromeliae* was recorded, its sympatric occurrence with *Ae. aegypti* and other potential sylvatic vectors was considered sufficient to sustain a YFV transmission. However, the established vector index thresholds were indicative of low-to-medium risk levels for urban YF for Kilifi and Kisumu, and low YF risk for Nairobi. *Aedes aegypti* in all three areas were mostly found breeding in jerricans, drums (which were particularly productive in all seasons), tyres, and discarded containers. The key containers utilized by this species for oviposition were water storage containers that can be effectively targeted to reduce vector numbers and, consequently, the risk of virus transmission through community mobilization and public health education. The oviposition site preference, indoor *vs* outdoor containers, between the study areas is suggestive of different behavioral adaptations between populations of the vector, in need of further exploration for a possible genetic basis. Overall, these findings provided the baseline for further studies to understand the observed differential risk patterns especially with respect to the vectorial capacity of the different populations of *Ae. aegypti* and *Ae. bromeliae* for DENV/YFV transmission.

Studies focusing on adult mosquito populations may provide additional informative estimates of risk of transmission of dengue and yellow fever viruses (Anders and Hay, 2012). Variation in abundance and diversity, both important epidemiological risk parameters, may impact differentially on transmission risk of DENV/YFV across seasons and cities. Corollary, the seasonal abundance and diversity of host seeking DENV/YFV vectors collected around homes were compared (Chapter 3). In addition, the resting preference of *Ae. aegypti* in and around homes in the three urban areas was assessed. Overall, the results confirmed the presence and abundance of *Ae. aegypti* near human habitations and dwellings especially in Kilifi/Kisumu, which is suggestive of increased DENV transmission risk due to higher prospects of human vector contact. Also, the abundance pattern suggests that the risk of DENV transmission is elevated during the long-rains (April-June), compared to the short-rains (October-December) and dry season (January-March, July-September). Despite low abundance of *Ae. bromeliae* suggestive of low YFV transmission risk, its proximity to human habitation as well as the observed diversity of potential YFV vectors should be of public health concern and monitored closely for targeted control. The largely outdoor resting behavior for *Ae. aegypti* provides insights for targeted adult vector control especially during emergency outbreak situations. Continuous vector surveillance should however be routinely performed for early detection of changing vector dynamics that may precipitate an outbreak of dengue/yellow fever.

Despite the presence and abundance of potential DENV vectors, if these vectors do not feed on humans and/or are not competent in transmitting the DENV, then occurrence of a dengue epidemic in the human population will be unlikely. This formed the basis of chapter 4, where the host feeding pattern as well as the ability to transmit DENV serotype-2 at selected temperatures of 22°C, 28°C and 31°C, by *Ae. aegypti* mosquitoes from all three cities was investigated. No difference was observed among the vector populations from the three cities in transmitting the DENV, although enhanced transmission at higher temperatures was evident. Interestingly, blood meal analyses revealed that field-collected *Ae. aegypti* from coastal Kenya (Kilifi, outskirts of Mombasa) exhibited higher human feeding than those from Kisumu but not Nairobi. Estimated vectorial capacity or potential of DENV

transmission to humans was about 9-fold higher in Kilifi than Kisumu, and 14-fold higher in Kilifi than Nairobi, at all three temperatures. Despite comparable transmission rates observed for *Ae. aegypti* across the cities, the higher human feeding substantially elevated the potential for DENV transmission in Kilifi as compared to Kisumu. Here it was concluded that recurrent outbreaks of dengue in Kilifi (Mombasa and coastal Kenya at large) and not in Kisumu and Nairobi, in spite of heavy population movements between these cities, is largely influenced by vector feeding behaviour for Kisumu and temperature for Nairobi. It is therefore worth noting that the DENV transmission potential in Kisumu could increase over time should the anthropophilic *Ae. aegypti* population increase. Also, with climate change and increasing potential for disease transmission (Jetten and Focks, 1997; Morin et al., 2013), the risk of DENV transmission in Nairobi will increase over time should temperatures in this area increase (Kramer and Ebel, 2003). This study provided evidence that risk factors such as host feeding pattern, environmental temperature, mosquito population density, extrinsic incubation period, and vector competence, when interpreted individually, are insufficient in assessing risk of transmission of DENV. An assessment of a combination of all risk factors is therefore more informative and highly recommended.

So far *Ae. aegypti* was the major vector for urban YFV recorded in these three cities, yet its ability to sustain YFV transmission is unknown. Therefore, in chapter 5, the vector competence of *Ae. aegypti* in the transmission of YFV was assessed. In addition, a mitochondrial marker (*CO*1) was used to characterize the genetic structure of *Ae. aegypti* specimens that were susceptible and non-susceptible to the YFV in all three cities. *Aedes aegypti* populations from all three cities were found to be susceptible to the YFV, but failed to disseminate the virus within 21 days of exposure, indicating they are refractory in transmitting the East African YFV genotype. This finding paves the way for further studies to investigate the different intrinsic and extrinsic factors that may be associated with *Ae. aegypti* refractoriness to the YFV. Also, the molecular phylogeny revealed the sympatric occurrence of two *CO1 Ae. aegypti* lineages in all three urban areas, although YFV susceptibility did not differ between the lineages. Therefore, it was highly unlikely that the sympatric existence of the different *Ae. aegypti* subspecies would impact urban YF epidemic patterns in Kenya. Based on these results, the risk of *Ae. aegypti* sustaining an urban YFV

transmission is considered to be low in Kenya. Although other potential vectors were identified in low numbers their potential to sustain YFV transmission need to be assessed to explain fully the risk associated with urban yellow fever emergence in Kenya.

This study represents the most detailed assessment of risk of DENV/YFV transmission in the urban areas of Kenya. With an investigation into the competence of the major/most abundant vector with a consideration of both the extrinsic and intrinsic factors affecting virus transmission, this study provided an entomological basis to explain the differential dengue epidemic pattern observed in the major urban areas of Kenya. Also, the absence of urban yellow fever epidemics in Kenya was partly explained. Vector control remains the mainstay in the control of most arboviral diseases. Therefore, with the improved understanding of the ecology and abundance of potential DENV/YFV vectors, as well as the risk of DENV/YFV transmission in the different urban areas of Kenya, this study paves the way for *Aedes* control programs and/or source reduction initiatives in Kenya to prevent the emergence of both dengue and yellow fever, as well as providing guidance for cost effective vaccination for yellow fever.

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Appendix

Table A1. Primers used for PCR amplification of the different gene fragments.

Target	Primer	Sequence (5'-3')	Annealing temperature (°C)
12S	12S3F[a]	GGGATTAGATACCCCACTATGC	56°C
	12S5R[a]	TGCTTACCATGTTACGACTT	
CO1	*CO1* FOR[b]	TGTAATTGTAACAGCTCATGCA	48 °C
	CO1 REV[b]	AATGATCATAGAAGGGCTGGAC-	48 °C

[a]Valinsky et al, 2014; [b]Paupy et al, 2012

Table A2. Sample area, name and code of *Aedes aegypti* mosquitoes analysed for blood meal in this study.

Sampling Area	Sample name	Sample code	12S
Kilifi	BG 29	BF1	-
Kilifi	BG 29	BF2	x
Kilifi	BG 75	BF3	x
Kilifi	BG 75	BF4	x
Kilifi	BG 101	BF5	x
Kilifi	BG 101	BF6	x
Kisumu	BG 129	BF7	x
Kisumu	BG 165	BF8	x
Kisumu	BG 184	BF9	x
Kisumu	BG 185	BF10	x
Kisumu	BG 198	BF11	x
Kilifi	BG 316	BF12*	x
Kilifi	BG 316	BF12*	x
Kilifi	BG 342	BF13	x
Kilifi	BG 402	BF14	x
Kilifi	BG 402	BF15	_
Kilifi	BG 402	BF16	x
Kilifi	BG 402	BF17	_
Kilifi	BG 415	BF18	x
Kilifi	BG 471	BF19	x
Kilifi	BG 471	BF20	x
Kilifi	BG 478	BF21	x
Kilifi	BG 486	BF22	-
Kilifi	BG 510	BF23	x
Kilifi	BG 510	BF24	x
Kilifi	BG 503	BF25	x
Kilifi	BG 514	BF26	x
Kilifi	BG 514	BF27	x
Kilifi	BG 527	BF28	x
Kilifi	BG 548	BF29	x
Kilifi	BG 553	BF30	x
Kilifi	BG 568	BF31*	x
Kilifi	BG 568	BF31*	x
Kilifi	BG 568	BF32	x
Kilifi	BG 568	BF33	x
Kilifi	BG 577	BF34	-
Kilifi	BG 580	BF35	x
Kilifi	BG 591	BF36	x
Kilifi	BG 596	BF37	x
Kilifi	BG 596	BF38	x
Nairobi	BG 1402	BF39	x
Nairobi	BG 1472	BF40	x

Sampling Area	Sample name	Sample code	12S
Nairobi	BG 1492	BF41	x
Nairobi	BG 1493	BF42	x
Nairobi	BG 1494	BF43	x
Nairobi	BG 1515	BF44	x
Nairobi	BG 1516	BF45	x
Nairobi	BG 1517	BF46	x
Nairobi	BG 1587	BF47	x
Nairobi	BG 1591	BF48	x
Nairobi	BG 1609	BF49	x
Nairobi	BG 1630	BF50	x
Nairobi	BG 1673	BF51	x
Nairobi	BG 1700	BF52	x
Nairobi	BG 1679	BF53	x
Nairobi	BG 1548	BF54	-
Nairobi	BG 1643	BF55	x
Nairobi	BG 1135	BF56	x
Nairobi	BG 1120	BF57	x
Nairobi	BG 1155	BF58	x
Kisumu	BG 1017	BF59	x
Kisumu	BG 1017	BF60	x
Kisumu	BG 1017	BF61	x
Kisumu	BG 1017	BF62	x
Kisumu	BG 1017	BF63	x
Kisumu	BG 1042	BF64	x
Kisumu	BG 1057	BF65	x
Kisumu	BG 885	BF66	x
Kisumu	BG 972	BF67	x
Kisumu	BG 975	BF68	x
Kisumu	BG 954	BF69	x
Kisumu	BG 1112	BF70	x
Kisumu	BG 1108	BF71	-
Kisumu	BG 1108	BF72	-
Kisumu	BG 1108	BF73	-
Kisumu	BG 1094	BF74	x
Kilifi	BG 1358	BF75	x
Kisumu	BG 737	BF76	x
Kilifi	BG 1334	BF77	x
Kisumu	BG 1245	BF78	x
Kilifi	P 1200	BF79	x
Kilifi	BG 273	BF80	x
Kilifi	BG 273	BF81	x
Kilifi	P 1775	BF82	x
Kilifi	BG 279	BF83	x

*Samples with multiple blood feeding; x, amplified samples/samples sequenced; - no amplification/samples without sequence.

Table A3. Yellow fever virus susceptible and non-susceptible *Aedes aegypti* mosquito samples sequenced in this study.

Sample Area	Sample name	*CO1* code
Kilifi	YF POSITIVE	YF-106
Kilifi	YF POSITIVE	YF-123
Kilifi	YF POSITIVE	YF-128
Kilifi	YF POSITIVE	YF-134
Kilifi	YF POSITIVE	YF-144
Kilifi	YF POSITIVE	YF-148
Kilifi	YF POSITIVE	YF-150
Kilifi	YF POSITIVE	YF-152
Kilifi	YF POSITIVE	YF-158
Kilifi	YF POSITIVE	YF-191
Kilifi	YF POSITIVE	YF-194
Kilifi	YF POSITIVE	YF-753
Kilifi	YF POSITIVE	YF-757
Kilifi	YF POSITIVE	YF-758
Kilifi	YF POSITIVE	YF-771
Kilifi	YF POSITIVE	YF-776
Kisumu	YF POSITIVE	YF-201
Kisumu	YF POSITIVE	YF-211
Kisumu	YF POSITIVE	YF-213
Kisumu	YF POSITIVE	YF-220
Kisumu	YF POSITIVE	YF-241
Kisumu	YF POSITIVE	YF-244
Kisumu	YF POSITIVE	YF-248
Kisumu	YF POSITIVE	YF-251
Kisumu	YF POSITIVE	YF-252
Kisumu	YF POSITIVE	YF-272
Kisumu	YF POSITIVE	YF-274
Kisumu	YF POSITIVE	YF-279
Kisumu	YF POSITIVE	YF-873
Kisumu	YF POSITIVE	YF-881
Kisumu	YF POSITIVE	YF-1233
Kisumu	YF POSITIVE	YF-1234
Nairobi	YF POSITIVE	YF-302
Nairobi	YF POSITIVE	YF-303
Nairobi	YF POSITIVE	YF-304
Nairobi	YF POSITIVE	YF-314
Nairobi	YF POSITIVE	YF-318
Nairobi	YF POSITIVE	YF-319
Nairobi	YF POSITIVE	YF-327
Nairobi	YF POSITIVE	YF-329
Nairobi	YF POSITIVE	YF-346
Nairobi	YF POSITIVE	YF-352
Nairobi	YF POSITIVE	YF-370

Sample Area	**Sample name**	***CO1* code**
Nairobi	YF POSITIVE	YF-903
Nairobi	YF POSITIVE	YF-904
Nairobi	YF POSITIVE	YF-907
Nairobi	YF POSITIVE	YF-978
Kilifi	YF NEGATIVE	YF-102
Kilifi	YF NEGATIVE	YF-103
Kilifi	YF NEGATIVE	YF-104
Kilifi	YF NEGATIVE	YF-105
Kilifi	YF NEGATIVE	YF-107
Kilifi	YF NEGATIVE	YF-108
Kilifi	YF NEGATIVE	YF-109
Kilifi	YF NEGATIVE	YF-111
Kilifi	YF NEGATIVE	YF-749
Kilifi	YF NEGATIVE	YF-750
Kilifi	YF NEGATIVE	YF-751
Kilifi	YF NEGATIVE	YF-752
Kilifi	YF NEGATIVE	YF-754
Kilifi	YF NEGATIVE	YF-755
Kilifi	YF NEGATIVE	YF-756
Kilifi	YF NEGATIVE	YF-759
Kisumu	YF NEGATIVE	YF-202
Kisumu	YF NEGATIVE	YF-204
Kisumu	YF NEGATIVE	YF-205
Kisumu	YF NEGATIVE	YF-206
Kisumu	YF NEGATIVE	YF-207
Kisumu	YF NEGATIVE	YF-208
Kisumu	YF NEGATIVE	YF-212
Kisumu	YF NEGATIVE	YF-214
Kisumu	YF NEGATIVE	YF-861
Kisumu	YF NEGATIVE	YF-862
Kisumu	YF NEGATIVE	YF-863
Kisumu	YF NEGATIVE	YF-864
Kisumu	YF NEGATIVE	YF-865
Kisumu	YF NEGATIVE	YF-866
Kisumu	YF NEGATIVE	YF-867
Kisumu	YF NEGATIVE	YF-868
Nairobi	YF NEGATIVE	YF-301
Nairobi	YF NEGATIVE	YF-305
Nairobi	YF NEGATIVE	YF-306
Nairobi	YF NEGATIVE	YF-307
Nairobi	YF NEGATIVE	YF-308
Nairobi	YF NEGATIVE	YF-309
Nairobi	YF NEGATIVE	YF-310
Nairobi	YF NEGATIVE	YF-353
Nairobi	YF NEGATIVE	YF-911
Nairobi	YF NEGATIVE	YF-912

Sample Area	**Sample name**	***CO1* code**
Nairobi	YF NEGATIVE	YF-913
Nairobi	YF NEGATIVE	YF-914
Nairobi	YF NEGATIVE	YF-915
Nairobi	YF NEGATIVE	YF-916
Nairobi	YF NEGATIVE	YF-917
Nairobi	YF NEGATIVE	YF-918