Comparative evaluation of the mosquito magnet® trap and the CDC light trap as sampling tools for outdoor mosquitoes in Kenya

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A thesis submitted in partial fulfillment for the Degree of Masters of Science in Medical Parasitology and Entomology in the Jomo Kenyatta University of Agriculture and Technology.

2010

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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To my mother Jane Wanjiku for her love, prayers and great interest in my study.To my brothers and sisters as well for their encouragement and moral support during the time of this study.

ACKNOWLEDGEMENT

I am greatly indebted to a number of people for their various roles played to enable me undertake and accomplish these studies. I am sincerely thankful to my supervisors; Dr. Charles Mbogo, Dr. Charles Mwandawiro and Prof. Japheth Magambo for their patience, valuable scientific and technical guidance and advice all throughout the study.

Special thanks go to Dr. Peter Luethy (Bio-Vision Switzerland) for his valuable advice and critical evaluation of my results.

I would like to express my gratitude to Dr. Joseph Mwangangi and Dr. Janet Midega for their critical evaluation of the thesis before submission to the university. Dr. Mwangangi's guidance and advice on statistical analysis is highly appreciated. Also greatly appreciated is Dr. Midega's time and advice given during study site preparation. I am also grateful to the scientific team in Entomology Department in Kilifi particularly Lydia Kibe, Joseph Nzovu, Festus Yaa, David Shida, Gabriel Nzai, Rosemary Wamae and Pamela Kipyab for their encouragement and support both in the field and in the laboratory. I was extremely fortunate to obtain the services of Mr. Christopher Nyundo and to whom I am greatly indebted for producing the maps of my study area. Special thanks go to the department of Entomology in KEMRI-Wellcome Trust research Laboratories in Kilifi for generously providing the laboratory space, transport, supplies and equipments, without which this study would not have happened.

I would like to acknowledge the help extended to me by Dr. Luna Kamau and Dr. John Githure for allowing me to use their laboratories and reagents for PCR analyses.

I wish to acknowledge the great interest and enthusiasm shown by my family members and friends for this study.

Finally, I would like to record the pleasure it has been working with the entire scientific community in KEMRI-Wellcome Trust in Kilifi. Their cooperation is much appreciated and admired.

These studies could not have been possible without the generous financial assistance from The Darwin Initiative to me through Dr. David Harper of Leicester University UK. I am greatly indebted for this assistance.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABC	American Biophysics Corporation
AgREPOT	Anopheles gambiae resting pot
AR	*Anopheles arabiensis*
BB	Blocking buffer
BC	Boiled casein
BP	Base pairs
BSA	Bovine serum albumin
CDC-LT	Centre for Disease Control light trap
CGMRC	Centre for Geographic Medicine Research Coast
CO_2	Carbon dioxide
CPS	Circumsporozoite proteins
dH_2O	Deionised water
DNA	Deoxyribonucleic acid
dNTPs	nucleotides
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological inoculation rate
ELISA	Enzyme linked immunosorbent assay
ESACIPAC	Eastern and Southern African centre of International Parasite Control
ETOH	Ethanol
ft	feet
g	grams
H_2O	Water
HBC	Human bait catches

HCL	Hydrochloric acid
in.	inches
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
Mab	monoclonal antibodies
$Mgcl_2$	Magnesium Chloride
Min	Minutes
MMLP	Mosquito magnet liberty plus
Nacl	Sodium chloride
Nonidet	Non-ionised detergent
PBS	phosphate buffered saline
PBS-TW	Phosphate buffered saline –Tween
PCR H_2O	PCR Water
PCR	Polymerase chain reaction
PSC	Pyrethrum spray catches
PVC	polyvinyl chloride
s.l.	Sensu lato
s.s.	Sensu stricto
SDS	Sodium dodecyl sulfate
Taq	Thermo aqueous
TBE	Tris base boric acid EDTA buffer
V	Volts
WHO	World Health Organisation
µl.	Microlitre

ABSTRACT

Sampling for surveillance of adult stages of mosquitoes is a necessary process in any disease control program. It provides important information for ecological, taxonomic as well as faunastic studies necessary in disease control attempt. There is, therefore, need for a continuous development as well as evaluation of new vector sampling systems for more accurate surveillance. Studies to evaluate the efficiency of a new trapping system known as the mosquito magnet trap® model liberty plus (MMLP) were undertaken in Jaribuni, a rural area in Kilifi district, coast province, Kenya. Its efficiency was compared with that of the standard centre for disease control light trap (CDC-LT). Two mosquito magnet traps and four CDC light traps were employed for this study. Mosquito sampling was done outdoors for a period of three months. All collected mosquitoes were morphologically identified to species level and counted. Members of the two principal malaria vectors in Kilifi namely, *Anopheles gambiae* and *An. funestus* were further identified to their sibling species by a polymerase chain reaction (PCR) assay. All mosquitoes belonging to the genus *Anopheles* were examined for infection with the human malaria parasite *Plasmodium falciparum* by an enzyme-linked immunosorbent (ELISA) assay. Bloodmeals from the fed mosquitoes were also analysed to determine the preferred hosts.

A total of 1192 mosquitoes belonging to 21 species were collected during the sampling period. Out of the total, 158 mosquitoes representing 13.3% were captured by the CDC-LT traps while 1,034 representing 86.7% were captured by the MMLP traps. The MMLP traps collected a significantly higher mean number of mosquitoes (12.93±2.51) compared to 0.99±0.18 in the CDC-LT traps (p=0.05). Each kind of

trap captured mosquitoes belonging to five genera. With a total of 18 species, the MMLP traps did not capture a significantly higher species diversity ($p < 0.05$) than the CDC-LT traps which had 15 species. Molecular identification of *An. funestus* using PCR assay yielded four sibling species namely *An. funestus s.s., An. leesoni, An. parensis* and *An. rivulorum*. PCR identifications of *An. gambiae* on the other hand yielded two species namely *An. arabiensis* and *An. merus.*

A single specimen belonging to *An. parensis* captured by MMLP trap tested positive *P. falciparum* circumsporozoites. The capture of this *P. falciparum* positive mosquito in a carbon dioxide baited trap was an indication that it was host seeking. This observation suggested that malaria transmission does occur outdoors.

In total, 13 blood-fed mosquitoes were captured. Sixty two percent (n=8) of the bloodmeals were identified, as bovine suggesting that majority of the outdoor captured mosquitoes were zoophagic. Human immunoglobulin G (IgG) was not detected in any of the mosquitoes.

This study demonstrated the superiority of the mosquito magnet traps over the CDC light traps for outdoor mosquito sampling. The large numbers of unfed host seeking mosquitoes suggested that carbon dioxide, heat and moisture baited traps can be considered as an ideal substitute for human bait catches which are increasingly raising ethical issues. These findings also suggest that mosquito control strategies targeting outdoor adult populations should be employed to reduce outdoor disease transmissions. A high potential of zoo prophylaxis is also demonstrated by this study.

1 CHAPTER ONE: GENERAL INTRODUCTION

1.1 Mosquito sampling and surveillance

Sampling and surveillance of adult stages of mosquitoes is an important and necessary process in disease control. It provides important information for ecological, taxonomic as well as faunastic studies. All this information is necessary during vector abatement operations (Blackmore and Dahl, 2002). Vector sampling and surveillance is a prerequisite to most vector population studies (Githeko *et al.*, 1994, Davis *et al.*, 1995, Mathenge *et al.*, 2005, Harbison *et al.*, 2006, Odiere *et al.*, 2007). Mosquito surveillance refers to a continuous process of sampling for the purpose of monitoring changes in mosquito population densities. This is of importance during control programs. A basic surveillance exercise tries to address a number of things which include adult mosquito population density, species composition, prevailing weather conditions as well as habitat identification.

Great efforts have been made to control mosquitoes compared to any other biting insect and a vast literature on control operations accumulated (Lounibos, 1981, Furman and Catts, 1982, Anon, 1995, Service, 2002). The success of any mosquito control operations depends upon regular and efficient surveillance of the mosquito populations. Mosquito surveillance is therefore a basic function of any organised mosquito control program as it provides data on which all control operations are based for example, determining the need, the timing and/or the location of pesticide application. In fact, without good surveillance data, it is practically impossible to conduct an efficient and effective control program (William, 2005). Surveillance is

not only applied in control exercises but also in gauging the effectiveness of control measures and hence can be used as an evaluation tool to measure the success of a control program.

Different mosquito species behave differently. For examples while some will be diurnal in their activities, others will be nocturnal, while still others will be crepuscular (Service, 2000). They will also show a high variation as pertains to endophily, exophily, endophagy as well as exophagy. Mosquitoes also show variations in their response to different stimuli. While some species will be highly attracted to light, others are attracted to other stimuli (Service, 1977). The behaviour of the mosquito species being sampled determines the choice of the sampling method to be applied (Githeko *et al.*, 1994, Davis *et al.*, 1995, Mathenge *et al.*, 2005). This choice of an appropriate sampling method is also determined by the entomological parameters that are being studied (Githeko *et al.*, 1994). For instance, mosquitoes may be collected for virus isolation (LeDuc *et al.*, 1975, Sang *et al.*, 2008), for studying resting habits (Harbison *et al.*, 2006), host preference (Beier, 1988, Mbogo *et al.*, 1993a, Mwangangi *et al.*, 2003, Muriu, *et al.*, 2008), dispersal and life span studies (Midega *et al.*, 2007), degree of mosquito-human contact, estimation of relative or absolute population sizes and quantifying the effects of interventions directed against the vectors (Odiere *et al.*, 2007). They may also be sampled for assessment of the entomologic and related risk factors (Mbogo *et al.*, 1999) as well as vector incrimination (Nelson, 1994). However, due to the great degree of variations in behaviour of mosquito species, it is unlikely that any sampling technique will be completely unbiased (Service, 1977)

1.2 Classification and global distribution of mosquitoes

Mosquitoes belong to a family of flies called Culicidae. According to Knight and Stone (1977), this family consists of 3 sub-families: Anophelinae, Culicinae and Toxorhychitinae. However, Harbach and Kitching (1998) considered the family as having two sub-families; Anophelinae and Culicinae with Toxorhychitinae having been treated as a tribe within Culicinae (Huang, 2001). Of these, the sub-family Anophelinae and Culicinae contain many species of medical and veterinary importance. Members of the sub-family Toxorhychitinae do not require blood meals and therefore are not of any medical importance. The sub-family Anophelinae contains 3 genera of mosquitoes: *Anopheles, Bironella and Chagasia*. The genus *Anopheles* has a worldwide distribution occurring not only in the tropical regions but also in the temperate regions (Warrell and Gilles, 2002). The other two are confined to Australasia, as well as in South and Central America respectively (Warrell and Gilles, 2002). The sub-family Culicinae is the largest consisting of approximately 2700 species, belonging to 34 genera (Service, 2002). However, medically important genera in this family are *Culex, Aedes, Mansonia, Haemagogus, Sabethes* and *Psorophora*. The genera *Culex, Aedes* and *Mansonia* are found in both the tropical and temperate regions of the world. *Psorophora, Haemagogus and Sabethes* are not found in Africa at all but are confined to the Americas with the latter two being restricted to Central and South America.

1.3 Major mosquito species in Kenya

The four major and common genera of mosquitoes in Africa: *Anopheles, Culex, Aedes* and *Mansonia* are well represented in Kenya. The most common members of the genus *Anopheles* include *Anopheles gambiae* (Giles), *Anopheles arabiensis* (Patton), *Anopheles merus* (Donitz), *Anopheles funestus* (Giles), *Anopheles coustani* (Laveran), *Anopheles squamosus* (Theobald) and *Anopheles pharoensis* (Theobald) (Gillett, 1972, Gillies and Coetzee, 1987).

An. gambiae s.l. has a wide distribution in Kenya. This species is known to breed in a variety of temporary habitats (Chandler and Highton, 1976). These include small, shallow and sunny water collections without vegetation, and in cavities such as hoof prints. A couple of man-made habitats are also suitable for *An. gambiae s.l.* breeding. These include ditches made by road constructions, flooded rice fields, as well as car tracks (Warren and Gilles, 2002). The open muddy pools such as those in rice irrigation schemes are a highly preferred breeding site for *An. gambiae s.s.* (Chandler and Highton, 1975). A recent report has shown that this species successfully breeds in tree holes in the western regions of Kenya (Omlin *et al.*, 2007). This species is highly anthropophilic (Mwangangi *et al.*, 2003), endophagic as well as endophilic but the feeding behaviour may change with local abundance of cattle (Service, 1970c, Warren and Gilles, 2002). Evidence of extensive animal feeding by this species has only been shown in selective samples such as those in outdoor resting sites or in compounds with abundance of cattle (Service, 1970c). Although it is highly endophagic, exophily of the same intensity as endophily has been reported in some places (Molineaux and Gramiccia, 1980)

An. arabiensis is also widely distributed in Kenya. The breeding habitats of this species are almost similar to those of *An. gambiae s.s.* (Service, 1970b; White and Rosen, 1973, Service, 1978). This species may sometimes breed in open water storage tanks as observed by Subra *et al.,* (1975) in Madagascar. Differences exist in the host preference among member species of the *An. gambiae* complex. While *An. gambiae s.s.* will be predominantly anthropophilic, several studies shown a very low Human Blood Index (HBI) in *An. arabiensis* (Ralisoa and Coluzzi, 1987, Mahande *et al.,* 2007). This is more so in areas where cattle are available in large numbers. However, where cattle are scarce, *An. arabiensis* has been reported to feed on humans (Krafsur, 1971). *An. merus* on the other hand breeds in brackish ponds along the coastal regions (Mosha and Mutero, 1982). However this species can be found at considerable distance towards the interior away from the coast, usually in areas with salt pans (Paterson *et al.,* 1964, Cross and Theron, 1983, Coetzee and Cross, 1983).

The differences in the biting behaviour of the members of the *An. gambiae* complex are not great. They have been shown to be active in the early part of the night although the biting activity is normally delayed for about 3 hours after sunset (Chandler *et al.,* 1975, 1976). The preferred resting places for members of *An. gambiae* complex are those with rough surfaces as opposed to smooth-textured ones (Hansell, 1970). The choice of either indoor or outdoor resting will so much depend on the sibling species. While *An. gambiae s.s.* will be endophilic, *An. arabiensis* shows a high degree of exophily (Service *et al.,* 1978). However, *An. gambiae s.s.*

have been shown to rest outdoors immediately before and after egg laying (Gillies, 1954)

An. funestus s.l. breeds in permanent or semi- permanent waters with herbaceous emergent or floating vegetation. They prefer waters with little organic matter and which is deep and clear (Gillett, 1972). One of the sibling species, *An. funestus s.s.* is highly endophilic such that very few will be collected from sites other than human dwellings (Garrett-Jones *et al.,* 1980). *An. coustani* is well distributed in Kenya to an altitude of close to 2000 meters above sea level. The adults are opportunists and readily bite humans in the absence of other hosts. Their breeding habitats include clear water with floating or emergent vegetation that are shaded by bush or trees. Another common species is *Anopheles pharoensis* Theobald. This species breeds in various types of habitats, which include river edges, ponds, lakeshores and irrigation ditches. Their larvae can tolerate certain levels of salinity. They feed both indoors and outdoors mainly on humans, cattle, horses and to a lesser extent on sheep, goats, camel and birds.

Some common members of the genus *Culex* include *Culex quinquefasciatus* say, *Culex poicilipes* Theobald and *Culex annulioris* Theobald. *Cx quinquefasciatus* is probably the most common. It is an exclusively nocturnal biting species and is a great biting nuisance at night especially in urban and semi-urban areas (Beier *et al.,* 1986). This species feeds mainly indoor where humans will be the preferred blood source. However cows will be the preferred hosts among outdoor biters of this species (Beier *et al.,* 1990). Its breeding habitats include drains, gulley-traps, pit

latrines, domestic water containers amongst others. This species thrives well in organically polluted waters (Service, 2000). Its common resting place is inside houses. *Cx. poicilipes* is another wide spread species which also occasionally bites humans mainly outside by day. This species breeds in ditches, pools in swamps and rivers where it is associated with clear and vegetated waters.

The genus M*ansonia* is represented in Kenya by two species which are the only representatives of this genus in Africa (Edwards, 1941). These are *Mansonia uniformis* Theobald and *Mansonia africana* Theobald. These two species bite humans and cattle outdoors (Beier *et al.,* 1990). The genus *Aedes* is well represented in Kenya and some of the most common species are *Aedes aegypti* Linnaeus, *Aedes bromeliae Theobald,* and *Aedes metallicus* Edwards. *Aedes aegypti* is cosmopolitan in distribution and is one of the most important human feeders in some regions (Gillett, 1972). Several forms of this species have been reported to occur (Gillett, 1972). In the coastal regions of Kenya, a pale form has been shown to be very common in populations close to human dwellings than those in the bushes (Van Someren *et al.,* 1955, 1958). In Australia, Hill (1921) observed a darker form breeding in the bushes away from human dwellings. The biting behaviour of this species so much depends on the form. The man feeding pale form tends to feed indoor just after dawn and late in the afternoon. The darker form tends to feed outdoors and mainly on non-humans (Gillett, 1972). Human-feeding paler form breeds mainly in man-made containers such as tins, water storage drums and pots, discarded tyres, flowerpots etc. They also breed in leaf axils. Non-human-feeding darker form usually breeds in tree-holes (Gillett, 1972).

Aedes bromeliae, (Huang, 1986) is widespread in Kenya. It feeds outdoors in full sunlight all day long. This species rests among the vegetation and breeds in tree holes, leaf axils mainly of bananas and bromeliads, bamboo stumps as well as tyres and fallen plant parts (Huang, 1986). *Aedes metallicus* is an outdoor biting mosquito and breeds in tree holes, leaf axils and coconut shells (Gillett, 1972).

1.4 Mosquitoes and mosquito borne diseases

Mosquitoes are the most important group of all arthropods of medical and veterinary importance (William, 2005) and probably with the exception of lepidopterans are the most studied group of insects (Lounibos, 1981). Currently there are well over 3500 known species worldwide (Service, 2002). They are found virtually everywhere in the world where suitable waters occur, which is essential for the development of their larval (immature) stages. Apart from the irritation and annoyance that mosquitoes inflict on humans and livestock, they also pose a great threat as disease vectors (Gillett, 1972, Meek *et al*., 1993, Gubler, 1998). The most important pests and vector species belong to the genus *Anopheles, Culex, Aedes, Psorophora, Haemagogus, and Sabethes.* The blood sucking habits of the female mosquitoes make them important disease pathogen carriers. Malaria, Yellow fever, Dengue fever, Rift valley fever, Lymphatic filariasis, Chikungunya, West Nile fever as well as Encephalitis are just but a few major representatives of a long list of diseases for which mosquitoes serve as vectors.

An. gambiae (Kamau *et al.,* 2006), *An. arabiensis* (Githeko *et al.,* 1996) and *An. funestus* (Kamau *et al.,* 2006, Temu *et al.,* 2007) are the main vectors of malaria in afro-tropical regions. Besides malaria, *An. gambiae* together with *An. funestus* also transmit arboviruses such as O'nyong Nyong to humans (Gillett, 1972), which is responsible for high morbidity in humans. The epidemics observed in 1960 in East Africa and between 1996 and 1997 in Uganda were due to these two species (Annon, 1998). Other arboviruses transmitted by *An. gambiae* include Bwamba, Middelburg, Orungo, Ilesha, Zika, Ngari and Tataguine (Gillett, 1972). These two species are also among the main vectors of bancroftian filariasis in Africa (Gillett, 1972, Wijers, 1977). *Anopheles gambiae* and *An. funestus* are the main transmitters of filariasis in the rural areas while *Culex quinquefasciatus* is the main transmitter in urban areas (Wijers and Kiilu, 1977). These diseases are especially severe in developing regions of the world where they cause early deaths and chronic debilitations (William, 2005). Of these, malaria is probably the greatest perennial threat in tropical Africa. Up to date estimates from World Health organisation (WHO) indicate that 1.7 billion people of the world's population are at risk of malaria, while 445 million are infected (WHO, 1990). With a good and reliable mosquito surveillance data, this risk and the consequent disease burden can greatly be reduced.

1.5 Problem statement

CDC light traps have been the sampling tool of choice in most of Africa for a long time. However, various research works have found that a number of mosquito species are not attracted to light and that some may actually be repelled by it (Pritchard and Pratt, 1944). Some major mosquito disease vectors such as *Aedes*

aegypti and *Aedes polynesiensis* (marks) are highly diurnal in behaviour thus making light traps totally inappropriate for their sampling (Russell, 2004). Urban sampling for malaria vectors in Kenya using the light traps has often been unrewarding despite hospital reports of disease incidences in these areas. This has in most cases been attributed to the ineffectiveness of light traps due to competing urban lighting. Use of alternative sampling tools such as those based on host attractants may provide better sampling results where light traps are not suitable.

1.6 Justification

Rapidly changing environments as well as an increase in human movement around the world have greatly contributed to a rise in new and emerging diseases most of them vector borne. This increase of both incidence and prevalence of vector borne diseases is common in tropical Africa owing to the great abundance and diversity of mosquitoes of medical importance. The recent reports of new cases of mosquito-borne infections in areas never reported before following establishment of the vectors, is a perfect example. Reports of resurgence of malaria outbreaks in the highlands west of the Rift valley of Kenya (Ernest *et al.,* 2006) and the wide spread outbreak of the viral rift valley fever in recent years in East Africa (Anyamba *et al.,* 2008) are cases in point. There has been an increase in frequency of some rare mosquito transmitted diseases in Africa and Kenya in particular such as the yellow fever and Chikungunya. This means that there is need for continuous development and evaluation of more efficient and reliable mosquito sampling and surveillance methods and tools. These improvements will not only assist in measuring, in a better way, important disease transmission parameters such as entomological inoculation

rates (EIR) but will also help in vector incrimination, determination of life cycles, host preferences, flight ranges, prediction of larval habitats and adult resting places. Equipped with this information from improved surveillance, preliminary recommendations for control programs will be made. This is because with a good surveillance data, the peak periods of mosquito abundance can easily be predicted and control measures implemented promptly. This research therefore sought to evaluate the commercially available mosquito magnet traps as a sampling tool with a view to providing a reliable surveillance data of the previously underestimated mosquito populations. It is also hoped that an efficient mosquito surveillance tool will provide an early warning system of vector-borne diseases and hence reduce the associated morbidity and mortality. The commonly used surveillance methods have certain limitations. These include lack of attraction to light by some species leading to a biased sampling as well as ethical issues for others. This therefore calls for alternative surveillance methods, which are cost effective, meets the ethical requirements and which are as close to the much efficient human landing collection as possible. For an outdoor collection, the mosquito magnet liberty plus through its carbon dioxide, heat and moisture production is hoped to provide this alternative.

1.7 Null hypotheses

1. The MMLP trap collects less species of mosquitoes than center for disease control light traps.
2. The mosquitoes collected outdoors in mosquito magnet traps have a lower sporozoite positivity rate than those collected in centre for disease control light traps.

3. Outdoor captured mosquitoes prefer humans to other hosts

1.8 Objectives of the study

1.8.1 General objective

To determine the efficiency of the mosquito magnet traps as a mosquito surveillance tool in the field compared to the CDC light traps.

1.8.2 Specific objectives

1. To determine the mosquito species diversity that will be attracted to octenol baited MMLP traps and CDC-LT traps.
2. To establish sporozoite infection rate in mosquitoes collected outdoors by the two methods.
3. To determine the host preference of mosquitoes captured outdoors

2 CHAPTER TWO: LITERATURE REVIEW

2.1 Overview

This section will seek to discuss various mosquito sampling tools and methods used globally, including those that have been used in Kenya, highlighting their target mosquito populations. Their functioning will be reviewed in details as is their pros and cons in mosquito sampling.

2.2 Mosquito sampling with light traps

Some types of insects including some species of mosquitoes appear to be highly attracted to light (Service, 1993). Light traps have been in use as insect sampling tools for many years, the earliest being paraffin or acetylene lamps. They have come in various designs and utilising different light sources. One of the simplest uses of light traps is to catch mosquitoes for laboratory studies such as virus isolations (Chamberlain *et al*., 1964). Light traps have also been used to study dispersal (Gillies and Wilkes, 1978). They may also be very useful in catching large numbers of certain mosquito species as well as in measuring relative changes in abundance of these species both in time and space (Service, 1993). Although several studies have used light traps with lots of successes (Odetoyinbo, 1969, Service, 1970, Chandler *et al.*, 1975, Joshi *et al.*, 1975), other studies have shown that they do have some limitations. One of them is that they are usually highly selective for species and also for physiological status as well as age of the females that they catch (Service, 1977). Again, some mosquito species become disoriented near light traps. Weakly flying insects such as mosquitoes often come within a short distance of the light trap but are ‘ repelled’ at the last minute. They may initially show some positive photo taxis and

then at some certain distance, varying according to the light intensity, show some negative photo taxis (Service, 1993). The distance from light source at which mosquitoes are influenced will vary according to several factors, which include the trap design, light type, mosquito species (Service, 1993) as well as their physiological status (Huffaker and Back 1943). Another limitation is that the working of light traps is affected by the moonlight (Service, 1993). There is a considerable difference in light intensity on nights with and those without moonlight. The number of mosquitoes caught in light traps has in many cases been found to be less at full than new moon (Bradley *et al.*, 1935, Bidlingmayer, 1967, Reisen *et al.*, 1983). This is due to the simple reason that the presence of the moonlight reduces the contrast between illumination from the light trap and the background resulting in an apparent reduction in the brightness of the bulb. This may in fact mean that light traps could have a reduced effectiveness when used in well-lit cities and towns. Some studies have also shown that light traps underestimate host seeking anophelines (Service, 1976, Hii *et al.*, 1986, Mbogo *et al.*, 1993b). A commonly used light trap is the CDC light trap.

2.3 Mosquito sampling using baited traps

Apart from human and animal baits, a few other attractants have been employed in traps to increase their attractiveness to mosquitoes. Some of these attractants include Carbon dioxide (CO_2), Octenol (1-octen-3-ol), and lactic acid. Rudolfs reported as early as 1922 that CO_2 was an attractant for mosquitoes and that CO_2 produced by breathing was an important factor in attracting mosquitoes to their host (Service,

1993). It has now been generally accepted that CO_2, together with other olfactory cues is an important attractant to virtually all haematophagous flies (Gillies and Wilkes, 1969, Snow, 1970). Field trials with human subjects breathing normally and others wearing breathing apparatus that removed approximately 95.5% of exhaled CO_2 showed that significantly fewer mosquitoes were attracted to the subjects wearing the apparatus (Snow, 1970). The incorporation of CO_2 into mosquito traps can be traced as long ago as 1934 when Head lee reported that delivering CO_2 gas over a New Jersey light trap for only 2 hours each evening, the numbers of mosquitoes increased by between 400 – 500% (Headlee, 1934). He later in 1941 reported that up to 19 times more mosquitoes were collected in a New Jersey light trap having dry ice in addition to light (Headlee, 1941). Huffaker and Back (1943) caught about 8 times as many mosquitoes in a New Jersey light trap baited with about 3 pounds of dry ice than in traps without. Another important observation that they made was that the relative order of abundance of the different species was altered with the addition of the dry ice. CO_2 can be added to traps mainly in two forms, gas form in cylinders or as dry ice. An obvious advantage of using the gaseous form in cylinders is that, its discharge can be regulated. This is considered important since different mosquito species are attracted at different emission rates (McIver and McElligott, 1989). More recently, traps employing the use of CO_2 have been shown to be an effective tool for sampling outdoor populations of important mosquitoes such as *An. gambiae*, *Culex quinquefasciatus* and *Aedes spp*. (Mboera *et al*., 2000, Kline, 2002, Blackmore *et al*., 2002, Sithiprasasna *et al*., 2004). Traps using CO_2 have been shown to be suitable for the collection of the day biting mosquito species such as *Aedes aegypti* and *Ae. taeniorhychus* (Wiedemann) which

are major vectors of yellow fever and lymphatic filariasis respectively (Kline, 1999, Burkett *et al*., 2001, Kline, 2002, Dennett *et al*., 2004). The surveillance of these vectors has been hindered by their relative lack of attraction to light traps which at times necessitates the undesirable use of human bait collections with the inherent risks of pathogen transmission (Russell, 2004). Since CO_2 has been shown to be a mosquito attractant, it has been incorporated in various traps mostly in form of dry ice since the mid 1970s (Service, 1993).

Certain esters of lactic acid have also been reported to increase the collection of mosquitoes in traps (Acree *et al.,* 1968). This report stimulated Stryker and young (1970) to undertake field trials in America to find out whether indeed esters of lactic acid increased catches of mosquitoes in New Jersey light traps. Large numbers of mosquitoes as well as species were caught. Octenol is another known mosquito attractant and it is indeed the most potent stimulant found in odours emitted by cattle (Service 1993). CDC light traps with some octenol placed on them have shown enhanced catches of *Culicoides furens* (Kline and Wood, 1988, Kline *et al.,* 1990) and *C. mississippiensis* (Kline and Wood, 1988) than in the same kind of traps using CO_2 only as an attractant. In another study, a combination of octenol and CO_2 increased catches of *C. furens* about a 100 times. These results led Kline and Wood (1989) to find out whether octenol would attract mosquitoes as well. They did this by comparing the efficiency of ten different trapping methods, a bulk of which incorporated CO_2 to trap *Ae. taeniorhynchus*. Other studies by Richie and Kline, (1995) and Van Essen *et al.*, (2008) have shown that the use of octenol increases collection of some mosquito species when used in conjuction with CO_2. However,

the effects of octenol just like that of CO_2 have been shown not to be uniform across species. For instance, while its addition increased the collection of *Ae. taeniorhynchus*, that of *Cx. nigripalpus* decreased even as the amount of CO_2 remained constant (Kline *et al.*, 1991).

2.4 Sampling of resting adult mosquitoes

Several mosquito sampling methods and tools have been developed in the last few decades, evaluated in many parts of the world and a lot of information generated about them (Nelson, 1994). The choice of a sampling method or tool will depend on the purpose of collection, the target species as well as the stage of the life cycle of mosquitoes to be sampled (Nelson, 1994). It is therefore important that sampling methods and tools are carefully chosen so that the most meaningful and pertinent information is obtained. Most of these tools will have some inbuilt biases and therefore it is worth using more than one sampling tool (Service, 1977, Nelson, 1994)

2.4.1 Sampling of indoor resting populations

Generally not many species of mosquitoes will be resting inside human and animal dwellings. But those few that do so are more often than not important vectors of diseases (Service, 1993). Some of the methods and tools used for sampling indoor resting mosquitoes include:

1. Pyrethrum spray collections (PSC)
2. Human bait collections (HBC)
3. Aspirators
4. Centre for Disease Control light trap (CDC-LT)

2.4.1.1 Pyrethrum spray collections (PSC)

Knockdown space spraying with pyrethrum also known as pyrethtum spray collection is now used as a standard, quick and easy method of catching mosquitoes resting indoors. Insecticides with a quick knockdown effect are sprayed in space inside human dwellings. It is usually the most efficient of the available methods for collecting mosquitoes (Service, 1993). It involves removing occupants, animals and easily removable objects such as tables and chairs, exposed food and drinking water from the house to be sprayed. The water in pots imbedded on the floor has to be covered with a lid since it cannot be removed. White sheets of strong fabric are then carefully laid over the entire floor, over beds, furniture and other objects that have not been removed. The doors and the windows are then closed and the space sprayed with pyrethrins using an appropriate pump. Knocked down mosquitoes are then collected from the sheets inside the shelter but weather permitting can be taken outside. A major limitation of this method is that its efficiency so much depends on the type of house to be sprayed (Service, 1993). For instance, its efficiency decreases in houses with many open eaves through which many mosquitoes escape before being knocked down.

2.4.1.2 Human bait catches (HBC)

This method is also known as the human landing collection. It simply involves the use of humans as mosquito baits. The mosquitoes use compounds emitted by the human (hosts) known as kairomones to locate them. These emanations from the hosts include heat, water vapour, carbon dioxide and various host odours (Service, 1993). Human bait catches can be applied in situations whereby a quick and almost instant

surveillance of mosquitoes in an area is needed (Mulhern, 1934). A common procedure is for a person to serve both as bait and the collector. Two people may also be involved: one serving as the bait, while the other serves as the collector. The person acting as the bait sits on the ground or stool and allows hungry mosquitoes to alight on his/her clothing or his/her exposed skin which are collected by an aspirator or by use of vials. Various kinds of drop-nets may be employed by some collectors to descend at intervals and enclose the mosquitoes attracted to stationary bait (Kloch and Bidlingmayer, 1953). This may be advantageous for catching the so-called 'shy' species, which hover near a bait for some time before finally landing. HBC have another advantage in that it remains useful for sampling anthropophilic mosquitoes than any other method especially when the mosquito populations are very low such as during the dry seasons or after effective control measures. No other sampling method gives such a reliable estimate of mosquito-human contact as HBC (Warrell & Gilles, 2002).

However, HBC has some limitations. In some situations, there may be such overwhelming numbers of biting mosquitoes at the bait that it is impossible to collect for more than a few minutes, and the catchers have to wear protective clothes. This makes it statistically not desirable to interpret biting behaviours from such brief and usually biased collections. The HBC will invariably be predominated by unfed females. This is commonly taken to indicate that these unfed females have been attracted for the purpose of taking a blood meal (Service, 1993). It may therefore not be a preferred sampling method when mosquitoes are required in a certain physiological state such as blood fed. HBC are also increasingly raising some ethical

objections due to the fact that, although collectors are expected to catch mosquitoes before they bite, it is almost inevitable that they are sometimes bitten. This exposes them to the risk of acquiring malaria as well as other vector borne infections (Warrell and Gilles, 2002). The mosquito landing is usually followed by a short initial period of apparent inactivity, which is in turn usually followed by a short exploratory period before the mosquito probes the skin. It is advisable to catch the mosquitoes before they have a chance of biting. This is done either by aspiration or collected using tubes. Collecting mosquitoes before they bite not only avoids unpleasant irritations caused by the bite but also eliminates the risk of acquiring mosquito borne infections. It is also advisable for collectors to take prophylactic anti-malarial drugs and be immunised against viral infections (Service, 1993). Lastly, HBC is also labour intensive and expensive.

2.4.1.3 Oral aspiration

Although most trapping and sampling procedures are oriented to catching actively flying mosquitoes, the adults probably pass more time resting in natural or man made shelters (Service, 1993). Collections of such resting populations usually provide more representative samples of the population as a whole than most other method (Service, 1993). Mosquitoes found resting on various surfaces are usually collected with an aspirator, which is sometimes referred to as a pooter. The use of a dim torch is employed to locate the indoor resting mosquitoes. Outdoor collections can also be done in natural resting sites. The mosquitoes are sucked into the aspirator using the mouth. Several mosquitoes can be sucked before being emptied into a suitable container such as a paper cup. Blood fed mosquitoes are gently sucked to avoid

smashing. The sucking end of an aspirator is kept closed when sucking has stopped to prevent mosquitoes from escaping. This, although a good method, has some limitations. For example, searches for outdoor resting mosquitoes have often proved time consuming and unrewarding (Service, 1993) while indoor resting collection is subject to consent by house owners.

2.4.1.4 CDC light traps (Sudia and Chamberlain, 1962)

The CDC light traps can be used both for indoor and outdoor sampling. The first CDC light trap was conceived by Sudia and Chamberlain in 1962.These traps have dominated mosquito surveillance activities for nearly half a century (Kline, 1999). There have been many changes to their original design leading to development of newer models. One of such modern models is Model 512, John W. Hock Company Gainesville, Florida.

2.4.1.4.1 CDC Light Trap (Model 512 John W. Hock Company Gainesville, Fl.)

CDC-LT Model 512, John W. Hock Company Gainesville, Florida is the model that was used in this study. It is also the most commonly utilised model in Kenya. It employs a more efficient miniature motor and multi-bladed fan to ensure a better flow of air through the trap. This trap also attracts mosquito with a white incandescent light and capturing them with the down draft produced by the running fan. Another modification from its original design includes the substitution of the original motorcycle battery with the dry cell and Nickel-cadmium rechargeable batteries. This model utilizes lightweight components: 6-volt batteries and a live capture net. It has a plastic or metallic cover to protect it from the rains. This cover

can be removed when being used indoors. This model can be supplemented with CO_2 (Plate 2.1). This involves the release of gaseous form from a cylinder or the use of dry ice in Styrofoam containers with holes punched in the bottom. Weighing at less than two pounds, the CDC-LT has been adopted as a standard trap in the collection of mosquitoes for arbovirus analysis (Mcnelly, 1989)

Plate 2:1: A carbon dioxide baited CDC light trap 512 John W. Hock Company Gainesville, Fl

2.4.2 Sampling of outdoor resting mosquitoes

Mosquitoes resting outdoors and especially those resting among the vegetation are usually difficult to locate. This is due to the fact that they are often scattered over a wide area (Service, 1993). To overcome this problem, artificial shelters have been constructed in an attempt to try and attract them to specific areas from where they can conveniently be collected. The idea of using artificial shelters to sample mosquitoes was first suggested by Nuttal and Shipley in 1902. Since this time, different artificial resting shelters have been developed and evaluated. Loomis and Aarons (1954) coined the term "Artificial resting unit" mainly to differentiate between an artificial shelter specifically made to sample mosquitoes from other man-made structures such as buildings and culverts which also harbour mosquitoes. Examples of these artificial resting units include:

1. Walk-in red box
2. Earth lined box shelter
3. Earth pots

2.4.2.1 Walk-red boxes (Nelson and Spadoni, 1972)

This is a simple trap consisting of 6 ft long, 4 ft wide and 6 ft tall box which is painted red both in and outside. Meyer (1985) made a slight modification to this trap by adding a curtain that can be pulled across the entrance as a person aspirates the catch. This trap has successfully been used to sample *Cx. quinquefasciatus, Cx. stigmatosoma* Dyar (Reisen *et al.,* 1990) and *An. freeborni* Aitken (McHugh, 1989)

2.4.2.2 Earth-lined box shelters (Russell and Santiago 1934)

It was first developed in the Philippines to collect *An. flavirostris* (Ludlow) and other *Anopheles* species. It consists of a 3 ft long and 2 ft square or larger in cross section wooden framework. 1-inch thick layer of earth is placed against the walls and the roof with the help of a screen. It also has a black cloth that hangs down over the entrance to within ½ ft of the ground. This trap has no bottom side. The earthen walls are kept moist by a drip that slowly releases water onto them.

In some environments, this trap can be a very useful tool for sampling exophilic mosquitoes. In a study carried out in an arid region in Tanzania, 3000 *An. gambiae* were collected from 23 box shelters over a 10 days period during peak populations. Occasionally over 100 *An. gambiae* were collected from a single shelter (Gillies, 1956). A major disadvantage of this kind of trap is that it is bulky and therefore not easy to transport to the field. Also a lot of care is needed when handling it to avoid damaging the earthen walls.

In general, all these tools for sampling outdoor resting mosquito populations are important in that, they collect species or stages of development poorly represented when collecting using other sampling methods (Service, 1977). However, they all have disadvantages in that; they are prone to destruction by termites and animals such as goats, vandalism and theft of the black cloth and being occupied by other dangerous organisms such as wasps, spiders, scorpions and snakes. These also predate on the collected mosquitoes.

2.4.2.3 Earthen pots

Clay pots, traditionally used for cooking and water storage can serve as useful resting places for mosquitoes. They have recently been modified to act as mosquito sampling tools. One such modified pot is *An. gambiae* Resting Pot (AgREPOT) (Odiere *et al.*, 2007). AgREPOT are just normal round bottom clay pots. The modification is that, a 2 cm diameter hole has been placed into the centre of the base. This is for the purpose of making the pots unable to hold water, making them useless and therefore reducing the chances of theft. They are normally set outdoors for mosquitoes to take shelter. To empty the resting mosquitoes, a cloth mesh is put and secured on the opening. The mosquitoes are then forced out by exposing the opening to the sun and agitating them by blowing into the small hole at the bottom. These traps have an advantage in that besides sampling both sexes of mosquitoes, they also sample all the three physiological states (unfed, fed and gravid). A disadvantage of AgREPOT traps is that besides mosquitoes, they also provide resting places for other animals such as lizards, spiders and scorpions, which are potential predators of mosquitoes.

2.4.2.4 Sweep – netting

A good number of mosquitoes rest among the vegetation. These mosquitoes have been collected by walking slowly through the vegetation and capturing them with a sweep net as they fly out when disturbed (McClelland, 1957, Copeland, 1986). For those species not readily flushed out by walking through vegetation, they are caught by vigorously sweep-netting through the vegetation (Van Someren, *et al.*, 1958). The most suitable net consist of a strong calico bag fastened to a metal frame to which a 2-3 ft wooden handle is attached. A number of swift forward and backward strokes

are made without interruption through the vegetation. The net is then quickly folded over to prevent escaping. It is then sprayed with chloroform and placed in a large plastic bag for about 2 minutes after which the collection is poured into a tray and sorted. Sweep netting has been used in many parts of the world to collect blood fed females for blood meal identification (McClelland *et al.,* 1963, Service, 1971, Takahashi, *et al.,* 1971). The main limitation of sweep-netting is that the mosquitoes tend to become denuded of scales and setae (Service, 1993). This in turn makes identification very difficult and impossible in some cases. Again if done on wet vegetation, the net becomes soaked with water and the collection becomes a mass of sodden leaf litter and specimens

2.5 Sampling outdoor active Mosquitoes

2.5.1 Hand-net collection

This involves the use of hand - nets for collecting active mosquitoes. Hand - nets can also be suitable for collection of active indoor mosquitoes. A small hand net 15 cm in diameter is occasionally used. This method is mainly suitable for collecting those mosquitoes that have not landed on the collectors but are hovering around. The best procedure may be to make a number of figure 8 sweeps around the head of the collector or his/her colleague especially when the numbers are overwhelmingly numerous. It is therefore a good substitute in situations whereby it is impossible to perform the conventional stationary human landing counts due to biting nuisance (Service, 1993). The collected mosquitoes can either be killed by freezing the whole net or by inserting and covering it in a big container with a killing agent.

2.5.2 The mosquito magnet traps (ABC Corporation, East Greenwich RI)

These are a relatively new class of traps that are manufactured by the American Biophysics Corporation (ABC) East Greenwich RI. There are several models of these traps and all which are commercially available. They currently include the Counter flow geometry, mosquito magnet professional, mosquito magnet defender, mosquito magnet liberty and mosquito magnet liberty plus. Instead of light, these traps use carbon dioxide, heat, moisture and optional slow-release of Octenol (1-Octen-3-ol) as an attractant. They utilize patented counter airflow technology in which a larger outer pipe directs air inside while an inner smaller pipe expels CO_2 rich plume to the outside. Both pipes are made of PVC (polyvinyl chloride). The larger pipe which is 11.4 cm in diameter opens at the bottom of the trap. Using a larger fan situated at the top of the trap, it sucks air in through vacuum creation. The smaller inner pipe is 6 cm in diameter and runs inside the bigger pipe, opening at the bottom of the trap but some distance beyond the outer larger pipe. It also has a fan as well which exhausts air containing CO_2 to the outside when running. Octenol, formulated as a solid is positioned just inside the base of the inner pipe for mixing with the CO_2 before release.

2.5.2.1 Mosquito magnet liberty plus (MMPL)

This is one of the several mosquito magnet trap models and is the model that was used in this study (Plate 2.2). Its performance as a sampling tool, not evaluated in Kenya before, was done by comparing it with that of the commonly used CDC light traps. MMLP is made of furniture-grade plastics and measures 25in. by 18in. by 33in. It utilizes no external power as it generates its own power through the conversion of heat energy from the burning gas to electrical energy. This power is

utilised to run the fans for expelling CO_2 from the trap and sucking the attracted mosquitoes into the collecting net of the trap. This gives enormous flexibility in the placement of the traps. It catalytically converts propane/butane to CO_2, heat and water vapour. The warm, moist plume of carbon dioxide that is given out attracts the mosquitoes to the MMLP trap by mimicking a human breath (Mcnelly, 1989). As the mosquitoes approach the source, they are vacuumed by one of the running fans into a collection net inside the trap. MMLP is powered by a 13 kilogram propane/butane gas cylinder, which is enough to continuously power and bait the trap for 28 days.

Plate 2:2: Mosquito magnet trap – model liberty plus ABC Corporation, East Greenwich RI

2.6 Sampling of emerging adult mosquitoes

Various kinds of emergence traps exist for sampling aquatic insects. These traps can broadly be divided into 2 categories

(a) Those that are completely submerged in the water

(b) Those that either float or are positioned just above the water surface.

Of these two, only traps that are positioned over the water surface are used to sample mosquitoes (Service, 1993). These traps are important in that they have often been used to collect mosquitoes in habitats that are otherwise difficult to access such as crab holes, pit latrines and deep wells (Evans, 1962, De Meillon *et al.,* 1967, Curtis and Hawkins, 1982). They have also proven quite necessary in the studies of seasonal emergence patterns as well as adult productivity estimation. Examples of emergence traps are:

1. Bed nets

3. Emergence light traps

2.6.1 Bed nets

Normal bed nets have successfully been used to sample emerging mosquito adults (Service, 1993). To collect emerging adults, the nets are suspended over the water from supports such as tree branches or vertical poles with the help of strings. Nets with the lower part made of a fabric instead of the netting material are the most ideal. These bed nets are equipped with strings on the lower part, which are fastened to pegs to prevent lifting and blowing in the wind. The advantage of using nets is that they are already made, readily available and very easy to transport to the field.

2.6.2 Emergence light trap (Chandler and Highton, 1975)

This is a very simple trap consisting of an 22.2 cm diameter elbowed metal cylinder to which a large white terylene netting bag for mosquito collection is tied to one end and the other end is positioned about 10cm above the water surface with the help of a support. The light source, a 3.8v torch bulb is operated from a 12v car battery with the help of a resistor. It also carries a 3 bladed fan that sucks mosquitoes up when running, delivering them into the collection net. This is a simple trap, which is efficient in sampling high numbers of emerging adult mosquitoes of various species (Chandler and Highton, 1975). Its disadvantage comes in when only newly emerged mosquitoes are required as there is no guarantee that the sampled adults are all newly emerged (Service, 1993).

2.7 Mosquito taxonomy and identification

Taxonomy refers to the theory and the practice of classifying organisms (Mayr and Ashlock, 1991). Correct identification of organisms is critical since so much biological research depends upon it (Harbach and Sandlant, 1997). Medical Entomologists for instance, will depend on it for success of intervention during outbreaks of mosquito borne diseases and in the associated epidemiological and ecological studies (Black and Munstermann, 1996). The need to correctly identify mosquitoes dates back to the early last century when it was discovered that malaria and yellow fever were both transmitted by mosquitoes (Harbach and Sandlant, 1997). There are several techniques available for mosquito identification. Among them are;

(a) Morphological identification techniques

(b) Biochemical techniques

(c) Molecular techniques

2.7.1 Morphological identification of mosquitoes

This involves the use of observable morphological characters to tell apart different species of mosquitoes. It is made possible by the use of identification keys. Identification keys are normally the starting point for obtaining information about a given taxon and therefore the basis of all biological studies (Harbach and Sandlant, 1997). This is particularly so for mosquitoes due to their great importance. Although the bulk of identifications can be done relying on morphological characters, for some taxa, problems do arise. This is especially so among very closely related species commonly referred to as sibling or cryptic species. Sibling species are those that are morphologically similar but reproductively isolated (Hill and Crampton, 1994, Black and Munstermann, 1996). A group of these sibling species make what is known as a species complex. Important disease vectors such as *An. gambiae* (Davidson, 1964, Davidson *et al.*, 1967, Davidson and Hunt, 1973, Paskewitz and Collins, 1990), *An. funestus* (Gillies and Coetzee, 1987) and *Ae. aegyti* (McClelland, 1971) consist of species complexes. *An. gambiae* complex for instance consists of six formally recognized species at present. These are *An. gambiae s.s., An. arabiensis, An. quandriannulatus* Theobald, *An. merus, An. melas* Theobald and *An. bwambae* White. *An. funestus* complex consists of 9 presently recognized sibling species (Gillies and De Meillon, 1968, Gillies and Coetzee, 1987). They are *An. funestus s.s.* Gillies, *An. vaneedeni* Gillies & Coetzee, *An. leesoni* Evans, *An. parensis* Gillies, *An.*

rivulorum leeson, *An. fuscovenosus* leesoni, *An. brucei* Service, *An. aruni* Sobti and *An. confusus* Evans and Leeson There is need to correctly identify members of the complexes in the control programmes. This is because different sibling species may differ in terms of their ecologies, vectorial capacity, host preferences, geographical and seasonal distribution. (White, 1982, Warrell and Gilles, 2002).

2.7.2 Biochemical mosquito identification techniques

Several biochemical techniques have been developed and applied in an attempt to identify different species. However, biochemically based keys for identifying mosquitoes are quite rare (Black and Munstermann 1996) and have mainly been applied in only a few species in a closely related group in which the morphological keys are not of much help. A good example is the use of chromatography of the eye pigments to differentiate between members of *An. gambiae s.l.* (Micks *et al.*, 1966). Electrophoresis of proteins extracted from freshly laid eggs has identified a protein that is present in *An. gambiae s.s.* but absent in *An. arabiensis* (Ross, 1968).

Another biochemical species identification technique that has been used for a long time is Isoenzyme typing (Mahon *et al.*, 1976, Miles, 1978). This is the only biochemical technique that is routinely used in some laboratories for species identification. This technique relies on different species having different electromorphs of a number of enzymes. However, the most reliable and widely used of these techniques is the polytene banding technique (Coluzzi and sabatini, 1967, Davidson and Hunt, 1973). This technique has been used successfully to separate members of the *An. gambiae* complex. These member species have three pairs of synapsed polytene chromosomes which are seen as six arms weakly attached in the

region of the centromere. The banding of the X chromosome is sufficient enough to distinguish *An. gambiae s.s., An. arabiensis* and *An. quandriannulatus.* This method was used to successfully distinguish between *An. gambiae* and *An. arabiensis* from Kisumu area in Kenya (Highton *et al.,* 1979). Bryan *et al.,* (1987) also used it to separate *An. gambiae* and *An. melas* in The Gambia. More recently an ELISA (Enzyme linked immunosorbent assays) technique that uses immunoaffinity-purified antibodies has been developed (Ma *et al.,* 1990). In one application of this, the egg yolk proteins known as vitellogenin and vitellin of *An. gambiae s.s.* and *An. arabiensis* have been used to distinguish the two species.

2.7.3 Molecular mosquito identification techniques

The shortcomings associated with the morphological and biochemical identification techniques, such as their failure to tell apart all the various sibling species within a complex, led to development of molecular methods of identifying mosquitoes. These techniques involve the use of the genetic material (DNA), which is the 'blueprint' of each living organism. The advantages of using the DNA are many. First, it remains constant irrespective of the life stage of the mosquitoes. Again the stability and durability of the DNA presents few problems for storage and handling making it possible to use dried, frozen or even alcohol preserved specimens. Molecular techniques mainly rely upon two methods, DNA probe hybridization and Polymerase Chain Reaction (PCR) (Black and Munstermann, 1996)

A DNA probe is basically a single stranded DNA sequence to which a detectable moiety e.g. radioactivity or enzyme ligands have been tagged and which recognize specific single stranded target DNA of the species in question (Hill and Crampton, 1994). In DNA probe hybridization, the probe is mixed with the target DNA that is immobilized on a nylon or nitrocellulose filter. Successful hybridization is detected by looking for the radioactively labelled probes through X-ray examination or by using enzymes for ligand labelled probes. A species is therefore identified by the fact that hybridization of the probe occurred.

Polymerase chain reaction on the other hand is an enzymatic amplification of the DNA using a thermostable DNA polymerase and single stranded DNA primers, which are sequences flanking the targeted fragment (Paskewitz and Collins 1990, Hill & Crampton, 1994). Amplification happens through successive cycles of thermal denaturation, annealing and synthesis using a thermostable DNA polymerase and species specific primers. The results are visualized on an ethidium bromide-stained agarose gel under ultra violet illumination. The species identity is shown by the presence of a PCR product or a PCR product of a given size. Paskewitz and Collins (1990) successfully used this method to distinguish *An. gambiae* from *An. arabiensis*. Modifications of the original PCR procedures can now be found in almost all fields of science e.g. genetics (Black and Munstermann, 1996). Besides taxonomic and systematic application of PCR, it also provides other necessary epidemiological information. For instance it has been applied to identify *P. falciparum* Welch within the vectors (Tassanakajon *et al.*, 1993, Tirasophon *et al.*, 1994). In addition, PCR has been used to determine the source of the bloodmeals

(Coulson *et al.,* 1990) as well as insecticide resistance in mosquitoes (Frank *et al.,* 2000, Kamau and Vulule, 2006)

2.8 ELISA techniques in entomology

ELISA was pioneered by Van Weemen and Schuurs (1971) and Engvall and Perlmann (1971). It is a highly sensitive and specific immunoassay. This high sensitivity is provided by a label such as an isotope or a dye while the high specificity is provided by an antibody-antigen reaction (Voller and Savigny, 1981). In ELISA, either the antigen or the antibody is attached to a solid phase, usually a plastic made of polystyrene, polyvinyl or polypropylene in the form of beads, tubes or microplates. Enzymes are normally conjugated to the antibodies using cross-linking agents such as glutaraldehyde (Avrameas and Ternyck, 1971) and Sodium periodate (Wilson and Nakane, 1978). Suitable enzymes include β-galactosidase, peroxidase, alkaline phosphatase and glucose-oxidase. They are all suitable since they all have good chromogenic substrates that yield coloured products upon enzymatic degradation (Voller and Savigny, 1981). Just like the PCR techniques, ELISA techniques have found application in many scientific areas such as bloodmeal source identification (Tempelis, 1975, Washino and Tempelis, 1983) and sporozoite infectivity analysis (Burkot *et al.,* 1984, Wirtz *et al.,* 1985). These yield important entomological indices such as human blood index (HBI) (Warrell & Gilles, 2002) and sporozoite rates both which are important in the study of disease transmission and epidemiology (Beier *et al.,* 1988).

For Bloodmeal identification, two ELISA procedures are commonly used; direct and indirect ELISA (Beier *et al.,* 1988). The difference between the two is that in indirect ELISA also known as “sandwich ELISA”, host specific antiserum is incubated first. This is followed by the addition of the homologous immunoglobulins from the bloodmeal sample, which is in turn captured by anti IgG. A washing step to get rid of non-antigenic material follows after which an enzyme-conjugate of the antibody specific to the host in which the antiserum was produced is then applied. An appropriate substrate to produce a colour reaction is then added to detect specific reactions. Direct ELISA on the other hand, uses a host specific antibody-antigen conjugate to detect homologous IgG in the bloodmeal sample. ELISA techniques for bloodmeal identification have proved to be very useful for field studies (Service *et al.,* 1986). For example in Iran, Edrissian *et al.,* (1985) used direct ELISA to screen more than 5000 *Anopheles* mosquitoes for human blood meals. In another study in Kilifi in coastal Kenya, Mbogo *et al.,* (1993a), using a direct ELISA for bloodmeal identification detected positive reactions in 95.1% of all *An. gambiae s.l.* tested (n=540) and in 90.8% *An. funestus s.l.* tested (n=76)

3 CHAPTER THREE: MATERIALS AND METHODS

3.1 Description of the study area

This study was carried out in Jaribuni village, Kilifi District in coastal Kenya. It lies between latitude 03^0 37 S and longitude 039^0 44 E and is situated about 70 kilometres north of the city of Mombasa (Fig.1). The vegetation mainly consists of shrubs and bushes, which have replaced large areas of natural forest following charcoal burning. To the south of Jaribuni is the only remaining natural forest known by the locals as "Kaya Kauma" and regarded as a sacred shrine. Jaribuni has a relatively hilly terrain. This, together with the sandy soil conditions, drain the area easily soon after the rains, making river Jaribuni which traverses the area the main breeding site for mosquitoes all year round (Plate 3.2). When the rains subside, the river is very slow moving making it very suitable for mosquito breeding. Cattle hoof prints as well as sand harvesting activities leave side pockets of water that are preferred for breeding by mosquitoes. Jaribuni is malaria (Newton *et al*., 1997, Mbogo *et al.,* 2003, Mwangangi *et al.,* 2004) and lymphatic filariasis endemic area (Wijers and Kiilu, 1977, Mukoko *et al*., 2004) The area receive between 400 and 1200mm of rainfall in a year with two mosquito peak seasons, which follow the April – June long rains and October – November short rains. The mean annual minimum and maximum temperature are 33°C and 21°C respectively.

3.2 Study population

Jaribuni is predominantly inhabited by the Kauma people, a sub-ethnic group of the Mijikenda who occupy much of the Kenyan Coastal strip. The main economic activities include agriculture, sand harvesting from river Jaribuni as well as charcoal

burning from the nearby natural forests. The cash crops include cashewnuts, coconuts and mangoes while subsistence crops include maize, cassava and cowpeas. Traditional breeds of animals, which include cows, goats and chicken, are also kept. Homesteads in Jaribuni are in clusters of extended families, which are sparsely distributed along both sides of the river (Plate 3.1). Majority of the houses consist of mud walls with coconut fronds locally known as makuti as roofs (Plate 3.3).

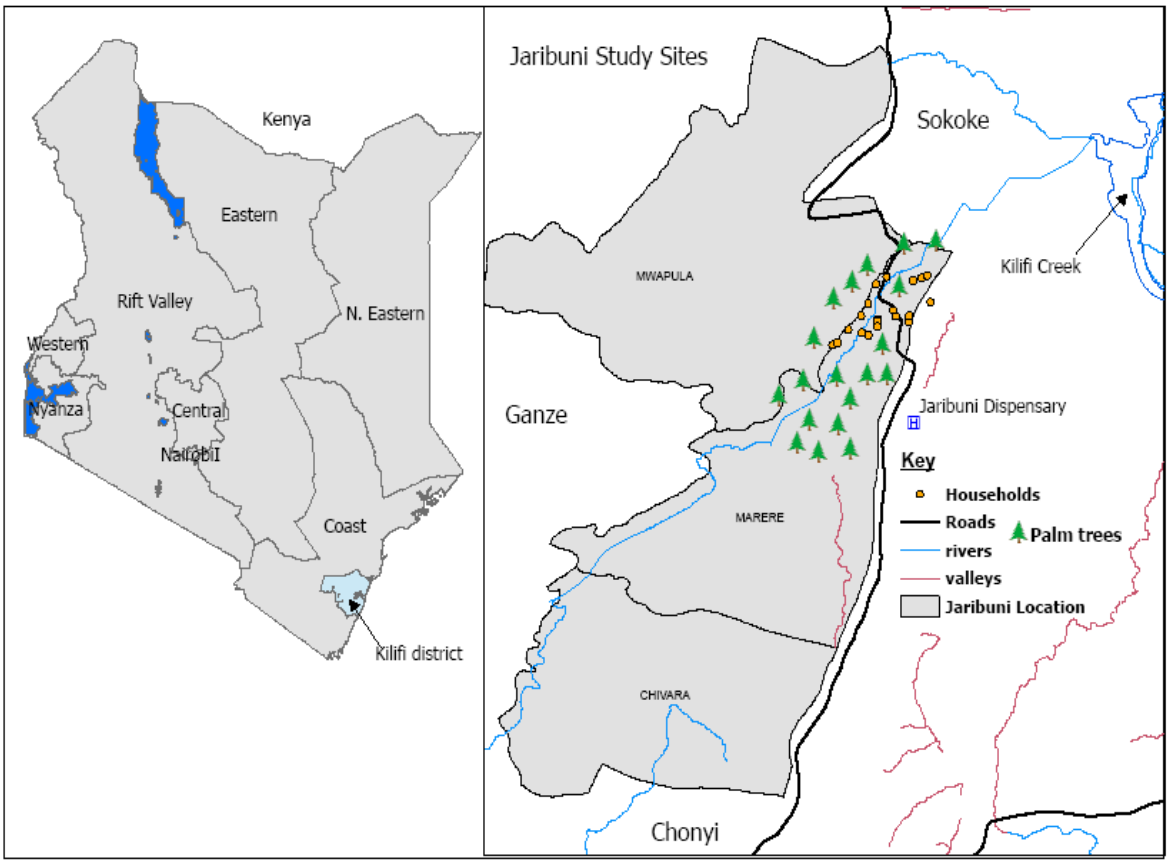


Figure 1: Map of Kenya showing the position of Kilifi District and the study area

Plate 3:1 A general view of Jaribuni showing the topography. Note the palm lined river Jaribuni with human settlements along it on both side.

Plate 3:2 A section of river Jaribuni near Kilifi creek where it drains. This is the main mosquito breeding habitat in Jaribuni

Plate 3:3 A traditional Kauma house made of mud walls and coconut fronds as roof

3.3 Mosquito collection methods and sampling design

Sampling for mosquitoes outdoor was done using CDC light traps and the mosquito magnet traps. The light traps that were used belonged to model 512, John W. Hock Company, Gainesville, FL while the mosquito magnet traps that were used belonged to the liberty plus model (American Biophysics Corporation (ABC) East Greenwich RI). Their designs as well as their functioning are described above. Two MMLP traps and four CDC-LTs were used in this study. The operation and positioning of the two trap models is described in details below.

3.3.1 Mosquito Magnet liberty Plus (MMLP)

The MMLP traps were operated to catalytically produce carbon dioxide from butane gas supplied in a 13.5 kg gas cylinder. This was supplemented with a solid formulation of Octenol, which is an insect attractant and supplied by the same manufacturer as the MMLP traps. The Octenol tablets were placed at the base of the inner Polyvinyl chloride (PVC) pipe that takes the warm, moist, carbon dioxide plume out. The octenol tablet was replaced after every 21 days as per the manufacturer's instructions. The butane gas cylinder was replaced with a new when it was depleted. Permission to collect mosquitoes from the homesteads was sought from the household heads after thorough explanation of the study. Individual consent was obtained as explained in Appendix 1. Mosquito collection was done outdoors near human habitations (Plate 3.4). All the twenty homesteads along the accessible stretch of river Jaribuni and within 500 metres from the river were selected for the study and their position marked using a hand held navigational system global positioning system (GPS) Garmin International Inc. Olathe KS. Their scattered

nature provided the required distances for trap separations. During each trapping night, two houses within the homesteads were chosen and used as the base for positioning the traps. The two MMLP traps were placed within 15 metres of the two chosen houses; one trap per house. Two CDC-LTs were placed at least more that 65 metres away from each MMLP trap. On the second night of trapping the MMLP traps and CDCLTs were swapped in position to cater for position effect. Mosquitoes collected in the MMLP traps were carried in the trap's collecting nets and a replacement net provided for the next trapping night. The collections were disqualified when any of the MMLP traps malfunctioned or the gas was depleted within the sampling period. Mosquito collection was done for 3 days in a week over a period of 3 months from July to October 2006. Collections were done from 1800 to 0600 hrs on each trapping night.

Plate 3:4 A mosquito magnet liberty plus trap set outdoors in a well vegetated area in Jaribuni, Kilifi.

3.3.2 Centre for Disease Control light trap (CDCLT)

The CDC-LTs were assembled and set outdoor. Four dry cells of 1.5 volts each were used to operate the fan and to light the bulb. The batteries were used for two nights of trapping before being replaced with new ones. A lid was screwed to the top of each trap to shelter the traps and the collected mosquitoes from rains. Using a pre-measured rope, the two CDC-LTs were placed more that 65 metres away from the MMLP as recommended by the manufacturer to avoid interference. There were two CDC-LTs for each MMLP trap. The two CDC-LTs for each MMLP trap were in turn placed 15 metres from each other. They were hung about 1.5 metres above the ground on natural supports such as trees where available (Plate 3.5). Artificial stands were provided where natural supports were not available. On the second night of trapping the CDC-LTs were swapped with the MMLP traps in position to cater for position effect. All mosquitoes collected in the CDC-LTs were aspirated with an aspirator and then emptied into paper cups for transportation to the laboratory. Collections were disqualified if any of the CDC-LTs malfunctioned during the sampling period. After the first night of trapping and the second night of trap swapping in a given homestead, all traps were then moved to new homesteads for another round of trapping. This was repeated until all the 20 homesteads chosen for this study were covered. A repeat round of trapping covering all the 20 homestead was carried out after the first round was completed.

All traps were closely monitored overnight for any problems. The mosquitoes collected by the traps were picked at 0600 hours in the morning and transported to the laboratories in Kilifi town.

Plate 3:5 A CDC light trap hung outdoors on a cashew nut tree in Jaribuni, Kilifi

3.4 Mosquito identification and preservation

In the laboratory, all mosquitoes were killed by freezing at –20°C. They were then sorted out into their physiological status (blood-fed and unfed) and then identified morphologically to species using the keys of Gillies and Coetzee, (1987) for Anophelines and that of Edwards, (1941) and Tanaka (2003) for the Culicines. All Anophelines had their head and thorax detached from the body and individually stored in clean vials for sporozoite detection by ELISA techniques. The legs and wings from all *Anopheles gambiae s.l* and *An. funestus s.l.* as well as abdomens from the unfed specimens of the two species were cut off and individually stored in vials for species identification using PCR techniques. All blood fed mosquitoes had their abdomens cut off and stored individually for determination of blood meal sources using ELISA techniques. All these samples were stored at -70°C.

3.4.1 Species identification by polymerase chain reaction (PCR)

The ribosomal DNA technique was used to identify members of the *An. gambiae* and *An. funestus* species complexes. The DNA used from both species was extracted from the collected and preserved mosquitoes as described in Appendix 2. The DNA amplifications were done as follows:

3.4.1.1 PCR techniques for *Anopheles gambiae* species complex

Members of *An. gambiae* species complex were determined using the PCR procedures as described by Scott *et al.*, (1993) and was done as follows:

To analyse the PCR products, 14µl. of the ice preserved PCR master mix constituted as in Appendix 3 was aliquoted into each labelled PCR vial that were placed on the

PCR plates which were in turn placed on ice. This was followed by addition of 1µl. aliquot of the template DNA extracted from the field captured mosquitoes. Each mosquito's DNA was put into a separate vial. The plates carrying the vials were then tapped gently to mix the template DNA and the master mix solution. The primers included in the master mix were specific for *An. gambiae s.s.*, *An. arabiensis* and *An. merus*. After this mixing, the vials were then loaded onto a PCR machine. The mosquito DNA was amplified through alternating cycles of DNA denaturisation, annealing and extension. The cycles for members of the *An. gambiae* complex were: 94^0C (1 min) for denaturation, 60^0C (1 min) for annealing and 74^0C (1min) for DNA extension. The cycles were repeated 30 times taking a total of one and a half hours.

3.4.1.2 PCR techniques for *Anopheles funestus* species complex

Members of *An. funestus* species complex were determined using the PCR techniques as described by Koekermoer *et al.,* (2002) and was done as follows:

14µl. of the ice preserved PCR master mix constituted as in appendix 4 was aliquoted into each labelled PCR vial that were placed on the PCR plates which were in turn placed on ice. This was followed by addition of 1µl. aliquot of the template DNA extracted from the field captured mosquitoes. Each mosquito's DNA was put into a separate vial. The plates carrying the vials were then tapped gently to mix the template DNA and the master mix solution. The primers used for the members of the *An. funestus* complex were specific for *An. funestus s.s., An. rivulorum, An. parensis* and *An. leesoni* and *An. vaneendeni*. The PCR cycle conditions for *An. funestus* included an initial denaturisation step at 94^0C for 5 minutes. This was followed by 30

cycles of denaturation, annealing and extension at 94^0C (30 second), 45^0C (30 sec) and 72^0C (40 sec) respectively.

The amplification cycles for both *An. gambiae and An. funestus* were followed by a final DNA extension step at 72^0C for 5 minutes. After the DNA amplification was done, the plates were placed on ice and 3 µl of loading dye consisting of bromophenol blue added to each PCR vial. The mixture of the PCR products and the loading dye was then carefully loaded onto the 3% agarose gels containing ethidium bromide prepared as in Appendix 5. This was then placed on an electrophoresis tank containing electrophoresis buffer. The gel was run from negative to positive charge for 30 minutes for sufficient separation of the PCR products. However frequent checks were made to observe the movement of the dye. On expiry of the electrophoresis time, the gel was scooped and placed on an ultraviolet source slab to reveal the bands. The revealed bands were then viewed and photographed using a camera for documentation .

3.4.2 *Plasmodium falciparum* infection rate

All Anopheline mosquitoes collected in the two different traps were also tested for the presence of *P. falciparum* circumsporozoite proteins (CSP) using indirect ELISA techniques of Wirtz *et al.,* (1987). The head and thorax of all Anopheline mosquitoes were transferred separately into labelled PVC microcetrifuge vials. To each of these vials was added 50 µl. of boiled casein blocking buffer (BB) containing Nonidet P-40 (5 µl NP40/1ml BB) prepared as in appendix 6 for homogenisation. They were then left for 1 hour after which grinding of the specimens was done using pestles. A

separate clean pestle was used in each vial to avoid contamination. After finely grinding the specimens, each pestle was then rinsed with 200 µl of BB bringing the total volume to 250 µl.

For the test, polyvinyl, U-shaped, 96 well microtiter plates (Dynatech Laboratories, Inc., Alexanderia, VA.) were coated with 50 µl. of *P. falciparum* specific monoclonal antibody (Mab) solution. Mab 2A10 Nardin *et al.*, (1982) was used for this study. The plates were then covered and incubated for 30 minutes at room temperature (23 – 25^0C). After the incubation, the MAB solution was then dumped and the plates dried by banging on non-dust paper towels. After the banging, each well was then filled with 200µl. of BB and incubated for 1 hour. After the incubation, the BB was again poured and a 50 µl. aliquot of each mosquito triturate added to a separate well on the labelled microtiter plates and incubated for 2 hours at room temperature. The mosquito triturate was then poured and the plates washed twice with PBS-Tween 20 solution constituted as in Appendix 6. After washing and drying by banging, 50 µl. aliquot of peroxidase-conjugated MAB (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was then added to each well and incubated for 30 minutes at room temperature.

After this incubation, the Mab-peroxidase conjugate was then poured and the wells washed 3 times with 100 µl of PBS-Tween 20 solution. This washing and subsequent drying were followed by an addition to each well, of peroxidase-substrate (kirkegaard and Perry Laboratories Inc., Gaithersburg, Md.). Positive and negative controls were included in every sporozoite ELISA test plate. The negative controls

consisted of male Anopheline mosquito triturate while the positive controls consisted of commercially available synthetic peptide standardised against *P. falciparum*. Positive reactions were determined visually by the dark green colour change after 30 minutes

3.4.3 Bloodmeal analysis

A direct ELISA test was carried out on all blood-fed mosquitoes to test for origin of the bloodmeals using the technique of Beier *et al.,* (1988). The hosts tested included, human, cow and chicken, which were the most common hosts in Jaribuni. Goat, the other common host was excluded in this test, as the anti-goat immuglobulin-G showed a cross reaction with that of sheep serum. Bloodmeal source tests for human and bovine blood were carried out in the same plate while that of chicken was tested in a separate plate.

For the test, the abdomens from blood-fed mosquitoes were cut off and individually placed in vials, containing 100 µl. of PBS buffer prepared as in appendix 7 and finely ground using pestles. After grinding, 900 µl. of PBS buffer was added bringing the total volume to 1000 µl. 50 µl. aliquot from each mosquito triturate was then added to a separate well in the U-shaped, polyvinyl chloride, 96-well microtiter plates (Dynatech Laboratories, Inc., Alexanderia, VA.) and incubated overnight at room temperature (23 – 25°C). Three positive and three negative controls of 50 µl. each, were included in the microtiter plates in the first and second columns respectively. The positive controls consisted of freshly prepared host serum from human, cow and chicken while the negative controls consisted of ground male anopheline mosquitoes.

One blank control, consisting of PBS buffer was included for troubleshooting. After an overnight incubation, the mosquito triturate in the microtiter plates was then dumped and the wells washed twice with a PBS-Tween 20 buffer constituted as in appendix 7. To the washed wells was added 50 µl. of host specific Mab-conjugate and then incubated for one hour. This incubation was followed by another round of three washes, using PBS-Tween 20 buffer. Every dumping and washing stage was followed by banging the plates on dustless paper towels for drying. After this second round of washing, a peroxidase substrate (Kirkegaard and Perry laboratories, Inc., Gaithersburg, Md.) was then added and the plates incubated for 30 minutes. The dark green colour change, indicating a positive reaction, was determined visually.

3.5 Data management and analysis

All field data was recorded in a notebook while that from laboratory procedures was entered into laboratory processing forms. All data was entered into Microsoft excel and analysis was done using SPSS statistical software package version 11.5 (SPSS Inc. Chicago, IL, USA). A paired t-test was used to determine whether there was any significant difference in the mean number of mosquitoes captured by both traps models. A Chi-square test was employed to establish any differences in the species diversity between the two traps. Significant difference in the trapping of members of *An. funestus* group which was collected in considerable large numbers by the two traps was also tested using Chi-square.

4 CHAPTER FOUR: RESULTS

4.1 Species abundance and diversity

A total of 1192 mosquitoes were collected using the two trap models, CDC-LT and MMLP traps (Table 1). Out of the total, 158 mosquitoes representing 13.3% were captured by the CDC light traps while 1,034 representing 86.7% were captured by the MMLP traps. A significantly higher mean number of mosquitoes (12.93±2.51, Mean SE) were collected in the MMLP traps compared to CDC light traps which was 0.99±0.18 (t=2.09, df=20 p=0.05). About 12 times more mosquitoes were collected in the MMLP traps compared to the CDC-LT traps (Table 2).

A total of six genera of mosquitoes: *Anopheles, Culex, Aedes, Mansonia, Eretmapodites and Lutzia* were collected. Both the MMLP traps and CDC-LT traps captured mosquitoes belonging to five genera each, with *Lutzia* being absent in the MMLP traps and *Eretmapodites* in the CDC-LT traps. The relative percentages of these genera in both traps are shown in figure 2. A total of 21 mosquito species was collected: 18 species in the MMLP traps and 15 in the CDC-LT traps. There was no significant difference in species diversity captured by the two trap models $\chi^2 = 0.269$, df=3 p <0.05.

The predominant mosquito species in the MMLP traps included *An. funestus, An. coustani, An. squamosus, Cx. quinquefasciatus, Cx. thalassius, Ae. pembaensis, Ae. minutus and Mansonia africana*. These comprised 98% of the total mosquitoes collected in these traps. On the other hand the predominant species among the CDC-LT traps were *An. funestus, An. coustani, Cx. quinquefasciatus, Cx. sinaiticus and Cx. bitaeniorhychus* comprising 88% of the total collection in these traps. MMLP

traps collected higher numbers of all species represented in both traps than the CDC-LT traps with an exception of two species *Cx. bitaeniorhynchus* and *Ae. aegypti* which were higher in the CDC-LT traps.

Three species, *Culex sitiens, Cx. cinellerus* and *Lutzia tigripes* were not captured in the MMLP traps while six species; *Anopheles gambiae, An. swahilicus, Culex tritaeniorhynchus, Aedes metallicus, Ae. ochraeus* and *Eretmapodites intermedius* were not recorded in the CDC-LT traps.

Table 1: Abundance and the percentage (%) of mosquitoes collected using mosquito magnet and CDC light traps from Jaribuni, Kilifi district, Kenya between July and Oct. 2006

Species	Mosquito Magnet Trap(%)	CDC Light Trap(%)	Total
Anopheles funestus	196(70)	84(30)	280
An. gambiae	9(100)	0(0)	9
An. coustani	81(92)	7(8)	88
An. squamosus	16(94.1)	1(5.9)	17
An. swahilicus	1(100)	0(0)	1
Culex quinquefasciatus	320(90.9)	32(9.1)	352
Cx. sinaiticus	16(64)	9(36)	25
Cx. thalassius	104(98.1)	2(1.9)	106
Cx. tritaeniorhynchus	7(100)	0(0)	7
Cx. sitiens	0(0)	2(100)	2
Cx. bitaeniorhynchus	2(25)	6(75)	8
Cx. cinellerus	0(0)	2(100)	2
Cx. cinereus	1(50)	1(50)	2
Lutzia tigripes	0(0)	1(100)	1
Aedes aegypti	1(25)	3(75)	4
Ae. pembaensis	74(94.9)	4(5.1)	78
Ae. minutus	30(96.8)	1(3.2)	31
Ae. metallicus	5(100)	0(0)	5
Ae. ochraeus	1(100)	0(0)	1
Mansonia africana	164(98.2)	3(1.8)	167
Eretmapodites intermedius	6(100)	0(0)	6
Total	1034(87)	158(13)	1192

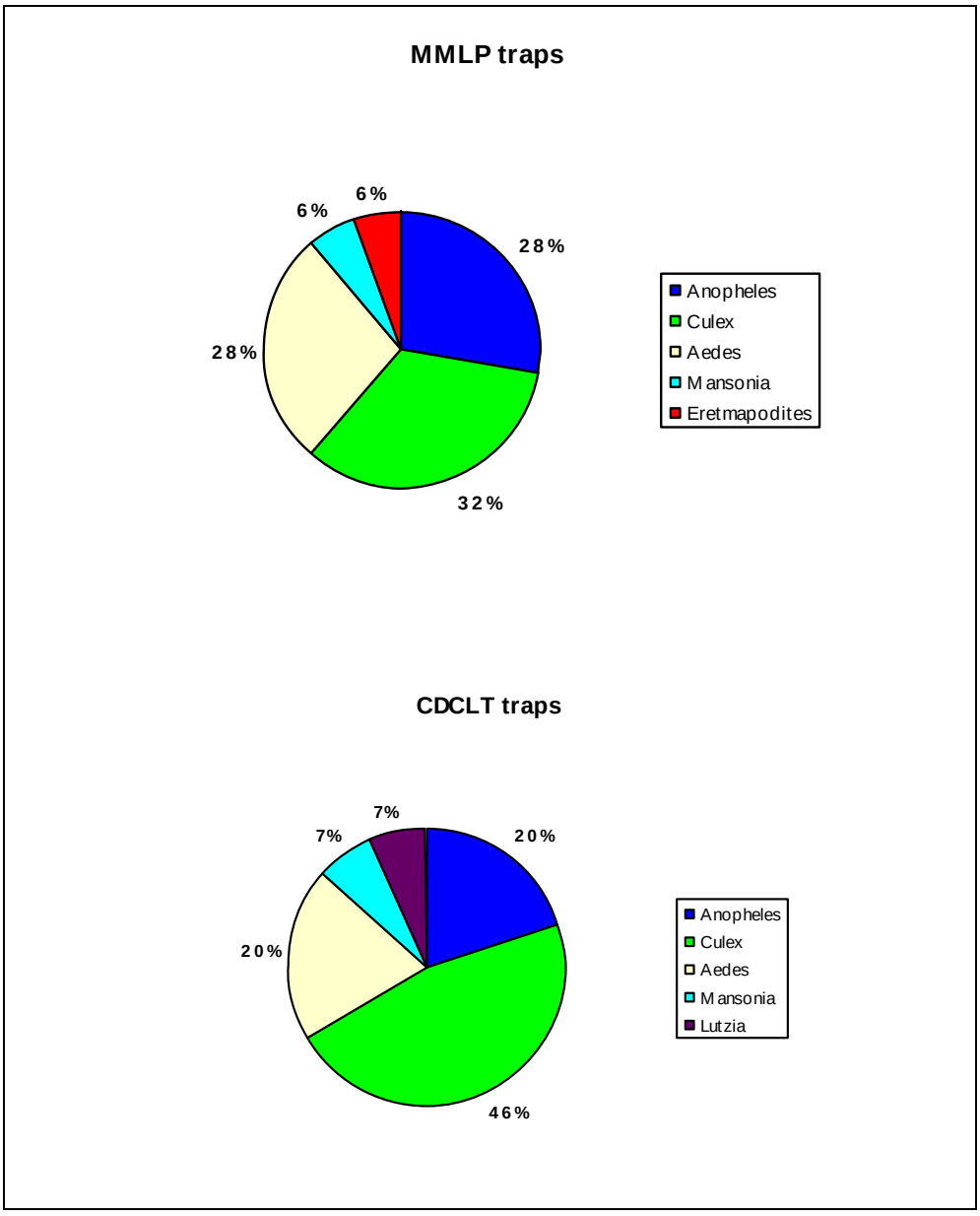


Figure 2: Mosquito genera represented in the two traps and their relative percentages

Table 2: Mean numbers (±SE) of mosquitoes collected using mosquito magnet traps and CDC light traps from Jaribuni, Kilifi district, Kenya between July and October 2006.

Species	Mosquito Magnet Trap		CDC Light trap		Total	
	Mean	SE	Mean	SE	Mean	SE
An. funestus	2.51	±0.49	0.54	±0.15	1.19	±0.20
An. gambiae	1.13	±0.13			1.13	±0.13
An. coustani	2.38	±0.31	1.00	±0.00	2.15	±0.27
An. squamosus	1.78	±0.36	1.00		1.70	±0.33
An. swahilicus	1.00	0.00			1.00	
Culex. quinquefasciatus	8.65	±4.81	2.91	±0.88	7.33	±3.71
Cx. sinaiticus	1.23	±0.12	1.00	±0.00	1.14	±0.07
Cx. thalassius	3.59	±0.92	1.00	0.00	3.42	±0.87
Cx. tritaeniorhynchus	1.17	±0.17			1.17	±0.17
Cx. sitiens			1.00	0.00	1.00	0.00
Cx. bitaeniorhynchus	1.00	0.00	1.20	±0.20	1.14	±0.14
Cx. cinellerus			2.00		2.00	
Cx. cinereus	1.00	0.00	1.00	0.00	1.00	0.00
Lutzia tigripes			1.00	0.00	1.00	
Aedes aegypti	1.00	0.00	1.50	±0.50	1.33	±0.33
Ae. pembaensis	2.47	±0.40	1.00	0.00	2.29	±0.36
Ae. minutus	3.00	±0.97	1.00	0.00	2.82	±0.89
Ae. metallicus	1.25	0.25			1.25	±0.25
Ae. ochraeus	1.00	0.00			1.00	0.00
Mansonia africana	3.35	±0.44	1.00	±0.00	3.21	±0.43
Eretmapodites intermedius	1.20	±0.20			1.20	±0.20
Total	12.93	±2.51	0.99	±0.18		

4.2 Outdoor captured cryptic species of *An. funestus* and *An. gambiae*

A total of 280 mosquitoes belonging to the *An. funestus* complex collected by the two traps were further identified using PCR technique. Of the total number tested, 194 (69%) were captured in the MMLP traps while 86 (31%) were captured in the CDC-LT traps (Table 3). Four sibling species were identified. They included *An. funestus s.s.*, *An. leesoni*, *An. parensis* and *An. rivulorum* (Plate 4.1). Ninety four percent of the specimens (n=262) were successively identified by PCR while 6% (n=18) failed to amplify. The sibling species composition within the MMLP traps comprised of 111 (61%) *An. rivulorum*, 44 (24%) *An. parensis*, 27 (15%) *An. leesoni*. No *An. funestus* s.s. was collected by MMLP traps. The composition within the CDC-LT traps comprised of 65 (81%) *An. rivulorum*, 9 (11%) *An. parensis*, 2 (3%) *An. leesoni* and 4 (5%) *An. funestus s.s.*. A significantly higher number of mosquitoes of *An. funestus* complex (χ^2 = 42.247, df = 1, p <0.001) was observed in MMLP traps compared to the CDC-LT traps. This was despite the fact that no *An. funestus s.s.* was collected in the MMLP traps. The abundance of the sibling species with an exception of *An. funestus s.s.* in both traps was, in ascending order, *An. leesoni*, *An. parensis* and *An. rivulorum* (Fig 3) Of all the sibling species, *An. rivulorum, An. parensis* and *An. leesoni* appeared to be more abundantly sampled by the MMLP traps than by the CDC-LT traps. A low number of *An. funestus s.s*, (n=4) was captured throughout the study.

A total of seven mosquitoes belonging to the *An. gambiae* complex were identified using the PCR techniques (Table 3). All specimens were successfully identified as *An. arabiensis* (n=4) and *An. merus* (n=3) all captured in the MMLP traps (Plate 4.2).

Anopheles gambiae s.s. was absent in both traps. The trapping of members of *An. gambiae* members by the two traps was not statistically compared as none was captured in the CDC-LT traps.

Table 3: Results of PCR analysis of *Anopheles funestus* and *An. gambiae* specimens collected from Jaribuni, Kilifi district in coastal Kenya between July - October 2006

		Collection method		
Species complex	Species	Mosquito magnet Trap (%)	CDC Light trap (%)	Total
An. funestus	*An. funestus s. s.*	0 (0.0%)	4 (100.0%)	4
	An. leesoni	27 (93.1%)	2 (6.9%)	29
	An. parensis	44 (83.0%)	9 (17.0%)	53
	An. rivulorum	111 (63.1%)	65 (36.9%)	176
	Unidentified	12 (66.7%)	6 (33.3%)	18
	Total	194 (69.3%)	86 (30.7%)	280
An. gambiae	*An.merus*	3 (100%)	0 (0.0%)	3
	An. arabiensis	4 (100%)	0 (0.0%)	4
	Total	7 (100.0%)	0 (0.0%)	7

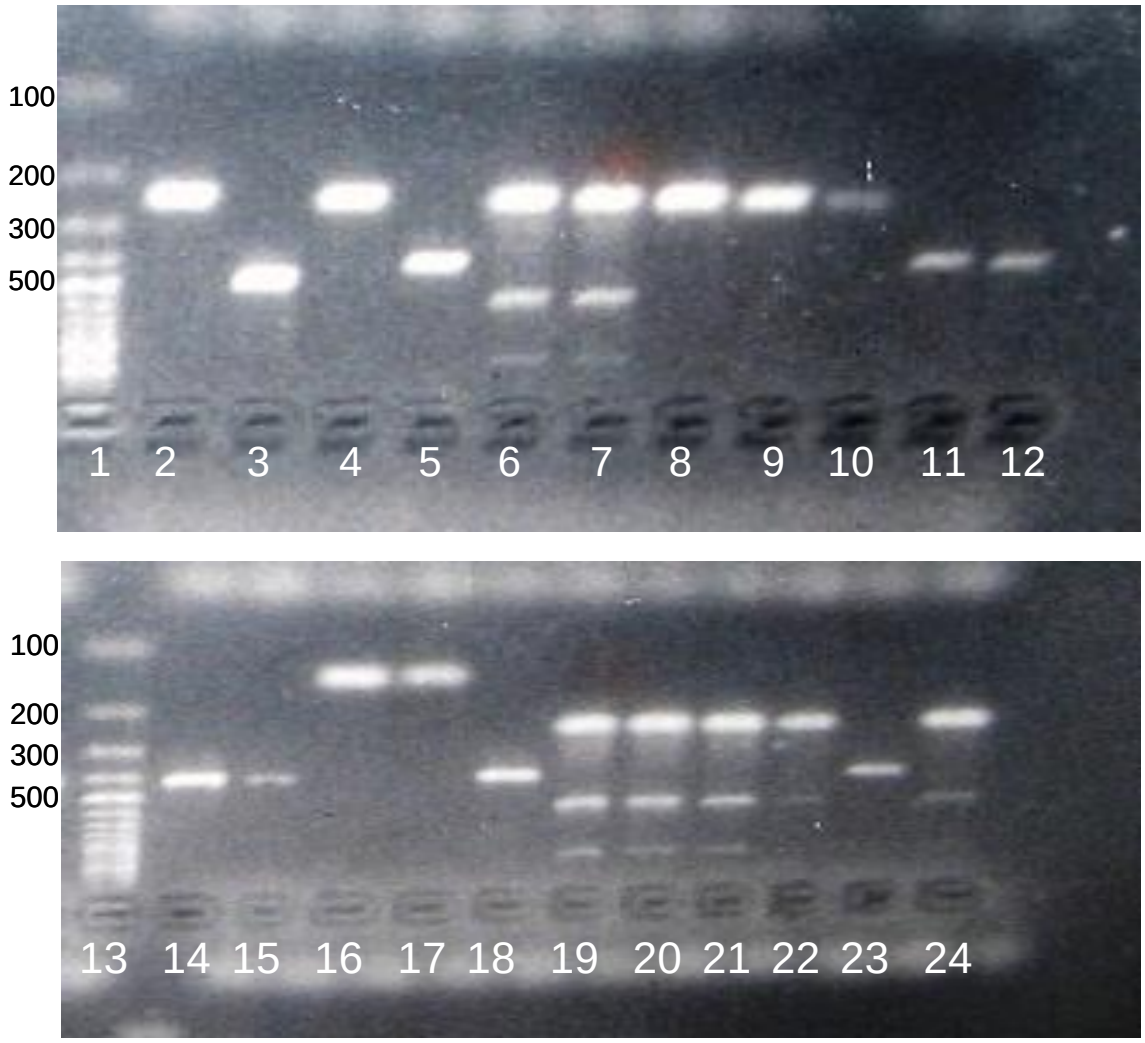
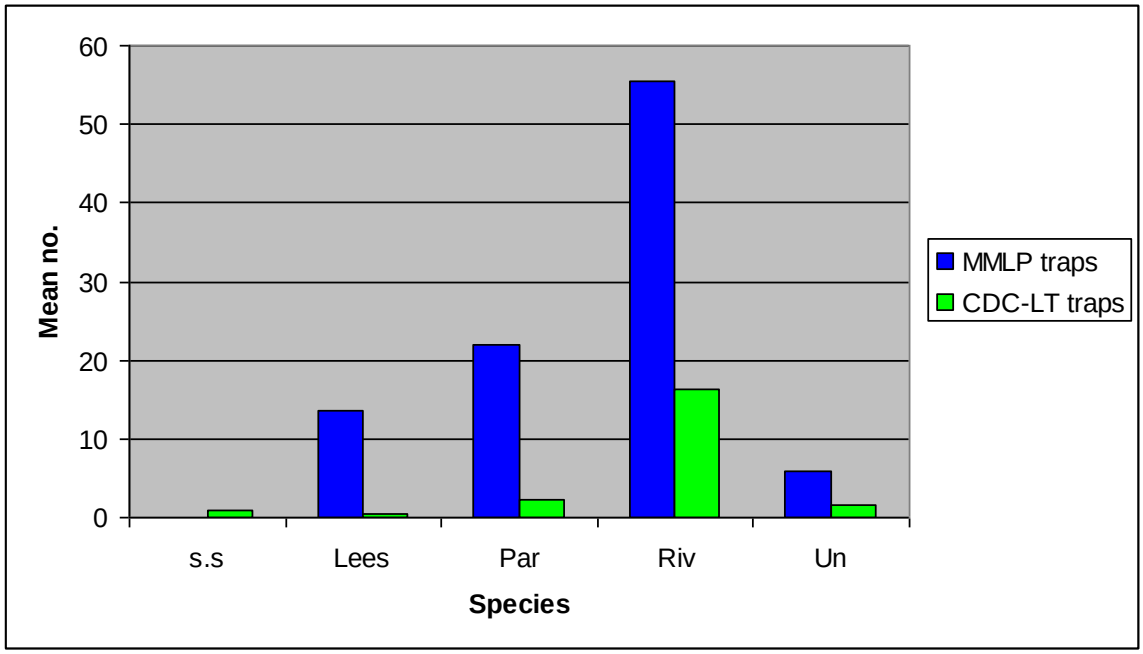


Plate 4:1 A representative of results of a PCR identification of members of the *An. funestus* group captured in Jaribuni, Kilifi, Kenya.
The plate shows amplified fragments. Lane 1 and 13: A 100 bp DNA ladder;Lanes 2,4,6,7,8,9,10,19,20,21,22,24: *An. parensis* (252bp);Lanes 3: *An. funestus* (505bp);Lanes 5,11,12,14,15,18,23: *An. rivulorum* (411bp) and lanes 16,17 *An. leesoni* (146bp)



s.s. - *An. funestus s.s.*

Lees - *An. leesoni*

Par - *An. parensis*

Riv - *An. rivulorum*

Un - Un-identified

Figure 3: Relative abundance and diversity of members of *An. funestus* group captured by CDC-LT and MMLP traps in Jaribuni, Kilifi, Kenya.

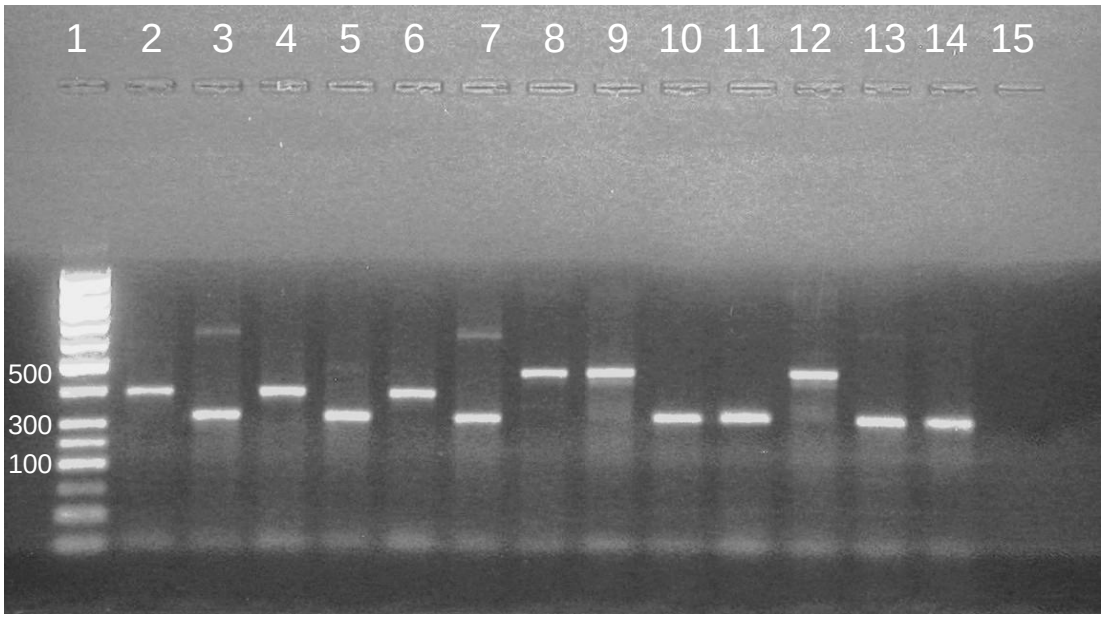


Plate 4:2 A representative of results of PCR identification of members of the *Anopheles gambiae* group from Jaribuni, Kilifi, Kenya.
The plate shows amplified fragments.Lane 1: A 100 bp DNA ladder. Lanes 2,4,6:*An. gambiae* controls; lanes 3,5,7: *An. arabiensis* controls(bp); Lane 8,9,12: *An. merus* (466bp);lanes 10,11,13 and 14: *An. arabiensis* (315bp); lane 15 is a blank control

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4.3 Sporozoite infectivity rates among outdoor captured mosquitoes

A total of 377 Anopheline mosquitoes were tested for *P. falciparum* circumsporozoites using sporozoite ELISA techniques. *Plasmodium falciparum* infection rates for *An. funestus s.l., An gambiae s.l., An. coustani* and *An. squamosus* caught by the two traps are shown in Table 4. None of the 92 Anopheline specimens captured in the CDCLT traps was positive for *P. falciparum* circumsporozites proteins. Of the 285 specimens captured by the MMLP traps, 0.4% (n=1) were positive for *P. falciparum* circumsporozoite proteins.

4.4 Analysis of bloodmeal among outdoor captured mosquitoes

Few mosquitoes across the species that were collected outdoors by the two methods were blood-fed. A total of 13 blood fed mosquitoes were collected by both traps, 5 in the MMLP traps and 8 in the CDCLT traps. Table 5 gives the analyses of bloodmeal origins. Overall, Sixty two percent (n=8) of the bloodmeal was identified as bovine.. Avian feeding was reported in seven percent of the specimen (n=1). Human immunoglobulin G (IgG) was not detected in any specimen. Bloodmeal origin was not identified in thirty one percent of the mosquitoes (n=4).

A number of other blood-seeking insects were also recorded in the MMLP traps. Those captured in considerable numbers were 4 species of Phlebotomine sandflies which included *Sergentomyia suberectus* (n=19), *S. schwetzi* (n=203), *S. antennata* (n=10) and *S. squamipleuris* (n=68). Also present in the MMLP traps were biting

midges belonging to *Culicoides adersi* (n=215) and blackflies belonging to *Simulium ruficorne* (n=6).

Table 4: Sporozoite infectivity rates for *Anopheles* species collected by CDC light traps and mosquito magnets traps in Jaribuni, Kilifi district, Kenya between July – October 2006.

Collection Method	Species	% Positive for *P. falciparum* sporozoite (n)
CDC Light trap	*An. funestus s.s.*	(0.0)4
	An. leesoni	(0.0)2
	An. parensis	(0.0)9
	An. rivulorum	(0.0) 65
	Unidentified	(0.0) 6
	An. coustani	(0.0) 6
	Total	(0.0) 92
Mosquito Magnet trap	*An. funestus s.s.*	(0.0) 0
	An. leesoni	(0.0) 27
	An. parensis	(2.3) 44
	An. rivulorum	(0.0) 111
	Unidentified	(0.0) 12
	An. arabiensis	(0.0) 4
	An. merus	(0.0) 3
	An. coustani	(0.0) 70
	An. squamosus	(0.0) 14
	Total	(0.4) 285

n = 377

Table 5: Bloodmeal sources and (proportions) for mosquitoes collected outdoors using CDC light traps and mosquito magnet traps in Jaribuni, Kilifi district, Kenya.

Collection Method	Species	No. tested	Human	Bovine	Chicken	Unknown
CDC light Trap	*An. funestus s.l*	6	0(0.00)	4(0.67)	0(0.00)	2(0.33)
	Cx. quinquefasciatus	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)
	Cx. thalassius	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)
Mosquito Magnet	*An. funestus s.l*	1	0(0.00)	0(0.00)	0(0.00)	1(1.00)
	Cx. quinquefasciatus	3	0(0.00)	1(0.33)	1(0.33)	1(0.33)
	Cx. thalassius	1	0(0.00)	1(1.00)	0(0.00)	0(0.00)
	Total	13	0(0.00)	8(0.61)	1(0.08)	4(0.31)

n=13

5 CHAPTER: FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

This study evaluated the mosquito magnet traps as a sampling tool for outdoor mosquito populations. Its performance was compared with that of the standard CDC-LT trap. The purpose of this study was to determine whether the mosquito magnet trap models could perform better than the unbaited CDC-LT traps which have traditionally been used in Kenya for mosquito sampling. As noted by Mboera *et al.*, (2000), traps based on host attractants such as carbon dioxide, heat, and moisture are likely to provide a more objective monitoring tool for the host-seeking fraction of mosquito vectors.

5.1.1 Efficiency of the MMLP traps for sampling outdoor mosquito populations

In this study, the superiority of the mosquito magnet traps in collecting a larger and more diverse mosquito population over the CDC-LT traps is evident. This great difference in the overall performance of both traps may have been caused by the fact that the two traps differed in their mode of attracting the mosquitoes. While the light traps used light as the only attractant, the mosquito magnet traps used CO_2 as the main attractant. In addition it produced heat, water vapour as well as other hydrocarbons, which as noted by Gillies (1980) might act as synergists in attracting mosquitoes. The differences were not only in the overall numbers and diversity captured by the two traps, but also within the same species collected by the two traps. These species-specific differences are likely to have been caused by the design of

each trap as well as the level of attractiveness of the main baits to these species. This study demonstrated that unbaited CDC-LT traps underestimated the mosquito abundance outdoors. Of the total mosquitoes collected during the study period, 86.7% were by the two mosquito magnet traps while only 13.3% were collected by a combined effort of four CDC-LT traps. This confirms an earlier observation in the same district by Mbogo *et al.,* (1993b) that the efficiency of light traps declines under conditions of low vector abundance.

Among the disease vectors sampled, *An. funestus*, *An. coustani, Cx. quinquefasciatus* and *Ma. africana* were recorded in high numbers by the mosquito magnet traps. This is an indication that, these traps can be suitable sampling tools for these vector species especially during disease outbreaks even in low mosquito density areas.

5.1.1.1 Efficiency of MMLP traps in collection of malaria vectors

Although the study was carried out during the peak period of *An. gambiae,* (Mbogo *et al.,* 2003) the number of *An. gambiae,* a principal malaria vector in Africa, caught by the two traps was unusually low compared to *An. funestus* the other principal vector. A mean of 1.3 mosquitoes for the mosquito magnet traps and zero for the CDC-LT traps was recorded in the entire period. This may in part be explained by the fact that Jaribuni has a greater abundance of *An. funestus* than *An. gambiae* (Wijers and Kiilu, 1977). A PCR procedure carried out on all the *An. gambiae* specimens revealed that they belong to the *An. arabiensis* and *An. merus* species. There was no *An. gambiae s.s.*. The absence of *An. gambiae s.s* was however an expected outcome since this was an outdoor collection and this species is known to

be highly endophagic as well as endophilic (Githeko *et al.,* 1996, Annon, 1998). This agreed with the finding of a separate study by Mbogo *et al.*,(2003) along the Kenyan coast, in which was found that of the total 5,476 *An. gambiae* collected indoors, 81.9% belonged to *An. gambiae* s.s., 12.8% *An. arabiensis* and 5.3% *An. merus*. This also agreed with the findings of Githeko *et al.*, (2006) in western Kenya in which they found that of all the indoor samples analysed by PCR (n=90), all were *An. gambiae s.s.*. *Anopheles arabiensis* and *An. merus* on the other hand have a varied behaviour. They can both be endophagic and endophilic and/or exophagic and exophilic. (Githeko *et al.,* 1996, Annon, 1998, Odiere *et al.,* 2007, Muriu, *et al.,* 2008). This may explain why only these two species were collected.

Anopheles funestus on the other hand was collected in significant numbers by both traps. Four sibling species, *An. funestus s.s*, *An. leesoni*, *An. parensis* and *An. rivulorum* were found co-existing in Jaribuni. These are however *An. funestus s.s.* was not recorded at all in the mosquito magnet traps and only four were caught in the CDC-LT traps. This species is known to be more endophagic than exophagic (Annon, 1998, Kamau *et al.,* 2003, Protopopoff *et al.,* 2007, Dabire *et al.,* 2007) and may explain its poor show in the traps set outdoors. An indoor collection on the other hand was likely to have yielded more *An. funestus s.s.*. In an indoor sampling in Bagamoyo Tanzania, Temu *et al.,* (2007) found that of the 649 *An. funestus* specimens positively identified by PCR, 84.3% belonged *An. funestus s.s.*.

An interesting observation from this study in Jaribuni was the order of relative abundance of the *An. funestus* sibling species. In both traps the order of abundance,

from the lowest to the most abundant was *An. leesoni*, *An. parensis* and *An. rivulorum*. This may indeed reflect the true order of their relative abundance in Jaribuni area. The only exception was *An. funestus s.s.* which may have been biased in its sampling due to its biting and resting behaviour.

5.1.2 Sporozoite positivity rates in outdoor collected mosquitoes

A sporozoite ELISA test revealed one specimen was positive for *Plasmodium falciparum* circumsporozoite proteins. This specimen belonged to the *An. funestus* group and was identified as *An. parensis* and was captured in a mosquito magnet trap. Its presence in a CO_2 baited trap in essence indicates that the specimen was host-seeking. This demonstrates that transmission of *Plasmodium falciparum* does occur outdoors. Not much is known about the vectorial capacity of *An. parensis* which has been all along regarded as generally zoophilic with no medical importance (Mouatcho *et al.,* 2007). In a study carried out in Mwea rice irrigation scheme in Central Kenya by Kamau *et al.,* (2003), it was shown that of all *An. parensis* specimens captured indoors, none was positive for *Plasmodium falciparum* circumsporozoite proteins. However, in another study in Kwazulu-Natal by Mouatcho *et al.,* (2007), it was shown that 13.5% of the *An. parensis* specimens tested for *Plasmodium falciparum* circumsporozoite proteins were positive

The finding of this study in Jaribuni calls for a re-thinking of the control strategies targeting the adult mosquitoes. A lot of effort has been directed towards the use of treated bed nets and indoor residual spraying. Other control methods targeting outdoor mosquito populations ought to be developed or enhanced. Methods such as

those targeting the immature stages of mosquitoes should be considered. This will control the disease vectors before they emerge into biting adults thereby reducing their risk of either indoor/or outdoor transmission.

5.1.3 Host preference in outdoor collected mosquitoes

Blood feeding behaviour of mosquito vectors is an essential parameter in the disease epidemiology (Mbogo *et al.,* 1993a). In this study a blood meal source investigation was carried out using ELISA techniques with a view of determining the preferred host of outdoor captured mosquitoes. Only 13 out of 1192 mosquitoes were blood-fed indicating that the majority of the disease vectors were host seeking. Analysis of the origin of the blood meal showed that none of the fed mosquitoes had human blood but a majority of them had fed on bovine blood. This shows a high degree of zoophily. This indicates a high potential for zoo-prophylaxis which should be considered for integration as part of the broader strategy to reduce disease transmission. This feeding preference was in total contrast to an indoor resting mosquito population in another area of the same district. As noted by Mbogo *et al.*, (1993a), indoor resting malaria vectors in Kilifi predominantly fed on human irrespective of the availability of cattle and other domestic animals.

Despite the high number of mosquitoes collected by the mosquito magnet traps only few were blood-fed. This finding was not unexpected since, being carbon dioxide traps; they are likely to get the attention of host-seeking fraction of mosquitoes such as the newly emerged as well as the older ones coming for another round of feeding following egg laying. This therefore indicates that these traps can be considered as a

replacement for human bait catches. A notable observation among the blood-fed mosquitoes in MMLP traps was that, almost all of them were less than half fed indicating that they were seeking for more blood following an interrupted earlier feeding. This differed with the ones captured in the CDC-LT traps which were fully fed.

5.2 Conclusions

The findings of this study show;

1. That carbon dioxide, heat and moisture baited traps are the sampling tool of choice for outdoor mosquito surveillance.
2. That having performed without any major breakdown, both traps are quite reliable for any mosquito sampling activity.
3. That the mosquito magnet traps are ideal for sampling host-seeking mosquitoes.
4. That the mosquito magnet traps can be ideal for sampling mosquitoes in low density areas or during low mosquito seasons.
5. Mosquito magnet traps can be effective for sampling other non-mosquito haematophagous disease vectors such as *Simulium* black flies, the biting midges and Phlebotomine sand flies.
6. The mosquito magnet traps through their own carbon dioxide and electricity production can be an ideal baited enhanced sampling tool especially in areas where dry ice and batteries are hard to supply.
7. The bulky nature and high initial cost of purchasing the MMLP traps may hinder their wide use in research.

Although the MMLP traps were found to be superior to unbaited CDC-LT traps, this study does not preclude the use of light traps in the sampling of mosquitoes.

5.3 Recommendations

1. A similar evaluation should be carried out in a high mosquito density area such as Western Kenya or Mwea rice irrigation scheme.
2. Known disease vectors collected in the mosquito magnet traps should be screened for other diseases like lymphatic filariasis as well as arboviruses such as Yellow fever, Rift valley fever and Chikungunya.
3. Important physiological status such as parity should be studied in mosquitoes captured by the mosquito magnet traps in order to understand what ages are attracted to them. This will be important if the sample is to be screened for diseases.
4. It is also recommended that, since both traps collected great numbers of *An. funestus* outdoors, a study ought to be done in a high malaria transmission area in order to better understand the out of door transmission especially where this species is a major vector.
5. Since both traps captured some species that did not appear in the other, it is therefore recommended that any mosquito sampling should incorporate various trapping tools in order to obtain more objective results.
6. Although the mosquito magnet traps have been shown to be a good sampling tool their cost may discourage their extensive use in research especially in developing countries. Modification of these traps to a simpler and cheaper

technology is therefore highly recommended in order to make it both less bulky and available to many researchers.

7. The mosquito magnet traps should be evaluated in well lit urban areas where certain species may have been missed in light traps due to competition with the background urban lighting.

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7 APPENDICES

Appendix 1: Consent seeking forms

Information and consent form for household heads for voluntary participation in the trap evaluation study in jaribuni in Kilifi district, Kenya.

Study Title:

The Use Of The Mosquito Magnet^{TM} Trap As An Alternative Surveillance Tool For outdoor Mosquito Populations In Kenya.

Introduction of the work of KEMRI

I am from the Kenya Medical research institute (KEMRI), Kilifi. KEMRI assists the Ministry of Health (MOH) with work pertaining to human health. The special work of KEMRI-Kilifi is to learn more about illnesses that affect the people of Kenya including residents of Jaribuni. Investigations involving various diseases that affect the community are carried out among communities and in the hospital; with learning about illnesses and their transmission sometimes requiring visits to the households and homesteads.

Purpose of the study

This research project is about assessing the working of a new trap in sampling mosquitoes outdoors within your homestead. This project aims at identifying a efficient trap for effective sampling of mosquitoes to better understand their dynamics; knowledge of which will be used to guide intervention as well as evaluating such interventions.

Procedures

The procedures for this study will involve trapping of mosquitoes using two type of traps outdoors within your compound. Before the study begins, three members of your community will undergo an education and training session on how to operate and empty the traps.

During the study, myself and together with my field assistants who are members of your community will visit your homestead four times for outdoor mosquito collection.

What I am requesting from you

I am requesting you for permission to allow your homestead to be used for this study. By accepting to participate in this study, you will permit us to visit your homestead periodically to capture mosquitoes outdoors.

Benefits of taking part in the study

There are no direct benefits associated with taking part in the study. However, the entire community will gain from your participation in the study through the positive application of the information being sort regarding the new trap. The knowledge will be applied in the control of mosquitoes and also in evaluating the effects of such control. All these will have a benefit of reduced mosquitoes and the diseases they transmit.

Foreseeable risks associated with the study.

There are no direct risks associated with your households' direct participation in this study.

Confidentiality

All the information collected from this research project will be kept confidentially. Information about your household that will be collected from the study will be stored in a file that will not have your name on it, but a number assigned to it instead. The name associated with the number assigned to each file will be kept under lock and key and will not be divulged to anyone.

Right to Refuse or Withdraw

You have the right to decide whether or not to allow traps placement in your compound. You may also stop participating in the research at any time without being penalized in any way.

Who to contact

If you have any questions, you may ask them now or later. If you wish to ask questions later, kindly address your questions to:

Dr. Charles Mbogo or Laban Njoroge,

KEMRI-CGMR-C KEMRI-CGMR-C

KILIFI, Kenya. KILIFI, Kenya

Phone: 41 5 22063 Phone: 41 5 22063

Name …… Hse. No……..…

Signature……

Appendix 1: Single mosquito DNA extraction solutions and procedure

Homogenization buffer:

0.10m Nacl	0.59g
0.20m sucrose	6.84g
0.01m EDTA	0.37g
0.03m Trizma base	0.36g
pH = 8.0	100ml sterile water

Lysis buffer:

0.25m EDTA	9.28g
2.5% (w/v) SDS	1.88g
0.5m Trizma base	6.03g
pH =9.2	100ml sterile water.

To make grinding buffer, add 4 parts homogenization buffer to 1 part lysis buffer.

1. Grind mosquito in 100µl grinding buffer in a microcentrifuge tube. Take care that no large fragments remain.
2. Place tube immediately in 65°c water bath for 20-40 minutes.
3. Add 14µl of 8m potassium acetate (58.89g in 75ml). Mix well.
4. Cool on ice water or crushed ice for 30 minutes.
5. Centrifuge at top speed for 10 minutes. Save supernatants in a new sterile microfuge tube taking care not to get any of the precipitate .If precipitate is taken with supernatant, re-spin the sample for an additional 5 minutes and place supernatant in a new tube.
6. Add 200µl cold 95% Ethanol. Sample can be stored in freezer overnight or longer at this stage.

7. Spin the sample in a centrifuge at top speed for 10 minutes.
8. Pour off ETOH. Add 200µl 70% ETOH to rinse, then pour off. Add 200µl 95% ETOH, and then pour off. Invert tube and allow drying completely (usually about 1 hour).
9. Re-suspend the pellet in 100µl sterile PCR water for at least 15 minutes. Use sterile pipette tips.

Appendix 2: PCR master mix for *Anopheles gambiae* for 15 µl reaction

		Single sample (µl)	1 5 samples (µl)
PCR H_2O		4.8	72.0
10_x PCR Buffer		1.5	22.5
$Mgcl_2$		1.8	27.0
dNTPs		0.3	4.5
Primers	GA	1.0	15
	AR	1.0	15
	ME	1.0	15
	UN	1.0	15
Taq		0.1	1.5
BSA		1.5	22.5
DNA template		1.0	

Appendix 3: PCR master mix for *Anopheles funestus* for 15 µl reaction

		Single sample(µl)	32 samples(µl)
PCR H_2O		8.15	260.8
10_x Buffer		1.5	48.0
$Mgcl_2$		1.2	38.4
dNTPs		1.25	40.0
Primers	Fun	0.3	9.6
	Lees	0.3	9.6
	Par	0.3	9.6
	Un	0.3	9.6
	Van	0.3	9.6
Taq		0.1	3.2
BSA		0.3	9.6
DNA template		1.0	

Appendix 4: Agarose gel preparation

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Made of Agarose and TBE or electorphoresis buffer

This is the gel making as well as running or electorphoresis buffer.

To make 20 litres of TBE (Tris Boric Acid EDTA) electrophoresis buffer:

Tris base	216g
Boric Acid	110g
0.5m EDTA (pH 8.0)	80ml.
Distilled water	20ltr.

For a big Gel:

% Gel	Agarose	1x Buffer	Ethidium bromide
3.0%	4.80g	160ml.	4µl.

Agarose and TBE are mixed on a hot plate while stirring with magnetic stirrer.

Ethidium bromide added just before pouring the molten but cooled gel into the electrophoresis tank.

Allow gel to solidify (takes 1- 1½hrs).

Ethidium bromide is highly **carcinogenic!** Avoid touching with uncovered hands, Wear gloves when handling it as well as when handling all gel containing it. To dispose it**,** incineration is preferred instead of pouring in the sink.

Remove the tank edge support blocks and push down edge slaps to leave gel edges free.

Add 210-220ml TBE buffer; cover the gel completely (don't allow solidified gel to sit for long without adding the buffer to avoid drying.)

Appendix 5: Procedure for sporozoite elisa solution preparation

Reagents preparation:

1. PBS (pH –7.4): Add one bottle Dulbecco's PBS to 1 litre distilled water mix and adjust pH if necessary. Store at 4°c. Shelf life is 2 weeks.

2. Boiled Casein, 0.5% (BC):

	500ml	1litre
Casein (Baker no. E397-07)	2.50g	5.0g
0.1N NaoH	50ml	100ml
PBS (pH 7.4)	450ml	900ml
Thermesol 1g/10ml dH_2O	0.5ml	1ml
or powder	0.05g	0.1g
phenol red 1gm/10ml dH_2O	0.1ml	0.2ml
or powder	0.01g	0.02g

-Suspend casein in 0.1N NaOH and bring to boil.

-After casein is dissolved, slowly add the PBS. Allow cooling and adjusting the pH to with Hcl. Add the thermersol and phenol red. Shelf-life one week

2. Blocking buffer (BB). Mix as follow:

BSA	10g
Casein	5g
PBS (pH 7.4)	1000ml
Thimersol	0.1g
Phenol Red	0.02g

Suspend BSA and casein in PBS and mix for 2 hours or until dissolved.

Add the Thimersol and phenol red. Shelf life at 4°c is 1 week. Solution may be frozen for later use.

3. Blocking buffer: NONIDET P-40 (BB: NP-40).

   To 1ml BB add 5µl NP-40

   To 5ml BB add 25µl NP-40

   Mix well to dissolve the NP-40 in the BB.

   Shelf life at 4°c is 1 week.

4. Wash solution (PBS-TW): PBS plus 0.05% TWEEN 20.

   Add 0.5ml. of Tween 20 to 1litre of PBS. Mix well. Store at 4°c. Shelf life 2 weeks.

5. 2A10 Monoclonal antibody (Capture MAb)

   Put 5 ml. of PBS plain into a tube (for only one plate)

   Add 20 µl of the capture MAb.

   Mix well and dispense 50 µl into each well of the PVC plate.

   Note: The amount is adjusted according to the number of plates.

6. Peroxidase labelled Enzyme

   Put 5 ml. of BB into a clear tube

   Add 10 µl of the 2A10 peroxidase labelled enzyme (Conjugate) into the tube

   And mix.

   Note: This only enough for one plate.

7. Peroxidase Substrate solution. Mix ABTS (Solution A) and hydrogen peroxide (solution B) 1:1 immediately before use. For each plate, use 5ml of solution A and

5ml of solution B. Add 100µl per well.

All solutions must be properly labeled showing their contents, date and initials of the person who made the solution. Refrigerate for storage.

Appendix 6: Procedure for bloodmeal ELISA solution preparation

1**. Phosphate buffered saline (PBS), pH 7.4:**

Use stock laboratory PBS or add 1 bottle Dulbecco1s BS to 1litre distilled water, Mix and adjust pH if necessary. Store all of the following solutions at 4°c

2. **Boiled Casein, 0.5% (BC):**

	500ml	1litre
Casein (Baker no. E397-07)	2.50g	5.0g
0.1N NaoH	50ml	100ml
PBS (pH 7.4)	450ml	900ml
Thermesol 1g/10ml dH_2O	0.5ml	1ml
or powder	0.05g	0.1g
phenol red 1gm/10ml dH_2O	0.1ml	0.2ml
or powder	0.01g	0.02g

-Suspend casein in 0.1N NaOH and bring to boil.

-After casein is dissolved, slowly add the PBS. Allow cooling and adjusting the pH with Hcl.

-Add the thermersol and phenol red. Shelf-life one week

3. **Enzyme Diluent (BC-Tween)**

100ml BC plus 25µl Tween 20. Do not store; make every day.

All solutions must be properly labeled showing their contents, date and initials of person who made the solution.