DISTRIBUTION PATTERNS, DISPERSAL AND POPULATION GENETICS OF ANOPHELINE MOSQUITOES ALONG THE KENYAN COAST

BY

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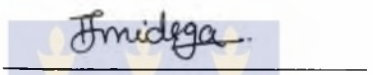
DECLARATION

I hereby declare that with the exception of references to other people's work which I have duly acknowledged all the experimental work described in this thesis was carried out by me and this thesis, either in whole or in part, has not been presented elsewhere for another degree.



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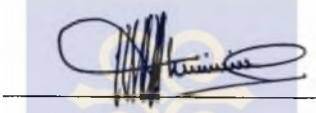

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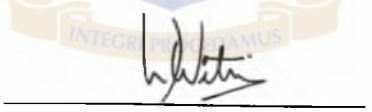

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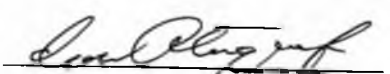

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DEDICATION

To Jerry and Jimmy

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LIST OF ABBREVIATIONS

bp	base pair
dATP	deoxyadenosine triphosphate
ddw	distilled de-ionized water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EDTA	Disodium ethylene diamine tetra acetate. $2H_2O$
EthBr	Ethidium Bromide
EtOH	Ethanol
GPS	Global positioning system.
H_2O	Water
KAc	Potassium acetate
kb	Kilobase
KOH	Potassium hydroxide
M	Molar
Mw	Molecular weight
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Hydrogen-ion exponent

RNA	ribonucleic acid
RNase	ribonuclease
rDNA	ribosomal DNA
rpm	revolution per minute
sddH_2O	sterile double distilled water
s.l	Sensu lato
s.s	Sensu stricto
TAE	Tris - Acetate EDTA
TEMED	N,N,N,'N'- tetramethyl ethylene diamine
Tm	Melting temperature
Tris	2 –amino-2-(hydroxymethyl)-1,3 propanediol
µl	microlitre
µM	micromolar

ABSTRACT

Studies on the distribution patterns, dispersal and population genetics of *Anopheles gambiae* (Diptera: Culicidae) and *Anopheles funestus* (Diptera: Culicidae) were conducted at two sites, i.e Jaribuni and Mtepeni in Kilifi, along the Kenyan Coast. Longitudinal sampling of mosquito populations was conducted during the period 2002 – 2003. Day resting indoor collections and all night human biting catches revealed the presence of *An. funestus, An. gambiae* s.s, *An. squamosus, An. coustani, An. nili* and *An. pharoensis. An. gambiae* was the most predominant specie at the Mtepeni site representing 91.8% of the total anophelines captured, with the least being *An. funestus* which accounted for to 6.7% of the total. At the Jaribuni site, *An. funestus* represented 85% of the total anophelines compared to 14.6% *An. gambiae.* Sporozoite ELISA tests on these species revealed no significant differences ($p>0.05$) in the sporozoite rates between *An. gambiae* and *An. funestus* from Jaribuni during 2001. In Mtepeni, sporozoite rates were significantly different ($p=0.02$) between *An. gambiae* and *An. funestus* in 2001 and 2002. Variations in *Anopheles* population density were associated with changes in rainfall, temperature and relative humidity. At Jaribuni, average relative humidity and daily rainfall were significantly ($P<0.05$) associated with *An. gambiae* abundance. *An. funestus* abundance was significantly associated with daily rainfall, and maximum and minimum temperatures. At the Mtepeni site, significant associations were observed between *An. gambiae* and rainfall ($P=0.02$), *An. funestus* and minumum temperatures ($P =0.04$) and *An. funestus* and maximum temperatures ($P =0.01$).

Mark-release-recapture (MRR) experiments were conducted with emergent *An. gambiae* s.l and *An. funestus* species to determine the vector dispersal, survival and population sizes yielded recapture rates of 24.6% and 4.33% at Jaribuni and Mtepeni respectively. Mean population size estimates for all *Anopheles* was estimated at 16,871 for Jaribuni, and 854 for Mtepeni. Daily survival probabilities were 0.96 for *An. funestus* and 0.95 for *An. gambiae* at Jaribuni, and 0.95 for *An gambiae* at Mtepeni. The highest mean distance of dispersal from the release point was 393 meters for *An. gambiae* and 384 meters for *An. funestus* at both sites. The population size estimates observed, coupled with the high estimates of survival probability of *An. gambiae* and *An. funestus*, facilitate the continuous transmission of malaria along the Kenyan coast.

Population genetics analysis using microsatellite DNA was conducted on samples of female *An. gambiae* from Jaribuni and Mtepeni; and *An. funestus* from Jaribuni. Eleven microsatellite markers were used to investigate *Anopheles* population genetic structure, gene flow and effective population sizes. These studies demonstrated that allelic composition and frequencies varied between the dry an wet seasons, suggesting the effect of temporal variations in environmental conditions on the genetic structure of vector populations. Higher inbreeding, confirmed by significant linkage disquilibrium was observed at both Jaribuni and Mtepeni. Effective populations sizes ranged between 11,427-33,067 for *An. gambiae* in Jaribuni; 4,595–17,968 for *An. gambiae* in Mtepeni, and 1,859-4,484 for *An. funestus* in Jaribuni. Overall, observations from this study suggest that *Anopheles* populations along the Kenyan coast consist of small locally inbred populations bearing a few genetic similarities spread cross seasons. Estimates of

effective population size indicate that even though population sizes drop considerably during the dry season, mosquitoes along the coast do not suffer severe population bottlenecks during the dry season, hence a small population of adults with a high survival probability sustains malaria transmission during the dry season. From this study, a better understanding of some aspects of malaria transmission patterns observed along the Kenya coast was derived and the information on population dynamics, population size, dispersal, survival and population genetics presented in the findings from this study will be very important to malaria control programme officers planning malaria control interventions based on vector control.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Insect vectors transmit some of the world's most important parasitic diseases. Tropical diseases such as malaria, yellow fever, lymphatic filariasis, dengue fever, the West Nile Virus (WNV) and other arboviruses are transmitted by mosquitoes. Of these, malaria is the most common and important; transmitted through the bite of an infected female *Anopheles* mosquito, leading to the infection of humans with either one or more protozoan parasites such as *Plasmodium falciparum, P. ovale, P. malariae* and *P. vivax.* Approximately 400 species of *Anopheles* mosquitoes have been identified and there is evidence of additional sibling species (White, 1982; Toure *et al.,* 1997), which are members of species complexes, such as the *An. gambiae* complex, *An. funestus* complex and *An. minimus* complex among others. Out of these, 60 to 80 species have been implicated in malaria transmission, but only 40 are of major importance (White, 1982).

In sub-saharan Africa, members of the *An. gambiae* complex and the *An. funestus* complex are the most efficient vectors of *P. falciparum* malaria. The highest rates of sporozoite development are in *An. gambiae,* the species that is widespread throughout tropical Africa (Coetzee *et al.,* 2000). The *An. gambiae* complex consists of six formally named species, and one unnamed species (Hunt *et al.,* 1998; Coetzee, 2004), and there exists evidence for at least six species within *An. gambiae s.s.* in West Africa (Fanello *et al.,* 2003) namely mopti, savanna, Bamako, Forest and Bissau forms (Bryan *et al.,* 1982; Coluzzi *et al.,* 1985; Toure *et al.,* 1997). Within the complex, *An. gambiae s.s.* is recognized as the world's most important vector of malaria, followed by *An. arabiensis*

in transmitting *P. falciparum*. The *An. funestus* complex consists of nine morphologically similar species namely; *An. funestus* Giles, *An. aruni* Sobti, *An. parensis* Gillies, *An. vaneedeni* Gillies and Coetzee, *An. rivulorum* Leeson, *An. confusus* Evans and Leeson, *An. fuscivenosus* Leeson, *An. leesoni* Evans and *An. brucei* Service (Gillies and Coetzee, 1987). *Anopheles funestus s.s.* is the most anthropophilic member of the group and is also a highly efficient vector of malaria, reportedly continuing malaria transmission during the dry season when *An. gambie* are less active in malaria transmission (Fontenille *et al.*, 1997).

Globally, 300-500 million clinical episodes of malaria are reported annually (UN, 2005). It is estimated that about 1 million deaths (range 744,000 – 1,300,000) from the direct effects of malaria occur annually in Africa; more than 75% of them are children less than 5 years of age (Collins and Paskewitz, 1997, UN, 2005). In all malaria endemic countries in Africa, 25-40% (average 30%) of all outpatient clinic visits is due to malaria and between 20 and 50% of all hospital admissions are a consequence of malaria (Snow, 1999). In Eastern and Southern Africa, the proportion of deaths caused by malaria has increased from 18% in the 1980s to 37% in the 1990s (Koenromp *et al.*, 2003). Overall, more than 41% of the world's population is at risk of acquiring malaria and the proportion increases yearly due to deteriorating health systems, growing drug and insecticide resistance, climate change and war (Breman, 2001).

In Kenya, more than four million cases of malaria are reported annually. Major malaria vector species in Kenya are members of the *Anopheles gambiae* complex and *An. funestus* (Mbogo *et al.*, 1993). *Plasmodium falciparum* is the species most frequently associated with severe malaria and accounts for 80-90% of cases in Kenya (Ministry of

Health Report, 1994), and a 5.1% mortality rate has been reported among patients admitted with severe malaria (Ministry of Health Report, 1994). Malaria is also responsible for the greatest number of consultations (30% of new cases in medical centres within the public health service) and is the most common reason for hospital admission (22,000 cases per year in public hospitals). Along the Kenyan coast, malaria is endemic and transmission has been shown to be over 50% in school children (Mbogo *et al.*, 2003).

The social and economic costs of malaria infection points to the need for more progress in malaria control efforts (Jones and Williams, 2004), at least to reduce the rates of disease incidences and mortality. Malaria control efforts are currently focused on case definition, accurate diagnosis, prompt and effective disease treatment, use of insecticide treated nets (ITNs) and vector control (WHO, 2000). However, these approaches have been met with many obstacles, such as the unavailability of proper health facilities/infrastructure, drug resistant parasites and insecticide resistance in vectors.
The launching of global malaria campaign initiatives, such as the Multilateral Initiative on Malaria (MIM), the Roll Back Malaria (RBM) Programme, the Malaria Vaccines Initiative the Presidents malaria initiative (PMI), the Bill and Melinda Gates Foundation's Grand challenges in Global Health Programme and the most recent Global Fund to fight AIDS, Tuberculosis and Malaria (GFATM), will hopefully contribute more resources for research towards the fight against malaria.

Efforts to promote the use of insecticide treated materials and insecticide treated nets (ITN's) have been shown to be effective in reducing childhood morbidity and mortality (Nevill *et al.*, 1996, Binka *et al.*, 1996, Lengeler *et al.*, 1996). Efficacy trials to evaluate

the potential of ITNs in countries with a wide range of epidemiological conditions and transmission intensities were conducted in Ghana (Binka *et al.,* 1996), Burkina Faso (Habluetzel *et al.,* 1997), Kenya (Nevill *et al.,* 1996; Philips-Howard *et al.,* 2003), and Gambia (D'Alessandro *et al.,* 1995). These were randomized-controlled trials, comparing ITN use with no net use and ITN use with use of untreated nets. The main conclusions drawn from these trials were that ITNs reduce overall mortality by about 20% in Africa (range=14 29%) and that, for every 1,000 children aged 1-59 months protected by ITNs, about six lives are saved each year. Increased access to insecticide treated nets requires major financial, technical and operational inputs. The increasing development of insecticide resistance to pyrethroids, the insecticide class mainly used for treating ITNs has hampered the use of impregnated bednets (Brooke *et al.,* 2001).

Malaria control is multi-faceted and a more integrated approach towards malaria control in sub-Saharan Africa may have a greater impact in reducing malaria morbidity and mortality. However, with the increase in human populations, rural to urban migration, urbanization and environmental degradation, there has been an increase in suitable mosquito breeding areas and a modified ecosystem giving the vector an even greater opportunity to evolve its efficiency in disease transmission. There is a need therefore for more research focusing on improving our understanding of malaria vector population ecology, reducing human vector contact and disease transmission. Vector control has been the means of eradication of malaria from numerous regions of the world, and has dramatically reduced its incidence in some countries (Bruce-Chwatt and de Zulueta., 1981). The vector remains the key link in the transmission of malaria, and hence, warrants research and control effort in areas where the disease is still a public health problem. Historically, success in combating malaria has been attributed to mosquito

control, a strategy which has largely failed due to various reasons including the development of insecticide resistance, economic limitations and gaps in the basic biological knowledge of these vectors (Lounibos and Conn, 2000; Shiff, 2002).

For any vector control effort to achieve a reduction in malaria transmission, it is important for control programme staff to have access to adequate information on the local malaria vector ecology, distribution, transmission patterns and the factors affecting transmission in order to design interventions suited to the area under consideration.

Along the Kenyan coast, the malaria vectorial system consists mainly of *An. gambiae s.s, An. funestus, An. merus,* and *An. arabiensis.* In some parts of the Kenya coast, *Anopheles funestus* is abundant and a major vector of malaria, while in other parts *An. funestus* and *An. gambiae* occur in sympatry with varying population densities and importance as vectors (Mbogo *et al.,* 2003). There exists a need to develop a long-term plan for malaria control through vector control along the Kenyan coast. A prerequisite for this is to obtain information necessary to develop a comprehensive picture of vector population ecology, genetics and the factors regulating it along the coast. This study, which is part of a larger study on anopheline larval ecology, was developed to explore the interrelationship between eco-climatic variables and *Anopheles* population dynamics as key determinants of malaria transmission along the Kenyan coast. Through mark-release-recapture (MRR) procedures the dispersal capability of the mosquito vector and its ability to cross geographic barriers has been examined as a possible factor contributing to the disease epidemiology. The study also investigated factors acting as major architects for the population structure of *An. gambiae* and *An. funestus* populations along the Kenyan coast. Experiments were formulated to determine how variations in

environmental parameters influence the population distribution, structure and abundance of malaria vectors at two sites along the Kenyan coast. Results from these studies are expected to describe the population composition, population size, dispersal, survival and population genetics of *An.gambiae* and *An. funestus* on the Kenyan Coast and form a basis for an intervention-planning framework, which will utilize the above information in planning future vector control activities.

The results of the present study are presented in three parts: Part 1 reports the distribution patterns of *An. gambiae* and *An. funestus* along the Kenyan coast; Part 2 reports on the use of mark-release-recapture methods to estimate the population size, dispersal capabilities and survival probabilities of *An. gambiae* and *An. funestus* and Part 3 is on the population genetics of *An. gambiae* and *An. funestus;* along the Coast of Kenya.

1.1.1 Study Hypothesis

The study tested three hypothesis and these were:

i) The dynamics of malaria vector abundance, distribution and *Plasmodium falciparum* transmission are influenced by environmental factors.

ii) The dispersal of Anopheline species is non random and it is closely related to the distribution of human habitations and

iii) The population structure and gene flow patterns of anopheline populations along the coast are influenced by seasonal changes in environmental factors.

1.1.1.1 Specific Objectives

The specific objectives set were to determine:

i) The spatial and temporal variations in the distribution patterns of *An. gambiae* s.l and *An. funestus* populations.

ii) The population density, dispersal capability and survival of *An. gambiae* s.l and *An. funestus* using Mark Release Recapture procedures.

iii) The population structure and gene flow of *An. gambiae* and *An. funestus.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria: The Disease and Symptomatology

Four species belonging to the genus *Plasmodium* namely *P. falciparum, P. vivax, P. malariae* and *P. ovale* cause malaria in humans. The species that causes the greatest illness and death in Africa is *P. falciparum* (WHO, 2000). *Plasmodium falciparum* occurs in most malaria affected areas of the world, in tropical Africa and Asia, 85-90% of malaria cases are due to *P. falciparum*. *Plasmodium vivax* is uncommon in sub-saharan Africa, but common in South Asia and Central America, and is predominant in South America. *Plasmodium ovale* is found mainly in tropical Africa, in West and South Africa, with sporadic reports from other continents, e.g the South Pacific islands. *Plasmodium malariae* is the least common species of malaria to infect humans, and is infrequent all over the world (Hombhange, 1998).

The general symptoms of malaria include headache, nausea, fever, vomiting and flu-like symptoms, although these symptoms may differ depending on the type of *Plasmodium* species that caused the infection (Schmitz and Gelfand, 1976; Wernsdorfer and Mc Gregor, 1986) and the immunity of the human host (Stevenson & Riley, 2004). *Plasmodium falciparum* normally takes 7 to 14 days for symptoms to show while *P. vivax* and *P. ovale* normally takes 8 to 14 days (but in some cases can survive for months in the human host) and for *P. malariae,* 7 to 30 days (Vinetz *et al.*, 1998; D'Avanzo *et al.*, 2002). However,

these figures are indications only - the onset of symptoms varies tremendously depending on the *Plasmodium* species infecting and the level of host immunity. Symptoms may appear and disappear in phases and may come and go at various time frames. These cyclic symptoms of malaria are caused by the life cycle of the parasites - as they develop, mature, reproduce and are once again released into the blood stream to infect more blood and liver cells.

Fever is the main symptom of malaria (Schmitz and Gelfand, 1976). A high swinging fever can develop, with marked shivering and intense perspiration. Further serious complication involving the brain and kidneys can then develop leading to delirium and coma. The most severe manifestations are cerebral malaria (mainly in children and persons without previous immunity), anaemia (mainly in children and pregnant women), kidney and other organ dysfunction (e.g respiratory distress) (WHO, 1986). Persons repeatedly infected with malaria will usually acquire a considerable degree of clinical immunity, which provides them with partial protection against future infections (Baird, 1995).

2.2 Vectors of Malaria

About 60-80 species of *Anopheles* have been implicated in malaria transmission. The importance of each species in malaria transmission however varies by region, as does their geographical distribution. For example, *An. Aquasalis* and *An. albimanus* complex are very important vectors in South America (Laubach *et al.*, 2001, Marquetti *et al.*, 1991). In sub-saharan Africa, the *An. gambiae* complex and the *An. funestus* complex are the most important vectors of malaria, transmitting *P. falciparum*, which is the most important parasite.

2.2.1 The *Anopheles gambiae* complex

The *An. gambiae* Giles complex comprises six sibling species, namely *An. gambiae* s.s, *An. arabiensis*, *An. melas, An. merus, An. bwambae* and *An. quadriannulatus*, one unnamed species and several incipient species all differing in various ways (Coluzzi *et al.*, 1985; Gillies and Coetzee 1987; Favia *et al.*, 1997; Hunt *et al.*, 1998). At least three species within *An. gambiae s.s.* have been reported in West Africa (Fanello *et al.*, 2003) namely Mopti, Savanna, Bamako, Forest and Bissau forms (Bryan *et al.*, 1982; Coluzzi *et al.*, 1985; Toure *et al.*, 1998). Members of the *An. gambiae* complex are morphologically indistinguishable, but exhibit distinct genetic and eco-ethological differences, as reflected in their ability to transmit malaria. *An. gambiae* sensu stricto is the most important vector of *P. falciparum* malaria in sub-Saharan Africa. This species is reported to be the most specifically adapted to humans and has the highest malaria parasite inoculation rates with entomological inoculation rates ranging from between <1 to >1000 infective bites per person per year (Trape and Rogier, 1996; Beier *et al.*, 1999). It is also remarkably stable in a wide

range of bio-ecological and seasonal conditions hence appears to be very flexible, both in exploiting new man-made environments and in their response to malaria control activities (Coluzzi, 1984; Pothikasikorn *et al.,* 2007).

Members of the *An. gambiae* complex have a wide geographical distribution and have been reported from most African countries (Coluzzi, *et al.,* 1979; Chinery, 1984; Coetzee *et al.,* 2000). *Anopheles melas* is confined to the West Coast of Africa (Coluzzi and Sabatini, 1968) whereas *An. merus* is confined to the coast of East Africa and islands off its coast (Pock Tsy *et al.,* 2003) , and has also been recorded in Somalia in the North to Natal in the South (Paterson *et al.,* 1964).

Two species of the *An. gambiae* complex namely *An. gambiae* s.s and *An. arabiensis* are the most broadly distributed and the most efficient vectors of malaria (Coetzee *et al.,* 2000). The distribution range and relative abundance of *An. gambiae* and *An. arabiensis* appear to be strongly influenced by climatological factors, especially total annual precipitation (Lindsay & Martens 1998). Whereas *An. gambiae* is dominant in humid zones (Lindsay & Martens 1998, Coetzee *et al.,* 2000), *An. arabiensis* tends to predominate in arid savannas. Where these two species occur in sympatry, changes in species composition usually occur, with *An. arabiensis* mainly predominant during the dry season and *An. gambiae* more abundant during the rainy season. However, this pattern may vary depending on local ecology and species adaptation. *Anopheles quadriannulatus* has been reportedly found in Ethiopia and Eritrea (Hunt *et al.,* 1998; Shililu *et al.,* 2003) and *An. bwambae* has been reported breeding in mineral water springs in Uganda (Davidson and Hunt, 1973).

In Kenya, *An. gambiae s.s.*, *An. arabiensis* and *An. merus* are the most common members of the *An. gambiae* complex, with the range of distribution and relative abundance of *An. gambiae* and *An. arabiensis* defined by climatic factors, mainly annual precipitation and annual and wet season temperatures (Minakawa *et al.*, 2002b). *Anopheles merus* prefers to breed in salty water habitats and is mainly found along the coastal strip, and is a minor vector in Kenya (Mwangangi *et al.*, 2003).

Studies in West Africa have provided evidence for further subdivision of the *An. gambiae* species. The evidence for this division is shown by the micro-morphological differences of the second chromosome in different populations, due to inversions. These chromosomal forms, which also carry different strain specific combinations have been well studied in Mali, West Africa where three chromosomal forms, 'Bamako', 'Mopti', 'Savanna', 'Forest' and 'Bissau' appearing to be reproductively isolated in nature have been reported (Favia *et al.*, 1997; Lanzaro *et al.*, 1998, Slotman *et al.*, 2007). None of these forms have been reported in Kenya, or anywhere else in the East African region.

2.2.2 The *Anopheles funestus* complex

Anopheles funestus Giles is one of the three major vectors of malaria in Africa, together with *An. gambiae* Giles and *An. arabiensis* Patton of the *An. gambiae* complex. *Anopheles funestus* is emerging as an important vector of malaria, with its major role sometimes being noticed during the dry season when *An. gambiae* are less numerous (Fontenille *et al.*, 1997). Taxonomically, *An. funestus* belongs to a group of nine morphologically similar species that

were classified by Gillies and De Meillon, (1968). The group consists of at least nine species. The first four species namely *An. funestus s.s., An. aruni* Sobti, *An. parensis* Gillies, and *An. vaneedeni* Gillies and Coetzee which belong to the *funestus* sub-group have been described on the basis of characters in the adult, whereas *An. confusus* Evans and Leeson, *An. fuscivenosus* Leeson, *An. leesoni* Evans and *An. rivulorum* can be distinguished from each other and from the *funestus* subgroup based on larval characters. *Anopheles funestus* is an adaptable species as shown by its wide distribution and ability to occupy regions ranging from lowland to high altitudes. This species is abundant, widespread and is highly endophilic and anthropophilic and is found in almost all bio-climatic areas near swamps or rivers (Faye *et al.,* 1995). The other species are more limited in density and distribution, are mainly zoophilic, but can avidly bite humans outdoors in the absence of other hosts (Gillies and De Meillon, 1968). *Anopheles rivulorum* is the second most abundant and widespread species in the group occasionally collected indoors along with *An. funestus s.s,* and has been shown to be a vector of malaria in Africa (Wilkes *et al.,* 1986). Other studies have shown that *An. vaneedeni* can be experimentally infected with *P. falciparum* in the laboratory (De Meillon *et al.,* 1977), but no evidence of its role in malaria transmission is available. This species has been recorded only from South Africa.

Studies in Kenya have indicated the presence of *An. funestus s.s., An. parensis, An. leesoni,* and *An. rivulorum* (Kamau *et al.,* 2002; 2003). *Anopheles funestus s.s.* has been found almost exclusively inside human dwellings while *An. rivulorum* was found exclusively outdoors, *An. parensis* both indoors and outdoors and *An. leesoni* was found indoors (Kamau *et al.,* 2003). Only *An. funestus s.s.* has been implicated as an important vector of

P. falciparum in Kenya, even though its importance in malaria transmission varies depending on locality.

The paucity of studies on this species group indicates the need for proper identification of members of the *An. funestus* species complex to make available data on biological and behavioural characteristics that will elucidate their role in malaria transmission, and that are relevant to vector control. Cytogenetic studies have shown that *An. funestus, An. rivulorum, An. leesoni, An. parensis* and *An. confusus* each possess unique chromosomal inversion arrangements that can be used to identify them (Green, 1982). *Anopheles vaneedeni* is homosequential with *An. funestus* differing from it only in the possession of a polymorphic inversion on arm 2 (Green and Hunt, 1980). However, if the inversion is homozygous for the standard arrangement, then these two species cannot be separated (Green and Hunt, 1980).

The use of cytogenetics for routine species identifications is however disadvantageous in various ways, for example, the inability to identify immature stages or males, large sample sizes are required, but this is limited by the requirement of half gravid females; hence the need for development of DNA testing methods, similar to those used for the *An. gambiae* complex. Molecular identification methods for species complexes have mainly used the ribosomal DNA locus because it is represented in multiple copies in the mosquito genome, and it contains highly variable regions (Collins and Paskewitz, 1997). Koekemoer *et al.* (1998) distinguished between *An. funestus* and *An. vaneedeni* using PCR primers developed from the D3 region in the 28S ribosomal gene, and amplified products were digested with

the restriction endonuclease *HpaII* producing distinct fragments on an agarose gel that could separate them. More assays have been developed to differentiate *An. funestus* and *An. rivulorum*, based on the second ribosomal DNA internal transcribed spacer region 2 (ITS2) (Hackett *et al.*, 2000). The single strand conformation polymorhism (SSCP) developed by Koekemoer *et al.* (1998) distinguishes between four species, namely *An. rivulorum, An. funestus, An.vaneedeni* and *An. leesoni.* Currently, the most reliable method for separating members of the *An. funestus* complex is a cocktail PCR assay (Koekemoer *et al.*, 2002), which separates the five most common members of the group.

Studies on variability between members of the *An. funestus* complex based on chromosomal inversions revealed variable levels of polymorphism: 9 inversions on chromosomal arm 2R [a, b, c, d, e and h (Green and Hunt, 1980), s, t, and u (Lochouarn *et al.*, 1998), 2 on arm 3R[a and b (Green and Hunt, 1980)], and two on arm 3L [a and b] (Green and Hunt, 1980)], three on chromosomal arm 3 (a, b, ab) and one on arm 5 (a). No inversions have been observed on the X chromosome and arm 4. Sharakov *et al.* (2004) have physically mapped 32 polymorphic *An. funestus* microsatellite markers to the polytene chromosomes, and based on this, 16 microsatellite markers have now been suggested as a reference set, which can be used by research groups to facilitate population genetics studies of the *An. funestus* group in parts of Africa.

2.3 Malaria Transmission

The female *Anopheles* mosquito transmits human malaria. When a mosquito bites an infected individual, it sucks the gametocytes, the sexual forms of the parasite, along with blood. These gametocytes continue the sexual phase of the cycle and the sporozoites fill the salivary glands of the infested mosquito. When this female mosquito bites the man for a blood meal, which it needs to nourish its eggs, it inoculates the sporozoites into human blood stream, where they may remain for about 30 minutes and then disappear.

Most of the sporozoites are destroyed by phagocytes whilst some enter the liver parenchymal cells, develop and undergo asexual multiplication (pre-erythrocytic schizogony). A large unpigmented schizont is formed containing several merozoites, which are released into the bloodstream to invade the erythrocytes. The merozoite attaches to an erythrocyte and is invaginated into the red cell through a parasitophorous vacuole, where it feeds and deposits a pigment called haemozoin as a by product (Aikawa, 1980). The ingested merozoite becomes a feeding trophozoite and in the early stages of an infection, the fully-grown trophozoite multiplies asexually to become a schizont (erythrocytic schizogony), producing a small number of merozoites (Aikawa and Seed, 1980).

Release of the merozoites from the erythrocytes brings on an attack of malaria, and the interval between attacks is the length of the schizogonic cycle. This may last several hours. The released merozoites repeat the cycle and invade other erythrocytes. After a number of cycles of schizogony, the trophozoites do not divide but become gametocytes, which

develop no further in man but circulate in the bloodstream until a mosquito takes them up during a blood meal (Kettle, 1995).

When in the *Anopheles* mosquito, microgametocytes exflagellate to become microgametes. The microgametes move away to find and fuse with macrogametes to form zygotes, which remain motionless for 18-24 hours then elongate to form ookinetes (Aikawa and Seed, 1980; Kettle, 1992). The ookinete penetrates the wall of the midgut and forms an oocyst and through sporogony, the oocyst develops into enlarged motile sporozoites, which burst through the ruptured wall of the oocyst and invade the body cavity of the mosquito. The sporozoites, which are now infective then migrate to the salivary glands of the mosquito and are injected into the wound when the mosquito takes the next blood meal.

2.3.1 Factors influencing malaria transmission

Malaria transmission is influenced by two main categories of factors which are intrinsic and extrinsic factors. Extrinsic factors are mainly climatic and environmental factors such as rainfall, temperature, and elevation whereas intrinsic factors include host, parasite and vector factors.

2.3.1.1 Climatic factors

Malaria, as a vector-borne disease, is particularly influenced by climatic factors, mainly rainfall, temperature and relative humidity (WHO, 1990). Rainfall affects the availability of breeding habitats. The oviposition of mosquito eggs by gravid females and their maturation to larvae and adults requires the availability of aquatic breeding habitats (Le Sueur and

Sharp, 1991; Molineux, 1988). Several studies have demonstrated the association between *An. gambiae* abundance and rainfall but no direct predictable relationship has been established. *Anopheles gambiae* prefers to breed in temporary turbid waters, whereas *An. funestus* prefers more permanent water bodies. However, the availability of both types of habitats depends on adequate rainfall, which is also related to the saturation deficit and affects mosquito survival (Molineux, 1988), providing a good basis for using rainfall as a predictor for the presence of vectors, their survival and possible malaria transmission. The natural boundaries of the geographical distribution of malaria are actually determined by temperature and rainfall.

Rainfall or precipitation is one of the important elements for the breeding and development of mosquitoes (McMichael *et al.,* 1996). Rainfall not only provides the medium for the acquatic stages of the mosquito life cycle but also increases the relative humidity and then the longevity of the adult mosquito. Hence, the association of malaria with rainfall is due not only to greater breeding activity of mosquitoes, but also to the rise in relative humidity and higher probability of survival of female *Anopheles*. The impact of rainfall on malaria transmission is however complicated, and varies with the circumstances of particular geographic regions and depending on local habits of mosquitoes. Rain may prove beneficial to mosquito breeding when it is moderate but it may destroy larval habitats and flush out the mosquito larvae when it is excessive. Considerable evidence has accrued to show that heavy rainfall and flooding can increase mosquito breeding (Ramasamy *et al.,* 1992) and therefore an increase in mosquito densities leading to a higher probability for mosquito bites, and disease transmission. It is not only the total amount of rainfall that is important but also its

weekly and monthly distribution. The rainfall patterns in known malaria and non-malaria regions indicate that the requirement for stable malaria transmission is around 80mm for 5 months and that neither 60mm for 5 months nor 80mm for less than 5 months is sufficient to sustain endemic malaria transmission (Lindsay & Birley, 1996).

Temperature, on the other hand plays an important role, driving both malaria vector and parasite development within the mosquitoes. After oviposition, the rate of egg development through the larval and pupal stages to the adult mosquito largely depends on temperature. As temperature increases, the time required for mosquito development shortens (Rueda *et al.*, 1990). For example at 16^0C, larval development may last more than 45 days, compared to only 10 days at 30^0C. Tekleheimanot *et al.* (2004) reports that at lower temperatures, the larval and pupal stages of mosquitoes take a longer time to complete – 47 days at 16°C – in Ethiopia and a small increase in temperature substantially shortens the duration of these phases to 37 days at 17°C. The effect of a long development cycle due to low temperatures is usually a reduction in the number of mosquito generations, in addition to putting larvae at the risk of predators, eventually reducing the adult population size. Thus, by affecting the duration of the aquatic stage in the mosquito life cycle, temperature determines the timing and abundance of mosquitoes following adequate rainfall. Following emergence, female adult mosquitoes seek a blood meal and in the process they ingest malaria parasites (gametocytes) with the blood. Martens (1995) reports that the blood feeding frequency of mosquitoes increases with temperature, leading to more host vector contact and resulting in increased proportions of infective mosquitoes. Very low temperatures limit vector development by lengthening the gonotrophic cycle and reducing the survival rate.

Temperature also affects vector survival and the sporogonic cycle of the parasite. *Anopheles gambiae* for example, only exists in frost-free regions (De Meillon, 1934; MARA, 1998) or where absolute minimum temperatures in winter remain above 5°C in Africa (Leeson, 1931). Empirical studies demonstrate that the duration of the sporogonic cycle varies inversely with environmental temperature. The duration of the extrinsic phase of the malaria parasite, which is the development of the ookinete in the midgut of the anopheline mosquito, also depends on temperature. As temperature declines, so does the development of malaria parasites and the biting activities of mosquitoes. In addition, the incubation time for *P. falciparum* is 26 days at 20°C but when the temperature is increased to 25°C, the incubation period is shortened to 13 days (Buynavanich and Landrigan, 2003). On average, the sporogonic cycle lasts about 10 days, but shortens as temperature increases becoming as short as five days when the temperature exceeds 30°C (Macdonald, 1957). These biological characteristics explain why effective malaria transmission can occur only in areas with a temperature higher than 20°C (Lindsay and Martens, 1998) and indicate the potential role that increasing temperature can play in the transmission of malaria and other vector-borne diseases.

Several studies have investigated the effects of climatic variables/weather on the transmission of vector-borne diseases. Mbogo *et al.* (2003) observed variation in the relationship between the mosquito population and rainfall in different districts of Kenya and attributed the variation to environmental heterogeneity. Similarly, Zhou *et al.* (2004) showed that there was high spatial variation in the sensitivity of malaria outpatient numbers to climate fluctuations in the East African highlands.

One of the most striking uncertainties in the literature on weather and malaria is the variability in the reported relationship between rainfall and malaria, with several studies showing the importance of rainfall as a precipitating factor for malaria transmission (Loevinsohn, 1999; Lindblade *et al.*, 1999, Kilian *et al.*, 1999; Bouma *et al.*, 1996) while other studies show negative or neutral effects (Lindsay *et al.*, 2000; Woube, 1997). For rainfall to have a positive association with malaria cases the temperature must be warm enough to support mosquito and parasite development thus, the impact of rainfall on malaria cases becomes more immediate in warmer temperatures. This is consistent with laboratory findings that a mosquito population peaks early at higher temperatures, while at low temperatures it experiences slow, steady growth with a delayed peak (Alto and Juliano, 2001).

On the other hand, data from studies on the timing of the mosquito life cycle suggest that malaria cases should follow periods of increased temperature and increased rainfall. Because temperature accelerates several steps in the process of mosquito and parasite development, the time lag between the appearance of suitable weather conditions and the appearance of new malaria cases should shorten as temperature rises. For example, at an average

temperature of 20^{0}C the aquatic phase of the mosquito will be completed in about 28days (5 days for the eggs to hatch and 23 days for the larva to develop to the adult stage); and sporogony is completed in about 28 days. At this temperature, malaria cases should appear 9-10 weeks following rainfall, assuming an average incubation period of about 10 – 16 days. The number of malaria cases should be positively related to increases in temperature i.e. when the mean temperature is higher, e.g 30°C, the aquatic stages of the mosquito and the sporogony cycle are completed in about 12 and 8 days respectively, and malaria cases should appear 4-5 weeks following rainfall and the time lag in the effect of temperature should also be shorter. An integration of the climatic and environmental factors governing the distribution of insect vectors and pathogens that they transmit provides a powerful system for assessing disease vector dynamics in relation to disease patterns and the impact of control measures.

Humidity is an important environmental parameter with respect to the survival of mosquitoes. Relative humidity is defined as the ratio of water-vapour content of the air to its total capacity at a given temperature (Woodward and Hales, 1998). Relative humidity affects dispersal, mating, feeding behaviour, and oviposition of vector species. Humidity also affects the rate of evaporation of water at breeding sites. High relative humidity favours metabolic processes in the vector, prolongs the survival of the mosquito vector and allows the parasite to complete the necessary life cycle so that it can transmit the malaria parasite to several hosts (Lindsay and Mackenzie, 1997). Low humidity levels cause the vectors to feed more frequently to compensate for dehydration. Under conditions of optimal humidity, mosquitoes tend to survive for a longer period, which allows them to disperse farther and to

have a greater opportunity to participate in malaria transmission cycles (Lindsay and Mackenzie, 1997; Liehne, 1998).

Anopheles mosquitoes transmit malaria parasites when the environmental parameters, such as water availability, temperature and humidity permit. In many parts of the world where temperature is not a limiting factor, malaria transmission is highly seasonal, with its peak following the period of peak rainfall. Understanding how malaria varies in the community as a result of seasonal or year-to-year changes in environmental factors is important for the planning of national malaria control programmes since it may allow interventions to be adapted to specific sites or times of the year. This is essential for effective disease control.

2.3.1.2 Host factors

The distribution of malaria infection within the human population is highly variable and is mainly dependent on factors such as age, degree of the risk of individual exposure to infective mosquito bites, acquired immunity to malaria from previous exposure, drug resistance, genetic and socioeconomic factors.

Children under the age of five have been observed to be at highest risk of malaria infection in sub-saharan Africa. In Kilifi District, it has been estimated that severe malaria attacks 1 in 15 children before their fifth birthday (Snow *et al.,* 1993; 1994) whereas in the Gambia, it has been reported that about 1% of children under the age of 5 years of age die each year from malaria (Greenwood *et al.,* 1987). This has been attributed to the low level or lack of immunity to malaria in infants, as compared to adults who due to repeated exposure to malaria infections, have developed immunity to the disease over time (Molyneux *et al.*,

1989). The immune status of individuals and the entire population plays an important role in the clinical response to infection and transmission. Adolescents and adults rarely develop severe disease or die after repeated infection with *P. falciparum* because they have presumably developed natural immunity that limits parasite replication and severe forms of malaria (Baird, 1995). In high transmission areas, age-specific peak mortality is high at a younger age as compared to low transmission areas where this occurs later in life (Greenwood, 1990).

Genetic factors such as possession of the sickle cell trait (AS) (Allison., 1954), the presence of the duffy blood factor on the surface of red blood cells (Miller *et al.,* 1976), β and α thalassemia (Weatherall and Clegg, 2002; Mockenhaupt *et al.,* 2004), and glucose-6-phosphate dehydrogenase deficiency (Luzzatto *et al.,* 1969) have also been associated with decreased susceptibility to malaria, whereas other factors such as a low frequency of the class 1 major histocompatibility complex molecule HLA–B53 has been associated with severe malaria in the Gambia (Hill *et al.,* 1992), and haemoglobin C protects against *P. falciparum* malaria (Wellems and Fairhurst, 2005).

Chances for the occurrence of malaria and outcomes are also likely to be influenced by the socioeconomic status of the individual/family concerned. Housing structure and type determines the level of personal exposure to infective mosquito bites; hence those living in unscreened houses appear to be more at risk compared to individuals living in screened houses (Lindasy *et al.,* 2003). Maternal education also has an influence on the outcome of malaria infection, as this affects the treatment-seeking behaviour. However, the few studies

that have been conducted in Africa on the relationship between socio economic status and malaria produced conflicting results (Koram *et al.,* 1995; Carme *et al.,* 1994). In rural areas, illiterate mothers visit traditional healers more often (Molineux, 1988) and they also have no resources for prevention or chemoprophylaxis. The observations indicate that education level and household wealth are both important factors in malaria transmission.

2.3.1.3 Vector factors

Vector efficiency in malaria transmission is determined by factors such as the degree of preference for human blood feeding (anthropophily) (Mbogo *et al.,* 2003), vector abundance (Minakawa *et al.*, 2002b) and the survival probability (Charlwood *et al.,* 1997) of female mosquitoes. Malaria transmission is directly proportional to vector abundance, the number of infective bites per day per mosquito and the probability of the mosquito surviving long enough to become infective (Trips and Hausermann, 1986). Mosquito survival through the sporogonic stages is most important because the successful completion of this stage ensures parasite development from gametocyte ingestion to inoculation, a process which can last as long as 8 to 30 days depending on ambient temperature.

In a highly anthropophilic vector population, with high adult mosquito population densities, the successful completion of sporogony is likely to result in a high entomologic inoculation rate (EIR). EIR is a measure of the intensity of malaria transmission, expressed as the number of sporozoite positive mosquito bites per year. The EIR is likely to be high if the vector density and man biting rate is high. In Africa, EIR's are highly variable, ranging from <1 to >1000 infective bites per person per year (Beier *et al.,* 1999). Generally, higher EIRs

are associated with stable and intense malaria transmission (Mbogo *et al.*, 1995). Beier *et al.* (1999) found that the relationship between EIR and malaria transmission in Africa is such that any detectable EIR is associated with prevalence rates of *P. falciparum* malaria, large enough to have an impact on public health. However, Kabiru (1994) and Mbogo *et al.* (1995) have recorded *P. falciparum* prevalence rates of 44.7% and 49.3% in two respective communities on the Kenyan coast where EIRs were 0.001 and another where no infected mosquitoes were found, indicating that high malaria prevalence rates can be seen with extremely low or non detectable EIRs. The implication of this for malaria vector control is that the extent of malaria control efforts employed in any epidemiological setting will have to be specific to the level of parasite transmission. Substantial reductions in the transmission intensity are necessary to reduce the prevalence of malaria infection in human populations.

2.3.1.4 Parasite factors

Four *Plasmodium* parasites namely *P. falciparum, P. vivax, P. ovale* and *P. malariae* are responsible for malaria infection in humans. Of the four, *P. falciparum* is the most virulent (Gupta *et al.*, 1994). The other *Plasmodium* species have also been reported to cause serious illness, for example *P. vivax* is widespread in Asia and Central and South America where it reportedly causes fewer severe complications but relapses months after infecting the host because of secondary exo-erythrocytic cycles. *Plasmodium malariae* can persist in humans as an asymptomatic erythrocytic disease for many years following an untreated or incompletely treated primary infection. This parasite has been associated with renal complications (Kulwichit *et al.*, 2000). *Plasmodium ovale* is the rarest of the four species and is apparently more restricted in distribution. However, it is common in the West African

countries of Ghana, Liberia, and Nigeria and in neighbouring areas (Chin and Contacos, 1966; Richter *et al.*, 2004).

The resistance of *Plasmodium* parasites to antimalarial drugs has complicated the treatment of malaria in sub-Saharan Africa and is one of the most serious threats to the control of malaria. Drug resistance has led to the withdrawal of cheap drugs such as chloroquine from the malaria drug regimen in almost all parts of Africa, necessitating the use of drugs that are more expensive and may have serious side effects. The *Plasmodium* parasites have extremely complex genomes and the ease with which they can switch between the microenvironments in different hosts, and the metabolic changes required illustrate the difficulty in studying the exact modes of action of the anti-malarial drugs on parasite metabolism (WHO, 1987). Resistance develops more quickly where large populations of parasites are exposed to drug pressure. The increasingly rapid spread of resistant malaria may be due to an increasingly efficient mosquito vector. This phenomenon may be explained by the increased oocyst formation efficiency that has been observed with resistant species and the reduction in gametocyte rates, and thereby transmission after treatment with co-artemether (Sutherland *et al.*, 2005).

2.4 Evaluation of Malaria Parasite Transmission

The evaluation of malaria parasite transmission rates in nature is performed by the identification of the source of blood meals using direct Enzyme-Linked Immunosorbent Assay (ELISA) (Beier *et al.,* 1988) and determination of the sporozoite loads in the salivary glands.

2.4.1 Detection of mosquito blood meal sources

The identification of blood meals taken by malaria vectors is an important procedure in malaria epidemiology because the degree of human feeding influences the probability that mosquitoes will come into contact with gametocyte carriers and thus acquire and transmit *Plasmodium* infections. Garret-Jones *et al.* (1980) demonstrated that the most successful vectors of malaria fed most commonly on humans and secondarily on cattle and other domestic animals, depending on host availability. In tropical Africa, it has been estimated that 80% of *Anopheles* species feed on any large mammal that is available. In areas where active malaria transmission is being reintroduced after a period of eradication or low transmission, the identification of blood meals from mosquitoes can provide important epidemiological information on host preference by different species, and a useful guide on which species to target for control. Along the Kenyan coast, a high degree of preference to human feeding by *Anopheles* vectors of malaria has been reported as a major factor contributing to their efficiency of *P. falciparum* transmission. Mbogo *et al.* (1993b) reported a human blood feeding rate of 94.4% in *An. gambiae* s.l and 90.8% in *An. funestus* Giles along the Kenyan coast. The high preference for human feeding, facilitating frequent vector contact with gametocyte carriers provides evidence for the high malaria transmission

observed along the Kenyan coast. Several methods have been developed for the detection of the different sources of blood meals in disease vectors. These include the use of enzyme linked immunosorbent assay (ELISA), precipitin tests, haemagglutination assays, counter current immuno-electrophoresis and immunoflourescence. The ELISA method developed by Beier *et al.* (1988) is the most commonly used test. The ELISA assay meets the criteria for a field operational kit for the detection of blood meals in mosquitoes and has been the most commonly used technique. A brief description of this method is given in the section on methods of this thesis.

2.4.2 Determination of sporozoite infection rates in wild mosquitoes

The rate of mosquito infectivity in the field has always been measured by determining the proportion found to be carrying *Plasmodium* sporozoites. Previously, the determination of sporozoites rates was done by the dissection of the salivary glands, and this was obviously a tedious, and labour intensive process, especially in areas where mosquito density was high. In addition, this process requires fresh female mosquito samples. This method was not easily applicable in the field. Wirtz *et al.* (1985) developed an ELISA method for the detection of the *P. falciparum* circumsporozoite protein in field-collected mosquitoes. ELISA combines the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. There are two main variations of this method: The ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen. In the sporozoite ELISA technique, monoclonal antibodies are used to detect circumsporozoite proteins of *P. falciparum* (Wirtz *et al.*, 1985) and the results are read

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visually (Beier and Koros, 1991) or by using an ELISA reader (Wirtz *et al.,* 1985; Beier *et al.,* 1988). The sporozoite ELISA technique is more rapid, and is very useful in detecting infectious mosquito species from either fresh or dry stored mosquito specimen. Sporozoite rates are then inferred from the proportion of human biting anophelines that test positive for *P. falciparum* circumsporozoite protein by ELISA.

2.4.3 Entomological inoculation rates (EIR)

The intensity of malaria parasite transmission in the field is estimated by determination of the entomological inoculation rate (EIR). EIR is the product of the mosquito biting rate and the proportion of mosquitoes carrying sporozoites in their salivary glands (Beier *et al.*, 1999). EIR can be used to estimate the level of transmission to individuals living in a particular location at a time and as a measure to differentiate transmission intensity between geographic areas (different villages or parts of villages) over a period.

In Africa, EIRSs in malaria endemic areas have been reported to range from between <1 to >1000 infective bites per person per year (Trape and Rogier, 1996). This reflects the extent to which the malaria transmission situation is variable in different parts of Africa, hence the need for more area specific control measures. Any control measures aimed at reducing malaria prevalence may need to substantially reduce EIRs in order to reduce the prevalence of malaria infection.

2.5 The Socio-economic Impact of Malaria

The burden of malaria has a potentially large economic impact, limiting the productivity of a country's two major assets, its people and its land. The cost of malaria prevention and treatment consumes the scarce household resources and in turn, the burden on one public health sector impacts on the allocation of already scarce resources. The annual economic burden of malaria in Africa is estimated at about $11.7 billion, or 1% of the Gross domestic product; the cost of treating a single case from a society's viewpoint is estimated at $9.84 or 12 days equivalent of active productivity, and the cost of treatment per household ranges from $ 0.2 -$15 per month (WHO, 1999). Highly malarious countries are among the very poorest in the world and typically have very low rates of economic growth (Gallup and Sachs, 2001).

The disease, which affects mainly children and pregnant women has lifelong effects on cognitive development and education levels through the impact of chronic malaria, induced anaemia and time lost or wasted away from the classroom due to malarial illness (Holding and Snow, 2001). Pregnant women are reportedly four times more likely to suffer from malaria attacks leading to the delivery of low weight babies and still births, endangering the health of the women and the prospects for the newborn (Brabin, 1983). Much of the mortality in endemic areas is concentrated among children under the age of five. In areas of stable endemic transmission about 25% of all-cause mortality in children aged 0 to 4 years has been attributed directly to malaria (Snow *et al.*, 1999). Malaria may have adverse demographic consequences as well. Malaria substantially raises the chances of infant and child mortality, and households respond to this increased risk by having more children,

thereby increasing the overall rate of population growth. In addition, investments which parents of many children can afford to make in the well-being of each child is limited — so that average levels of health care and education per child tend to be reduced. As a result, mothers of large numbers of children are less able to participate in the formal labour force, thereby also reducing the household income. Malaria imposes a heavy cost not only on a country's current income, but also on its rate of its economic growth, and therefore on the level of economic development in the long run. There is a need for more research to document the household and institutional impacts of epidemic malaria in sub-Saharan Africa where malaria epidemics are on the rise.

2.6 Entomologic Methods in Malaria Vector Population Studies

One of the fundamental requirements in the epidemiology and control of vector-borne diseases is information on the biology and ecology of the vectors. Two main methods; the direct (field-based) ecological methods and indirect (laboratory based) methods have been developed for vector population studies.

2.6.1 Direct ecological methods

The collection of certain types of information about insect populations always requires direct field measurements and experimentation. To enable the determination of sample sizes that will be representative of the entire population and be assured of good quality data a sound knowledge of the field site is necessary. In studies involving mosquito populations, direct ecological methods have been applied in the collection of information on the population sizes and species abundance, spatial and temporal distribution, movements and dispersal.

The abundance of vector populations can be measured in two ways, as absolute density, which is the number of organisms per unit area, or relative density which is the density of one population relative to that of another population. One of the most common methods applied to estimate population sizes are mark release recapture (MRR) experiments in which investigators capture and mark individuals from the population and then resample to establish what fraction of marked individuals are recaptured. The strength of mark recapture studies is that they can provide information on birth, death, and movement rates in addition to information on absolute abundance on any particular species of interest. Most

populations are constantly changing in size because of birth, death and movement rates; therefore the procedure for obtaining estimates from an open population is usually the mark-recapture method, with marked samples taken on more than one occasion. One critical assumption of mark recapture studies is that there is an equal probability of recapturing released individuals, so that marked individuals at any given sampling time have the same chances of capture as unmarked ones. However, this assumption is often violated in natural populations.

Understanding the dispersal patterns of vector mosquitoes in nature is essential to evaluating the risk of malaria transmission to humans, implementing surveillance and developing abatement strategies. It is known that malaria transmission is related closely to the population density and the flight range of vector mosquitoes (Trpis and Hausermann, 1986), and many studies have been conducted on the flight range of female anopheline species. In 1900s, Wright (1918) described the flight range of *An. sacharovi* to be 13km -14 km based on dispersal from the larval habitat to search for overwintering sites (Kligler & Mer, 1930; Kligler, 1932; Bruce-Chwatt, 1970). Rosenstiel (1947) reported that the flight range of *An. freeborni* is over 42 km before hibernation. It has also been reported that *Culex tarsalis* is able to fly at least 17.7 km per night (Bailey *et al.,* 1965) and *Aedes taeniorhynchus* can fly long distance up to 32 km from breeding sites, when it is wind assisted (Johnson, 1969). In Africa, the flight range of *An. gambiae* has been reported to range between <1 to 1.5km from the point of release (Thomson *et al,* 1995; Costantini *et al.,* 1996). However, none of the MRR studies conducted so far has included the dispersal of *An. funestus*. Information on vector dispersal is very important for the determinations of how far genes such as

insecticide resistance genes are exchanged within and between populations, and information on vector flight range can be very important in the planning of the geographical limits that any vector control intervention should cover.

Direct ecological methods however require tools such as geographic information systems (GIS) to allow the integration of data collected from direct measurements with other data such as weather data (rainfall and temperature), regional maps and landscape features (Omumbo *et al.,* 1998). GIS provides tools for the studies of population dynamics of disease vectors in association with environmental and habitat features on multiple spatial scales. A GIS is a computer-based system for automating, storing, manipulating and displaying mapped information and data (Chrisman *et al.,* 1989). It includes spatial data (locations) in forms of geographic coverage (maps) and descriptive data (attributes) of a relational database associated with the mapped features. GIS therefore allows for the overlaying of a variety of data (e.g. climate, vegetation type, habitat distribution, vector abundance and infection rates) to identify factors that may explain the spatial and temporal distribution patterns of vectors and disease. With the aid of spatial analysis, GIS can provide the means to identify and map the larval habitats of vector species, and their relationships to human settlements, thus predicting the potential risk for disease transmission.

The application of GIS and spatial analysis to define the epidemiology of vector-borne diseases has been documented for a number of diseases, such as Lyme disease (Kitron *et al.,* 1991), trypanosomiasis (Rogers & Williams, 1993) and malaria (Beck *et al.,* 1997; Omumbo

et al., 1998; Craig *et al.*, 1999). The study by Omumbo *et al.* (1998) however presents the first attempt to develop an empirical map of malaria endemicity for Kenya.

2.6.2 Indirect methods

Indirect methods of studying vector populations involve the analysis of genetic material to infer the population demographic structure, its history and current status by analyzing its genetic structure. Indirect methods provide information on the genetic basis of any population characteristics.

2.6.2.1 Molecular biology methods

The application of molecular biology and modern biotechnology to malaria vectors studies is currently playing a major role in the development and evaluation of new methodologies, which are expected to lead to the production of new tools for vector control. Examples of the applications of molecular biology in relation to vector biology and malaria control are the utilization of DNA technology to improve vector identification techniques with an aim to enhancing the efficiency of existing control measures by providing accurate data and information about vector populations. The novel approach to malaria control through the genetic manipulation of anophelines to disrupt the parasite transmission cycle (James *et al.*, 1999) and the application of molecular methods to characterize the level of genetic variation within and between populations (Tabachnick and Black, 1994) are examples of recent advances in malariology.

Anopheline mosquitoes are members of sibling species complexes. Sibling species are reproductively distinct but cannot be distinguished reliably by morphological methods alone and require alternative more specific methods for identification. The identification of these

species in a control programme is important as the different species within a complex may exhibit differences in ecology, vectorial capacity and response to control measures (White, 1982). Crossing experiments of the offspring from unknown specimens against laboratory colonies of known species (Davidson and Hunt, 1973) is one of the methods that have been of great value in the elucidation of species within species complexes. However, this method is laborious and time consuming for routine identification of specimen hence the development of more reliable methods such as the use of polytene chromosome banding techniques (Coluzzi *et al.,* 1979), isoenzyme typing (Mahon *et al.,* 1976); the use of cuitcular and internal hydrocarbons (Hamilton and Service, 1983) and most recently, the development and use of DNA based methods.

2.6.2.1.1 DNA Based methods

The advent of the polymerase chain reaction (PCR) has revolutionized the application of DNA technology in the study of living organisms. The shift from biochemical methods to DNA-based methods allows the identification of very old archived specimens, given that DNA is easily preserved by desiccation in alcohol. DNA based methods have the advantage of being applicable to all developmental stages of mosquitoes and with the PCR, very minute amounts of template DNA is required (Sambrook *et al.,* 1989). Several techniques have been developed for the application of DNA-based methods to study mosquito populations, and other organisms either through the amplification of anonymous regions of the genome such as the use of randomly amplified polymorphic DNA (RAPD-PCR) which requires no prior knowledge of the genome and tends to target repetitive and rapidly evolving genome regions (Wilkerson *et al.,* 1995) or targeting known specific sequences

such as ribosomal DNA (rDNA). Very basic, but important is the application of DNA methods in species identification, which was previously based on mating experiments. Currently, the identification of *Anopheles* species is based on a ribosomal DNA method. Some of the most widely applied PCR assays target nuclear rDNA, which is present as a single X-linked locus in anophelines (Rai and Black, 1999). The rDNA is organized as a tandem repeated array of conserved genes (18S, 5.8S and 28S) punctuated by rapidly evolving non-coding spacers such as the internal transcribed spacers '1 and 2' (ITS1 & ITS2) and the intergenic spacer (IGS). The most recently developed *Anopheles* species identification assays are based on differences within the ITS2 (Scott *et al.,* 1993; Koekemoer *et al.,* 2002; Proft *et al.,* 1999; Beebe *et al.,* 2001).

2.6.3 The population genetics of *Anopheles* vectors of malaria

Population genetics is the study of genetic variation in natural populations and the factors that influence this variation. Understanding genetic variation in vector populations provides a foundation for understanding the role of the vector in disease epidemiology. Random mating, population size, migration, mutation and selection are the factors that shape genetic variation in vector species and also influence variation in vectorial capacity and vector competence traits. Methods used to identify genetic variation in natural populations include the analysis of chromosomal variants, isozymes, DNA markers and morphometric methods, for identifying morphologic mutants. Some of the most commonly used methods that have been applied in studying genetic variation in *Anopheles* mosquito populations are the analysis of inversion polymorphisms in polytene chromosomes (Coluzzi *et al.,* 1985) isozyme electrophoresis and most recently there has been more focus on the use of DNA

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markers. DNA analysis is highly suitable for population studies because it is the most direct analysis of genetic material possible and provides an easy to use method (Saiki *et al.*, 1988; Sambrook *et al.*, 1989; Post *et al.*, 1991). The success of a genetic modification programme requires the precise identification of appropriate genes for introduction into natural populations, and a clear understanding of the population genetic structure of the vector. Through the use of various DNA markers, especially microsatellite DNA markers the *Anopheles gambiae* has been widely studied, and reports mainly on variations in the population genetic structure of this species in Africa are available (Lanzaro *et al.*, 1995; Lehmann *et al.*, 1996; 1997; Kamau *et al.*, 1998; 1999).

2.6.3.1 Microsatellite DNA analysis of *Anopheles* populations

Microsatellites are DNA fragments consisting of relatively short regions of tandemly repeated DNA sequences, such as dinucleotides or a trinucleotides, (e.g [GT]n, [GAC]n), etc. In most of the eukaryotic organisms, microsatellite sequences are abundant, widely dispersed throughout the genome and are usually highly polymorphic in length due to a variation in the number of repeats within a particular locus as a result of uneven cross over (Jeffery *et al.* 1985) or slippage of the DNA polymerase during replication (Tautz, 1989). Microsatellite loci have been described as powerful markers for mapping qualitative and quantitative traits of mosquitoes (Zheng *et al.*, 1993) and in the field for measuring intra-species differentiation because of their high polymorphism, co-dominance, abundance throughout the genome, and the relative ease of scoring.

It is known that microsatellite DNA sequences can differ in the number of repeats among individuals (Slatkin, 1995). With the advent of polymerase chain reaction (PCR) technology, this property of microsatellite DNA has been exploited and is currently a highly versatile genetic marker. Microsatellites are now important tools for application in determining the pattern of relationships between individual organisms and populations because they are capable of highly discriminating bi-parentally inherited co-dominant markers. The almost random distribution of microsatellites within the genome and their high level of polymorphism have greatly facilitated the construction of genetic maps (Sharakov *et al.*, 2004) and enabled subsequent positional cloning of several genes.

Microsatellite DNA analysis has also been applied in the development of an integrated genetic map of *An. gambiae* (Zheng *et al.*, 1991; 1993; 1996). The availability of the genetic map has made it possible to develop genetic markers evenly located in the *Anopheles* genome and has therefore enabled researchers to conduct further broadly based genetic studies of anopheline biology, which are a requirement for any meaningful population studies. For example, microsatellite DNA markers have been applied in the study of gene flow between *An. gambiae* populations (Lehmann *et al.*, 1999), comparing genetic differentiation between populations (Lehmann *et al.*, 1996; 1997; Kamau *et al.*, 1998; 1999) and in determining the population genetic structure of vector populations (Lehmann *et al.*, 1997). Allelic variation at microsatellite loci has been proven to be informative in studies of population structure of *An. gambiae* (Lanzaro *et al.*, 1995; Lehmann *et al.*, 1997). The availability of accurate genetic information on *An. gambiae* populations paves the way for

the future development and testing of novel vector control approaches, such as the introduction of genetically modified mosquitoes that are refractory to the malaria parasite. Most recently, microsatellite DNA markers for the malaria vector *An. funestus* have been isolated, and characterized (Cohuet *et al.,* 2002), enabling studies on the population genetics of this important vector (Braginets *et al.,* 2003).

CHAPTER THREE

GENERAL METHODOLOGY

3.1 The study area

Kenya is located on the East African Coast and the country covers an area of 582,000 square kilometres. It borders Ethiopia in the north, Sudan in the northwest, Uganda on the west, Tanzania in the south and Somalia in the east. Lying between 3^0 North and 5^0 South latitude and between 34^0 and 41^0 East longitude, it is entirely within the equatorial zone and almost bisected by the equator. It has 400 kilometres of the Indian Ocean shoreline (Ojany and Ogendo, 1988).

The study sites were located in Kilifi District, on the coast of Kenya. The district is one of the 6 districts that form the Coast Province of Kenya. It borders Malindi District to the North, Mombasa to the South, Kwale to the Southwest and Taita Taveta District to the West. To the East is the Indian Ocean. Kilifi lies between the latitude $2^0$20" and 4^0 South and between longitude 39^0 and $40^0$14" East. Kilifi District covers a total area of 4,779 square kilometers and according to the 1999 population census has a population of approximately 544,303 inhabitants (Central Bureau of Statistics, 2001).

3.1.1. Geographic location of the study sites

The study sites are located within the Kenya coastal plain, which is a narrow belt varying in width from 3 to 20km from the coastline. It is covered by dense forest and savanna type of vegetation. The natural vegetation varies widely from patches of rain forest in the Arabuko

Sokoke forest to dry thorn bushes. Most of the human populations live within the 10 km wide coastal strip and in the hilly areas. The altitude ranges from zero to 400m above sea level. The study area has been previously described for malaria epidemiological and demographic survey (Snow *et al.*, 1994) and for entomological surveys (Mbogo *et al.*, 1993a; 1995; 1996; 2003). Figure 3.1 shows a map of Kenya, and the location of Kilifi and the study areas in the District.

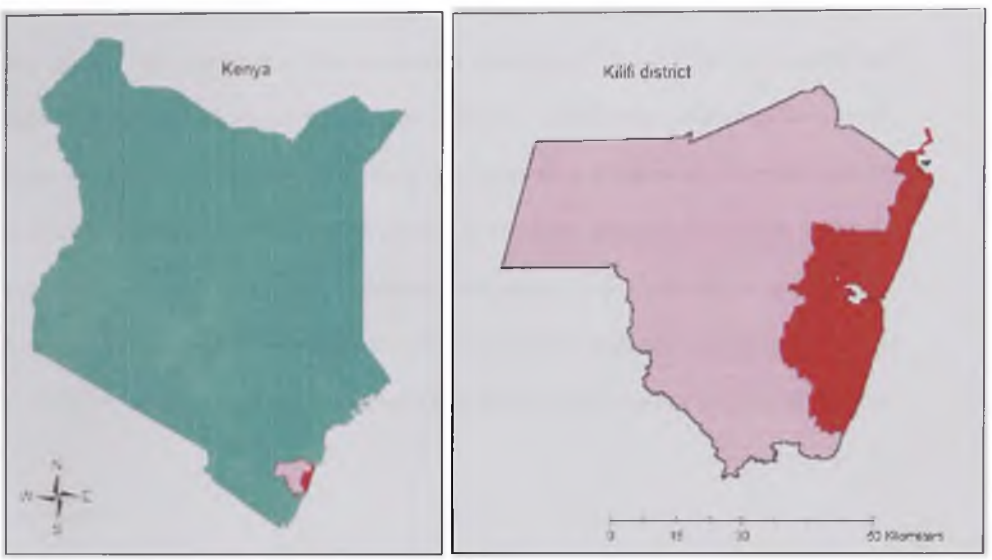

Figure 3.1: The map of Kenya showing the location of Kilifi District. The red area shows the Kilifi Demographic Surveillance System (DSS) area.

3.1.2 The inhabitants

Inhabitants of Kilifi District mainly belong to the Giriama tribe, a subgroup of the Mijikenda ethnic group. The majority of the population consists of rural farmers of coconut and cashew nut for cash crops and maize for domestic consumption. Mangoes, tangerines, oranges and paw paws also provide a source of income for subsistence. Domestic animals kept include cows, goats and sheep. A small fraction of the population is employed in the hotel and the construction industries, mining coral stones in quarries and as employees in government offices. Houses are mainly built from mud-filled walls and makuti (palm leaves) thatched roofs. Figure 3.2 shows an example of a typical residential house in the study area.

3.1.3 Climate

Meteorological data for Kilifi district during the study period was obtained from a weather station located at the Kilifi Agricultural Institute located approximately 30 – 35 kilometres away from the study sites. Daily records of data on rainfall, maximum and minimum temperatures and relative humidity were obtained for the years 2001-2002 and monthly averages calculated for each year. Average annual rainfall ranges from 400mm in the hinterland to 1,200mm at the coastal belt. The rains are bimodal with long rains falling in April-June (with a peak in May) and short rains during October-December. Seasonality of rainfall is quite marked in the coastal strip but barely noticeable at the hinterland. Average maximum temperature along the coastal belt is 30.8^{0}C (range 28-32^{0}C) and average minimum temperature is 23.1^{0}C (range 22-25^{0}C). The district is generally hot and humid all year round with average relative humidity ranging between 70-78%. The optimum

temperatures and relative humidity for the development of the malaria vectors are 20-30^{0}C and 70-80% respectively. Figures 3.3, 3.4 and 3.5 show the distribution of rainfall, temperature and relative humidity in Kilifi district during the year 2001 and 2002.

Figure 3.2: A typical residential house found in the study area in Kilifi District, Kenya.

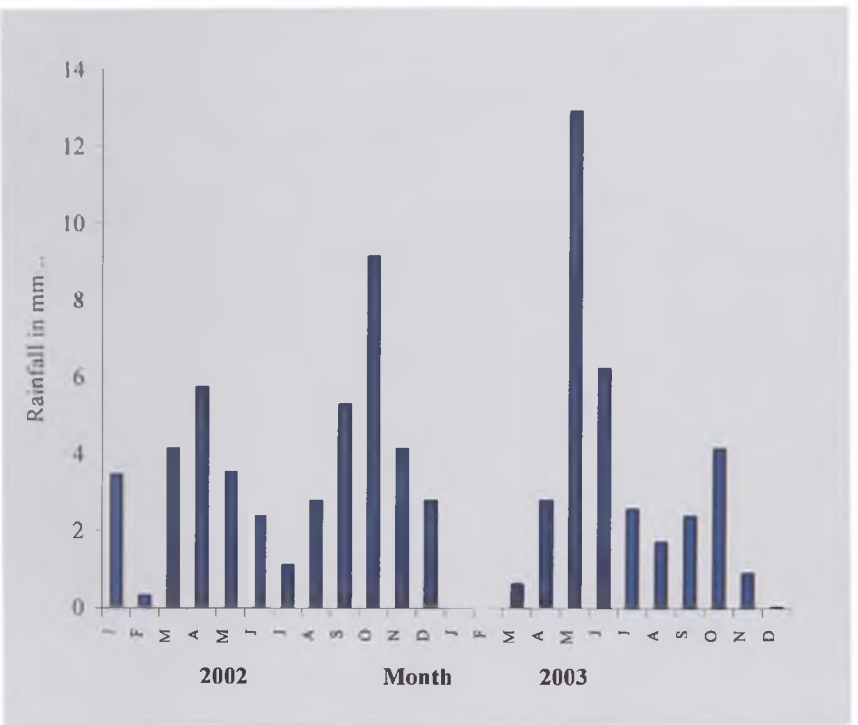

Figure 3.3: Total Monthly rainfall in millimeters, January 2002 – December 2003

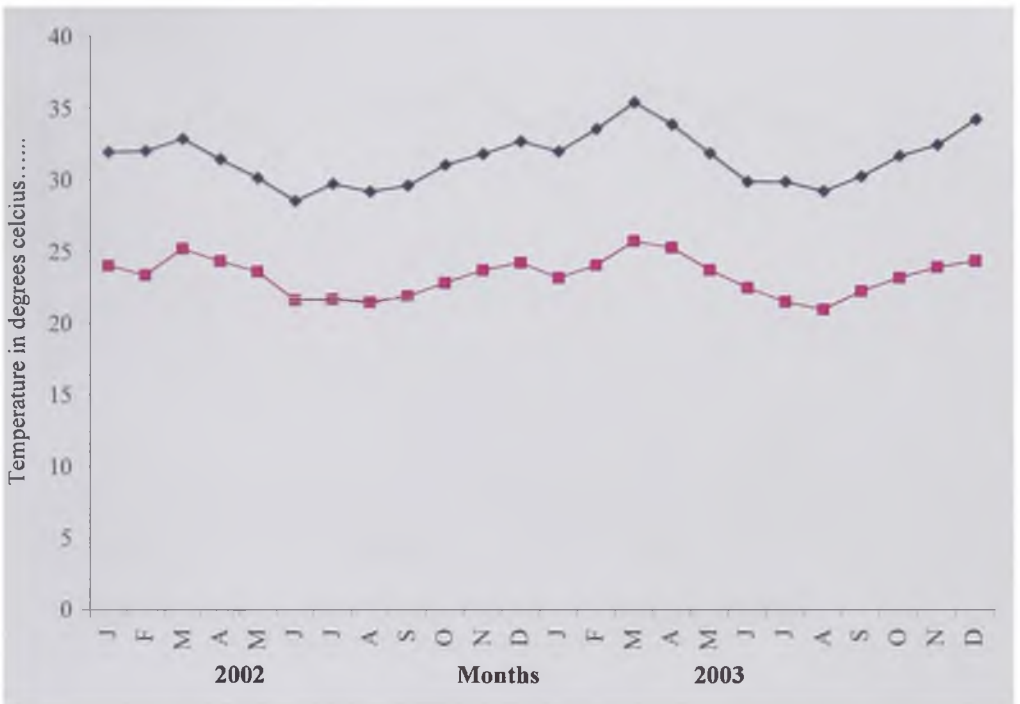

Figure 3.4: Mean monthly maximum and minimum temperatures recorded at Kilifi from January 2002 – December 2003

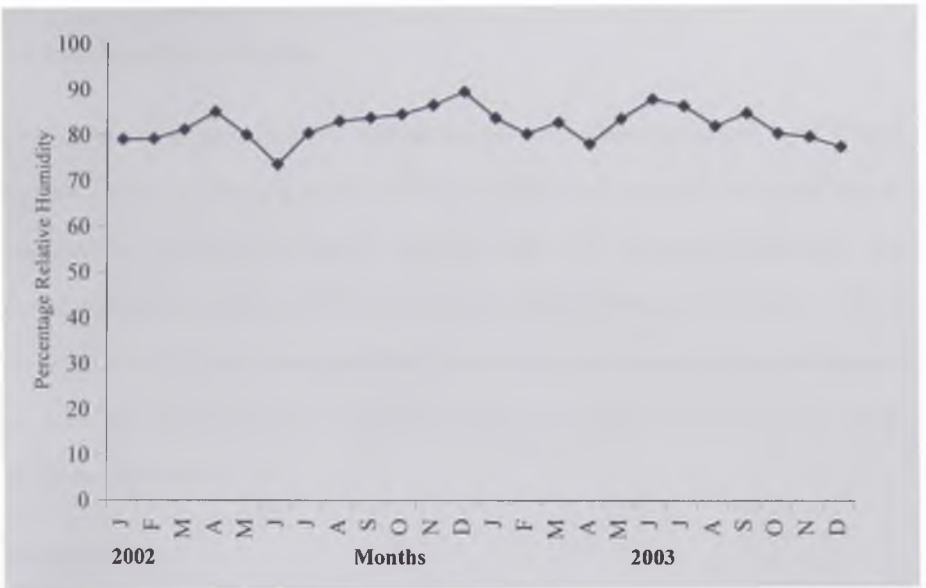

Figure 3.5: Mean monthly Relative Humidity at 1200 hours at Kilifi from January 2002 – December 2003

3.1.4 Selection of the study sites

Three representative sites; Jaribuni, Mtepeni and Majajani along the Kenyan coast (Figure 3.6) were selected for the study based on the fact that they are known to have many aquatic larval habitats of anopheline species, relatively high adult mosquito populations and endemic malaria transmission (>50% among school children) (Mbogo *et al.,* 2003). All the three sites lie in the foot plateau but differ in the terrain and seasonal rivers that transect them. Each site covers at least $4km^2$ and has a school at a central location. The three study sites are described below.

3.1.4.1 Jaribuni

Jaribuni is located 03° 37.3′′ South and 039° 44.6′′East. Jaribuni lies in the foot plateau, with a fairly flat terrain. Neighbouring the Jaribuni village is the Kaya forest, with numerous indigenous plants and trees. The soils are more sandy compared to the two other sites, Majajani and Mtepeni, and this provides better infiltration of ground water and less support for surface puddles. The Jaribuni River flows across the site and remains a permanent water body all year round. During the rainy season, water levels rise and temporary larval habitats are formed at the edge of the river. These habitats expand and contract with the rise and fall in water levels, and may disappear when the water reduces significantly. The dominant vectors of malaria are *Anopheles funestus* followed by *An. gambiae s.s.,* with *An. funestus* being found at a higher density than *An. gambiae* during the dry season (Mbogo *et al.*, 2003). Compared to Majajani and Mtepeni, compounds at Jaribuni are closer to each other except for a few, which are distances apart.

3.14.2 Majajani

This site is located on 03° 39.5′ South and 039° 47.2′ East, and in between Mtepeni and Jaribuni. It also lies in the foot plateau and the terrain is flat. The soil is rich in organic material and clay. The vegetation mainly consists of shrubs and short grass. *Anopheles gambiae s.s.* is the predominant vector of malaria in Majajani, where it has been shown to have a preference rate of 94.4% of feeding on humans (Mwangangi *et al.*, 2003). Compounds are sparsely distributed at this site.

3.1.4.3 Mtepeni

Mtepeni is located on 03° 54.5′ South and 039° 43.6′ East. This site also lies in the foot plateau. Compared to Jaribuni and Majajani, this site has a more hilly terrain characterized by broader plateaus at hilltops and rolling ravines. The soils are less sandy and thick bushes and shrubs characterize the vegetation. There are very few permanent larval habitats existing at the site, and the few that are available exist as swamps. *Anopheles gambiae* is the major vector of malaria and very few *An. funestus* have been recorded. (Mbogo *et al.*, 2003). Of all the three sites, compounds at Mtepeni site are the most sparsely distributed.

Figure 3.6: The base map of the three study sites showing sampling sites and ecoclimatic conditions at Jaribuni (1), Majajani (2) and Mtepeni (3).

3.1.5 Development of base maps, mapping of compounds and larval habitats

The geographic positioning system (GPS) [Garmin International Inc., Olathe, KS] was used to record the latitude and longitude co-ordinates of all the compounds and larval habitats within the study areas. Compounds refer to a collection of houses, ranging from a minimum of three houses, to a maximum of 10 or 12 houses. Using GIS methodologies and Arcview and ArcInfo software, base maps of the three study sites (Figure. 3.6) were produced to give a pictorial representation of the study area. It also showed the precise locations of all the compounds selected for entomological collections and the relative distances to the mosquito larval habitats. Geo-referenced layers of roads and major landmarks were overlaid onto the coverage to depict the distributions of larval habitats and households on a base map in ArcView 3.2a®. The point distance command in Arc Info was used to generate distances between larval habitats and the nearest neighbouring households.

3.2 Entomological Sampling Methods

Mosquito sampling from houses within the selected compounds was done with the assistance of 10 trained mosquito collectors using three techniques. These were day resting indoor collections (DRI), Human Biting Catches (HBC) and CDC light traps. Mosquito collections using these methods were done in a total of between 18 -26 households at each site.

3.2.1 Day resting indoor collections (DRI)

Day resting indoor collection of mosquitoes was done by the manual aspiration method. A light bulb torch was used to provide light in the room(s) when searching for mosquitoes resting on walls and dark corners. A glass aspirator was then used to carefully capture any mosquitoes seen. These were then released from the aspirator into a paper cup. The Paper cup was then stored in a coolbox and transported to the laboratory. The exercise was conducted by two collectors in each house, for a total of 15–20 minutes per house. The time for DRI collections was from 7.00 am to 11.00 am, and DRI was done once per week in every household. This method targeted female *Anopheles* who had taken a blood meal the previous night and were resting.

3.2.2 Human bait collections

Human bait collections were conducted at night, both indoors and outdoors. Collection times were from 7.00 pm to 1200 midnight. Two male collectors sat inside and outside the house respectively, with legs exposed to the knees. The collectors captured all landing mosquitoes using the aspirator aided by light from a torch. During each hour of collection, the collectors had a 15-minute resting period, after which they alternated their sitting positions. It was not possible to conduct human landing catches in all the compounds each night, and so a sequential sampling scheme was devised, whereby a total of 4 compounds were selected to be sampled each night. To determine which compounds to sample daily, the compounds were randomly selected then assigned a night for collection. Two houses were selected for sampling within each compound each night. The houses were selected based on the number of people sleeping in that house per night, and its productivity in terms of adult mosquitoes

as was seen during DRI collections from that house. Houses that had more than three sleepers were considered first. Four to five compounds were sampled each night and it took four sampling nights before repeat collections were done in each compound. All the mosquitoes collected were placed in paper cups labelled separately for indoor and outdoor collections and stored in coolers before transportation to the laboratory for processing.

3.2.3 Light Traps

CDC Light traps were placed in selected houses in compounds where human landing catches were not in progress. The light traps were placed in the houses at 1800h and were left on until 0600h. In each household, one trap was carefully hung in the room where residents were sleeping so as to capture blood-seeking mosquitoes.

Every morning, collections from the light traps were emptied into labelled petri-dishes, which were stored in a coolbox and transported to the laboratory for processing.

3.2.4 Pyrethrum Spray Catches

Randomly selected rooms (1 room/house) were sprayed with pyrethroid insecticide formulation (Raid® Insecticide: Tetramethrin, Allethrin, Deltamethrin) and 10 minutes after spraying, the knockdown mosquitoes were picked and placed from thw white sheet laid on the floors and laced onto moist filter paper in labeled petri dishes.

CHAPTER FOUR

THE SPATIAL AND TEMPORAL DISTRUBUTION PATTERNS OF *ANOPHELES GAMBIAE* AND *ANOPHELES FUNESTUS* ALONG THE KENYAN COAST

4.1 Introduction

Mosquitoes have an almost worldwide distribution, being found throughout the tropics and temperate regions, and beyond the Arctic Circle. They are absent only in the Antarctic Circle and a few islands (Service, 1993a). The colonization of many contrasting environments by mosquitoes has been enabled by the availability of a great diversity of habitats, their life history strategies and ability to adapt to various aquatic habitats.

Mosquitoes within the genus *Anopheles* are widely distributed. Members of the *Anopheles gambiae* complex have been reported from most countries in Africa and its adjacent islands including Madagascar as well as Saudi Arabia and Yemen (Coetzee *et al.*, 2000; Coetzee, 2004). The natural distribution pattern of disease vectors almost always determines the distribution of the disease, and the contribution of vectors to disease transmission is determined by several factors including environmental conditions prevailing at a given location, their degree of endophagic behaviour, vector presence and abundance at human habitations.

Climatic factors such as rainfall, temperature and relative humidity are fundamental factors that drive or limit the distributions of biologic systems. Many studies have shown that climate is a major factor governing the distribution of insects (Andrewartha and Birch, 1954; Southerst *et al.,* 1995) either by acting directly on insect populations themselves or indirectly by affecting the structure of the ecosystem they inhabit. For malaria vectors, each mosquito species has unique environmental tolerance limits (Martens *et al.,* 1995). Vector distribution is highly dependent upon the availability of suitable aquatic larval habitats and the proximity of the human host as a potential source of blood meals. The dependence of vector species on aquatic habitats is an invariant aspect of the mosquito life cycle, and the availability of an appropriate aquatic realm, necessary for egg, larval, and pupal development, critically determines the abundance of mosquito species (WHO, 1982; Service, 1993b; Kettle, 1995). Localized variations in the effect of climatic changes on the presence of suitable mosquito breeding conditions have been implicated in the variability observed in the ecology of the vectors and the distribution of the disease across different spatial scales. Changes in temperature and humidity, could impact the presence, development, activity, and survival of pathogens, vectors, zoonotic reservoirs of infection, and their interactions with humans which consequently lead to changes in their geographic distribution (Meade *et al.*, 1988; McMichael, 2003).

The availability of information on local malaria vector species distribution pattern provides important baseline information, which when incorporated into a GIS, can provide a systematic way to spatially link known epidemiologic data on disease systems with relevant

features in the environment, and to develop models that can then be used, by extrapolation to predict the risk of disease over broad geographic areas where data are not available. An accurate and predictive understanding of the geographic distribution of vector species provides valuable data for use in control programmes and permits the efficient planning of strategies for targeted vector control.

Along the Kenyan coast, malaria is transmitted by *An.gambiae* s.l. and *An. funestus* (Mbogo *et al.*, 2003). Other *Anopheles* species found along the coast include *An. nili, An. squamosus, An. pharoensis* and *An. coustani* (Mbogo *et al.*, 1995). Since the early 1990's a lot of field based research has been conducted on *An. gambiae s.l* and *An. funestus,* addressing the larval ecology of *Anopheles* and the dynamics of malaria transmission along the Kenyan coast (Mosha and Mutero, 1982; Mosha and Petrarca, 1983; Mbogo *et al.*, 1993b; 1995; 1996; 2003).

Environmental heterogeneity has been recognized as an important factor affecting the abundance and quality of mosquito larval habitats and hence the production of adult mosquitoes (Mwangangi *et al.*, 2004). Vector abundance is an important component of measuring transmission intensity. In an area where malaria prevalence is high, high vector densities are likely to increase the risk of malaria transmission. In an area where malaria is endemic, information on the vector species abundance, distribution and factors regulating the vector populations remains important for malaria epidemiology. This study was conducted to provide information on the spatial and temporal distribution patterns of *An. gambiae s.s.* and *An. funestus s.s.*, the main vectors of malaria along the Kenyan coast.

4.2 Materials and Methods

This longitudinal study was conducted over a period of two years, 2001 and 2003.

4.2.1 The study area

The study area has been described in detail in Chapter Three of this thesis. These studies on the spatial and temporal distribution patterns of *An. gambiae s.s.* and *An. funestus s.s.* mosquitoes were conducted at two sites, Jaribuni and Mtepeni.

4.2.2 Mapping of study compounds and the development of base maps

A survey of the sites was conducted to identify potential larval habitats and households located within a 1km radius. Larval habitats were selected for the study based on their larval productivity. The latitude and longitude of each productive larval habitat and households within the study areas were recorded using a hand held GPS (Garmin International Inc., Olathe, KS). Geo-referenced layers of roads and major landmarks were overlaid onto the coverage to depict the distributions of larval habitats and households on a base map in Arc View 3.2a®. These data and information on the distribution of larval habitats was also utilized for the studies. A total of 11 compounds were identified in Jaribuni, and 10 in Mtepeni for collection of adult mosquitoes. Using GIS methodologies (ArcView, ArcGIS and ArcInfo software), base maps of the study sites were developed to give a pictorial representation of the area, showing the precise locations of all the compounds selected for entomological collections (Figure 5.1).

4.2.3 Field sampling of *Anopheles* mosquitoes

Anopheles mosquitoes were sampled both indoors and outdoors, once weekly from January to December 2001-2002. During the year 2001, indoor sampling of female *Anopheles gambiae* s.l and *An. funestus* was conducted using the pyrethrum spray catch (PSC) method once weekly. In 2002, the indoor day resting adult collection method (DRI) was applied; whereas outdoor and indoor collections were conducted using human landing catches (HBO and HBI). These mosquito-sampling methods are described in Section 3.2 above. Sampling was conducted all year round. DRI collections were done once weekly in all the households from 8am to 11.00am while HBO and HBI was from 7.00pm till 12.00 midnight. All mosquitoes were kept in paper cups held in a cool box and transported to the laboratory for processing.

4.2.4 Morphological identification of *Anopheles* mosquitoes

All the mosquitoes were preserved by keeping them in a -20^0C freezer for approximately 10 minutes. They were then identified as *Anopheles* species using the morphological criteria (Gillies and Coetzee, 1987) outlined below:

Palps: Smooth, with 3 pale bands: The apical band broad and enveloping the whole of the 5^{th} and apex of the 4^{th} segment; median band narrow, less than half width of apical, involving apex of third and extreme base of 4^{th} segments; basal band as median, usually confined to apex of 2^{nd} segment.

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Thorax: Ground colour variable, usually light brown or grayish; medi-annally clothed with cream or yellow scales, pointed and moderately broad; laterally scales much broader and often rounded apically; anterior pronotal lobes with scales.

Pharynx: With 12-20 pairs of rods and cones, latter with deep roots and long flattish pointed filaments, which are somewhat fimbriated, crest not bifid in posterior view; rods with short filaments, post pharyngeal ridges with very long hair like processes.

Legs: Femora, tibiae and first tarsal segment speckled to a variable degree, sometimes with speckles fused to form short lines, rarely in the form of complete rings; tibiae rather narrowly pale apically; on fore and mid legs apices of first and fourth tarsi and apices and bases of tarsi 2 and 3 narrowly but distinctly pale, 5th all dark; hind tarsi similar but basal pale bands on 2 and 3 absent or reduced 5th tarsus usually all dark.

Wing length: 2.8-4.4 mm

Wings: Pale markings, yellowish or cream. Wing field largely pale and with pale costal spots fairly long. The illustration is that of an average type of wing but there is much variation in the relative sizes of the pale and dark markings. Some of this variation may be indicative of inter-specific differences. Fringe spots present opposite veins 3-6; in addition a pale spot present between 6 and 5.2 and between 6 and the base of the wing.

Abdomen: Light brown, mainly clothed with hairs but 8th tergite usually with some scales, which may extend onto 7th tergite. Scaly cerci. Sternites conspicuously marked with symmetrical grey patches.

4.2.5 Sample preservation

Each identified *An. gambiae* body was divided into two parts by separating the head from the thorax and the abdomen. Legs and wings were detached from the rest of the body and stored in absolute ethanol in labelled vials at -20^0C until needed for DNA extraction and further molecular studies. The head and the thorax and abdomen were also preserved in separate vials for sporozoite detection and blood meal source using ELISA methods.

4.2.6 PCR methods for species identification

The ribosomal DNA method developed by Scott *et al.* (1993) was used to identify members of the *An. gambiae* species complex. Primers used are specific for *An. gambiae s.s.*, *An. arabiensis* and *An. merus*. *Anopheles funestus* mosquitoes were identified by a rapid rDNA-PCR method developed by Koekemoer *et al.* (2002) to identify members of the species group. The cocktail PCR assay is based on species-specific primers in the ITS2 region on the rDNA to identify *An. funestus, An. leesoni* Evans, *An. rivolurum* Leeson, *An. parensis* Gillies and *An. vaneedeni* Gillies and Coetzee.

4.2.7 Determination of blood meal source using ELISA-based method

The abdomen from each bloodfed mosquito was first homogenised in 50µl of phosphate buffered saline (PBS) and then 950 µl of the buffer added after grinding. Blood meals were identified by direct ELISA anti-host IgG conjugate against human and cow in a single-step assay. Any non-reacting samples were subsequently tested using chicken and goat IgG. Fifty microlitres of the mosquito triturate was added to U-shaped bottom 96-well microtitre plates and incubated overnight at room temperature. Each well was then washed twice with

PBS containing 0.5% Tween 20, followed by the addition of 50µl host specific conjugate in 0.5% boiled casein containing 0.025% Tween 20. The ELISA results were read visually (Beier *et al.*, 1988).

4.2.8 ELISA detection of Plasmodium falciparum sporozoite infections

The head and thorax portion of each individual mosquito was homogenized in 50µl of boiled casein blocking buffer (BB) containing Nonidet P- 40 (5 µl NP40/1 ml BB). Two hundred microlitres of blocking buffer was then added, bringing the final volume to 250 µl. Fifty microlitre aliquots were used following the ELISA protocol developed by Wirtz *et al.* (1985).

4.2.9 Data analysis

Data were double entered in Fox Pro software to create the database and analysis was done using SPSS statistical software package version 11.5 (SPSS Inc., Chicago, IL, USA), and Stata version 8 data analysis software (StataCorp, Texas, U.S.A). The analysis focused on *An. gambiae s.s.* and *An. funestus s.s.*, which are the most important vectors of malaria in the study area. Spearman's rank correlation and multiple logistic regressions were used to detect if any of the climatic variables were significantly associated with the abundance of adult *An. gambiae* and *An. funestus* adults. For the regression analysis, population densities of *Anopheles funestus* and *Anopheles gambiae* were set as the dependent variables. ANOVA was used to determine the variability of species composition at the two study sites. The students't-test was used to determine if there were any significant differences between the sporozoite rates observed in *An. gambiae* and *An. funestus*.

4.3 RESULTS

4.3.1 Meteorological data

Figure 4.1 shows the rainfall, maximum and minimum temperature and relative humidity patterns recorded during the study period. The mean monthly minimum and maximum temperatures at the study sites were 29.9^{0}C (range: 20.7-25.1^{0}C) and 30.9^{0}C (range: 27.5 34.0^{0}C) respectively. The average monthly relative humidity was 80.3% (range 72.6% – 93.4%). There were no differences between the monthly minimum and maximum temperatures, at the sites (F=1.68, p= 0.11). Significant relationships were recorded between rainfall and relative humidity (F=2.51, P = 0.02), rainfall and maximum temperature (F=0.43, P =0.02) and rainfall and minimum temperature (F=0.25, P=0.0009).

4.3.2 Mosquito species

During 2001, a total of 4,016 and 281 mosquitoes were collected from Jaribuni and Mtepeni respectively. In 2002, 20,505 mosquitoes were collected from Jaribuni and 456 from Mtepeni. Mosquitoes were identified as belonging to the following five species: *An. gambiae s.l., An. funestus, An. nili, An. coustani,* and *An. squamosus.*

4.3.3 Relative abundance of Mosquito species

Figures 4.2 and 4.3 show the relative abundance of the *Anopheles* species in Jaribuni and Mtepeni during the years 2001 and 2002, expressed as the corresponding percentages of the total numbers of mosquitoes collected by different techniques. *Anopheles gambiae* and *An. funestus* were the most abundant species at both sites, followed by *An. squamosus, An. coustani* and *An. nili* at the Jaribuni site.

During the year 2001, mosquito collections using the pyrethrum spray catch method yielded a total of 3,141 *An. funestus* and 875 *An. gambaie* s.l mosquitoes from the 11 compounds in Jaribuni and 22 *An. funestus* and 259 *An. gambiae* from the 10 compounds in Mtepeni. In 2002, a total of 17,423 *An. funestus*, 3,002 *An. gambiae*, 62 *An. squamosus*, 16 *An. coustani* and 2 *An. nili* were caught at Jaribuni using the DRI, HBO, HBI, PSC and light trap methods. In Mtepeni, 31 *An. funestus*, 419 *An. gambiae* and 6 *An. coustani* were captured. During both years *An. funestus* was the most abundant species at the Jaribuni site, representing 85% of the total anophelines captured compared to 14.6% *An. gambiae*. In Mtepeni, 91.8% of them were *An. gambiae* and 6.7% were *An. funestus*.

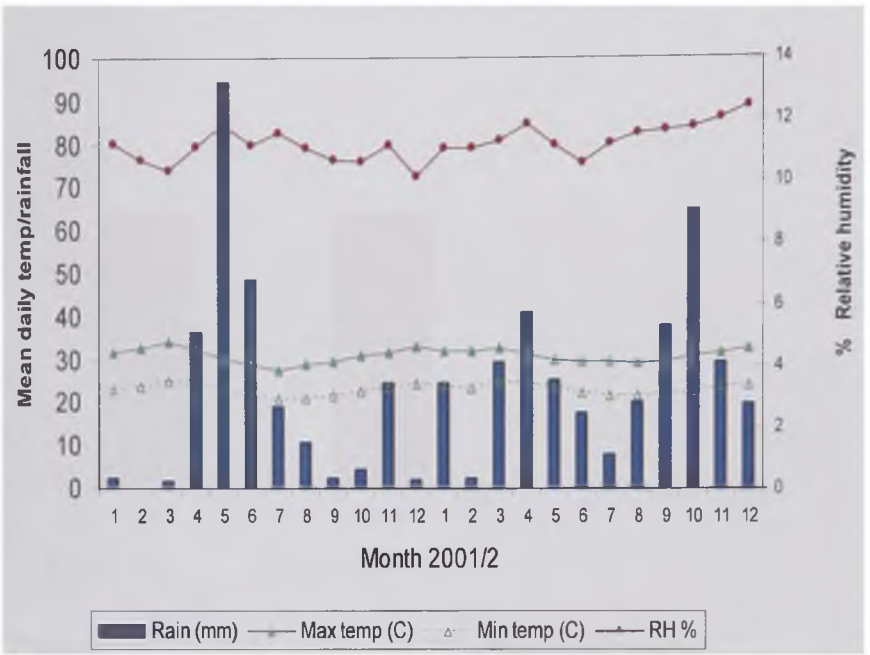

Figure 4.1: Distribution of rainfall, temperature and relative humidity in Jaribuni and Mtepeni, Kilifi during 2001-2002.

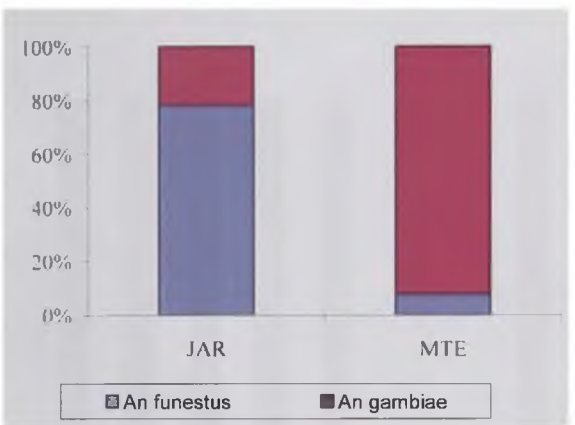

Figure 4.2: Proportions of *Anopheles gambiae* and *An. funestus* of the mosquito populations at Jaribuni (JAR) and Mtepeni (MTE) during the year 2001 using the PSC method.

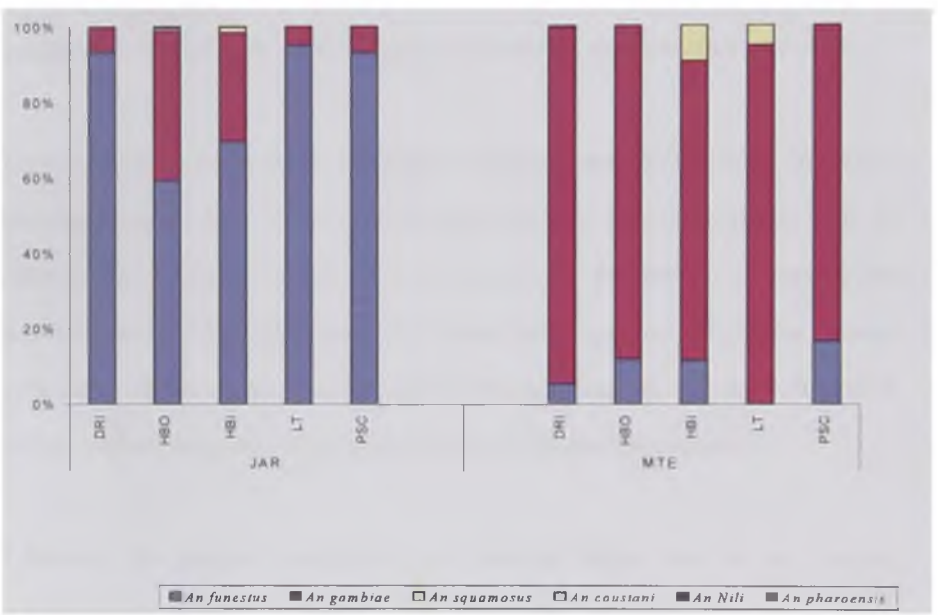

Figure 4.3: Proportion of *Anopheles gambiae* and *An. funestus* collected from Jaribuni (JAR) and Mtepeni (MTE) during the year 2002 using the various mosquito collection methods

4.3.4 Monthly changes in population fluctuation

Identifications by PCR revealed that the relative proportions of each species were 78.2% (n=3141) *An. funestus s.s.* and 19.8% (n=795) *An. gambiae s.s.* at Jaribuni; and 92.2% (n=259) *An. gambiae s.s.* at Mtepeni, and 7.8% (n=22) *An. funestus s.s.* The variations in population densities recorded for *An. gambiae s.s.* and *An. funestus* in Jaribuni and Mtepeni during 2001-2002 are shown in Figures 4.4 and 4.5 respectively. They also indicate the annual patterns of rainfall, temperature and relative humidity during the study period.

At Jaribuni, both *An. gambiae* and *An. funestus* populations were low during the first quarter of the year (January – March) 2001. With the beginning of the first rainy season in April, the number of adult mosquitoes caught started to increase. The first peak of *An. funestus* was observed in May (n=235), and the numbers increased until August (n=818). During the same period, an increase in *An. gambiae* populations was also observed, from n=79 in April to n=216 in June and dropped to n=96 in July, when the amount of rain reduced.

At Mtepeni, *An. gambiae* populations were generally higher than the *An. funestus* populations, even though the numbers were much lower compared to those observed in Jaribuni. The first peak in *An. gambiae* numbers was observed in February (n=145) and during the same period, it was zero for *An. funestus*. However, the *An. funestus* population began to increase in March (n=8) when that of *An. gambiae* had dropped (n=39). After May, no *An. funestus* were captured, but low levels of *An. gambiae* populations, which were observed between July –October.

During the year 2002, a similar pattern of species occurrence and abundance was observed; whereby at the Jaribuni site, very low numbers of *An. gambiae* were recorded (range: 2 – 40) between January and March, whereas during the same period, *An. funestus* numbers were moderately high (range: 182 - 336). The beginning of the long rainy season in April was marked by an increase in the numbers of both *An. gambiae* and *An. funestus* (n=5600 and n=2145 respectively), with *An. funestus* being twice as abundant as *An. gambiae*. The relative abundance of both species dropped with the reduction in the amount of rainfall. During the middle of the dry season in July, 1,119 *An. funestus* were caught as against 33 *An. gambiae*. Their numbers rose and peaked again in August but with much higher numbers of *An. funestus* (n=5243) than *An. gambiae* (n=152).

A similar trend is observed in Mtepeni, where peaks in *An. gambiae* populations were recorded from January – March, (n=69, 42 and 33) respectively. The data in Mtepeni from April to August 2002 were not sufficient to establish a seasonal pattern for that period during the year.

4.3.5 Relationship between climatic factors and anopheline species composition and abundance

Detailed results on the relative abundance of *An. gambiae* and *An. funestus* species in Jaribuni and Mtepeni during 2001 to 2002 are shown in figures 4.4 and 4.5. These figures illustrate the variability in the total numbers of *An. gambiae* and *An. funestus* species captured relative to the amount of rainfall, temperature and relative humidity recorded during the study period. Figure 4.4 shows that at the Mtepeni site, during January to March

2001and 2002 there was a peak in *An. gambiae* populations during January to March, a period that represents the first dry season of the year in Kilifi. *Anopheles funestus* populations were very low in Mtepeni within the period, and for most parts of the year. In 2002, *Anopheles* populations are minimal and irregular at the Mtepeni site.

In contrast to the observation made in Mtepeni, Figure 4.5 shows that in Jaribuni, *An. funestus* was the most abundant species compared to *An. gambiae*. Varying densities of *An. funestus* and *An. gambiae* were recorded throughout the year, with the populations rising following an increase in the amount of rainfall recorded, as is observed during April to July 2001 and April to June 2002. At the Jaribuni site, the presence of the river Jaribuni is an important feature in the ecology of anophelines at the site, and this might explain the variability observed in the population density of *An. gambiae* and *An. funestus* seen at the sites, given the differences in their population ecology. *An. funestus* were more abundant in Jaribuni compared to *An. gambiae* during both 2001 and 2002, even though the population densities vary between months. Species abundance was generally higher in 2002 compared to 2001 and the species composition in 2002 included other species such as *An. coustani, An. nili* and *An. squamosus* in low proportions.

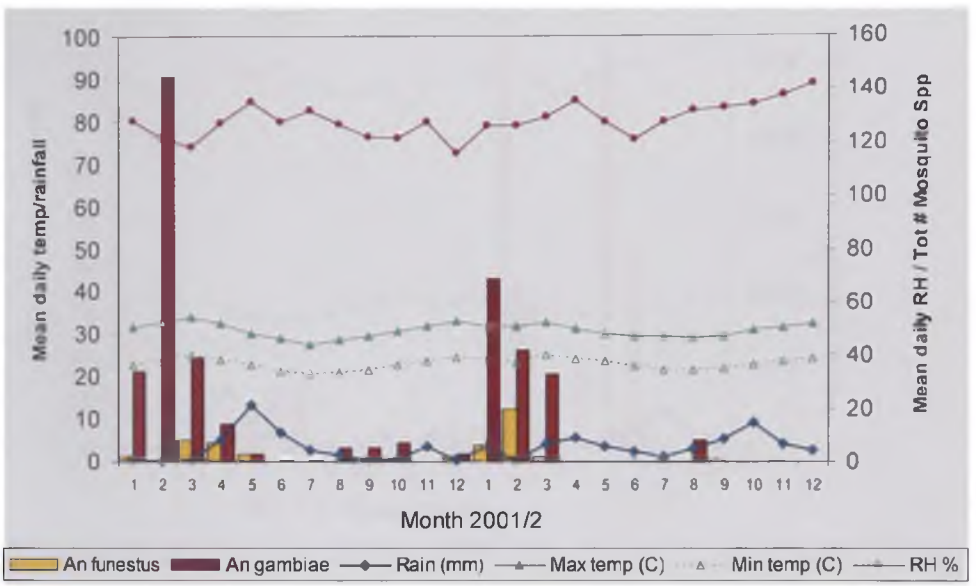

Figure 4.4: Distribution of rainfall, temperature and relative humidity, and *Anopheles* mosquito populations in Mtepeni, Kilifi during 2001-2002.

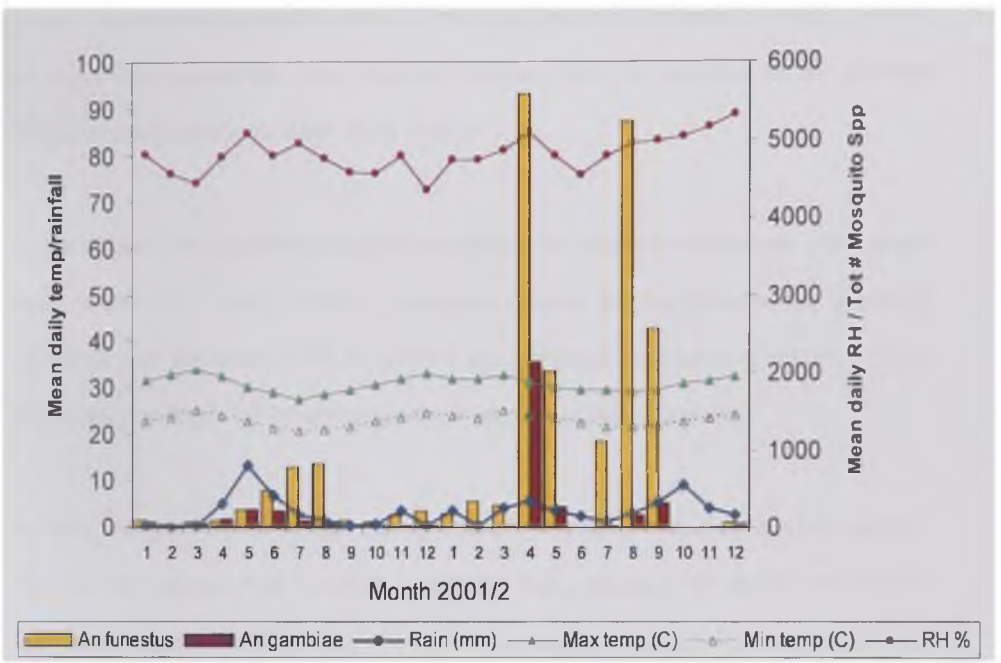

Figure 4.5: Distribution of rainfall, temperature and relative humidity, and *Anopheles* mosquito populations in Jaribuni, Kilifi during 2001-2002.

Average relative humidity and daily rainfall were found to be significantly associated with *An. gambiae* abundance in 2001. Maximum temperature, minimum temperature and daily rainfall were also significantly associated with *An. funestus* abundance ($p<0.05$). In 2002, no significant associations were observed between both *An. gambiae* or *An. funestus* abundance and climatic variables at the Jaribuni

At the Mtepeni Site, significant negative correlation was observed between *An. gambiae* and daily rainfall ($r^2 = -0.63$, $p=0.02$). *Anopheles funestus* was significantly and positively correlated with minimum ($r^2 = 0.59$, $p=0.04$) and maximum temperature ($r^2 = 0.66$, $p=0.01$) during the year 2001. All other associations were not significant ($p>0.05$).

In 2002, *An. gambiae* abundance was significantly correlated with daily rainfall ($p<0.05$). That of *An. funestus* was positively correlated with minimum ($r^2 =0.59$, $p=0.04$) and maximum temperature ($r^2 =0.66$, $p=0.01$). This association was not observed in the year 2002 when none of the climatic variables indicated association with mosquito species abundance. Tables 4.1 – 4.4 show the correlation co-efficients and p-values resulting from Spearman's correlation method on mosquito abundance and climatic variables.

Table 4.1: R^2 and p-values (in parentheses) resulting from Spearman's rank correlation analysis of mosquito abundance and climatic variables in Jaribuni during the year 2001. (Significant p- values are shown in boldface).

	An. gambiae	*An. funestus*	Average R.H	Max. Temp	Min. Temp	Daily rainfall
An. gambiae						
An. funestus	0.46 (0.13)					
Av. R.H	0.66 **(0.01)**	0.51(0.08)				
Max. Temp	-0.27(0.39)	-0.74**(0.005)**	-0.61**(0.03)**			
Min. Temp	-0.16(0.61)	-0.63**(0.002)**	-0.49(0.1)	0.97**(0.00)**		
Daily rainfall	0.65 **(0.02)**	0.57**(0.05)**	0.97**(0.00)**	-0.53(0.07)	-0.37(0.23)	

Table 4.2: R^2 and (p-values) resulting from Spearman's rank correlation method on mosquito abundance and climatic variables in Jaribuni during the year 2002

	An. gambiae	*An funestus*	Average R.H	Max. Temp	Min. Temp	Daily rainfall
An. gambiae						
An. funestus	0.82(0.0009)					
Average R.H	0.29(0.35)	0.02(0.94)				
Max. Temp	-0.18(0.58)	-0.29(0.35)	0.21(0.51)			
Min. Temp	0.11(0.73)	-0.15(0.63)	0.25(0.41)	0.84**(0.0006)**		
Daily rainfall	0.3(0.28)	0.08(0.79)	0.50(0.09)	0.04(0.89)	0.32(0.31)	

Table 4.3: R^2 and (p-values) resulting from Spearman's rank correlation method on mosqnito abnndance and climatic variables in Mtepeni during the year 2001

	An. gambiae	*An. funestus*	Average R.H	Max. Temp	Min. Temp	Daily rainfall
An. gambiae						
An. funestus	0.43(0.17)					
Av. R.H	-0.43(0.15)	-0.15(0.62)				
Max. Temp	0.51 (0.08)	0.59**(0.04)**	-0.61**(0.03)**			
Min. Temp	0.39(0.21)	0.66**(0.01)**	-0.49(0.1)	0.97**(0.00)**		
Daily rainfall	-0.63**(0.02)**	-0.02(0.94)	0.74(0.005)	-0.53(0.07)	-0.37(0.23)	

Table 4.4: R^2 and (p-values) resulting from spearman's rank correlation method on mosquito abundance and climatic variables in Mtepeni during the year 2002.

	An. gambiae	*An funestus*	Average R.H	Max. Temp	Min. Temp	Daily rainfall
An. gambiae						
An. funestus	0.72(0.081)					
Av. R.H	-0.47(0.1)	-0.27(0.38)				
Max. Temp	0. 39 (0.20)	0.54(0.06)	0.21(0.51)			
Min. Temp	0.17(0.58)	0.29(0.35)	0.25(0.41)	0.84**(0.0006)**		
Daily rainfall	-0.27(0.39)	-0.07(0.8)	0.50(0.09)	0.04(0.89)	0.32(0.31)	

4.3.5 Blood meal and sporozoite ELISA results

Blood meal ELISA results from *An. gambiae s.s.* and *An. funestus s.s.* during 2001 and 2002 indicated that both species preferred human hosts. Overall 95% of the *An. gambiae* s.l. and 92.4% *An. funestus* females tested positive for Human IgG. No positive reactions were detected for cow and goat. The sporozoite rates in *An. gambiae s.s.* and *An. funestus s.s.* from Jaribuni in the years 2001 were 0.36 and 0.15 respectively and in 2002 they were 0.17 and 0.16 respectively (Table 4.7). The observed rates of the two species however were not statistically different (Students t-test, t = 0.9; p=0.26). At Mtepeni, the sporozoite rates in *An. gambiae s.s.* and *An. funestus s.s.* were 0.14 and 0.05 respectively in 2001 and 0.22 and 0.12 in 2002, which were significantly different (Students t-test, t = -15; p=0.02).

Table 4.7: Proportion of *An gambiae s.l.* and *An. funestus* caught in Jaribuni and Mtepeni Kilifi, Kenya, infected with *Plasmodium falciparum* sporozoites.

		Sporozoite rates		
Site	Species	*An. funestus s.s.*	*An. gambiae s.s.*	P-value
Jaribuni	Year 2001	0.15	0.36	P=0.26
	Year 2002	0.16	0.17	
Mtepeni	Year 2001	0.05	0.14	P=0.02
	Year 2002	0.12	0.22	

4.4 The spatial distribution of *An. gambiae* and *An. funestus* in Jaribuni and Mtepeni, Kilifi.

Variability was observed in the spatial distribution of *An. gambiae s.s.* and *An. funestus s.s.* populations in Jaribuni and Mtepeni (Figure 4.6). A significantly higher proportion of *An. funestus s.s.* was observed in Jaribuni compared to *An. gambiae* ($p=0.03$) whereas in Mtepeni, more *An. gambiae s.s.* were observed compared to *An. funestus s.s.*

In Jaribuni, *An. funestus s.s.* formed >50% of the total mosquito population in 86% (13/15) of all the compounds sampled, while the remaining 13% were *An. gambiae s.s.* 1% were other species. In Mtepeni, the mosquito population in 80% (3/15) of the compounds was made up of >80% *An. gambiae* species. Compounds 5A, 004 and 006 in Mtepeni were predominantly *An. gambiae* sites, whereas at compound 3, only *An. funestus* populations were recorded. These results indicate clustering of *Anopheles* mosquitoes in compounds and microhabitats in the field.

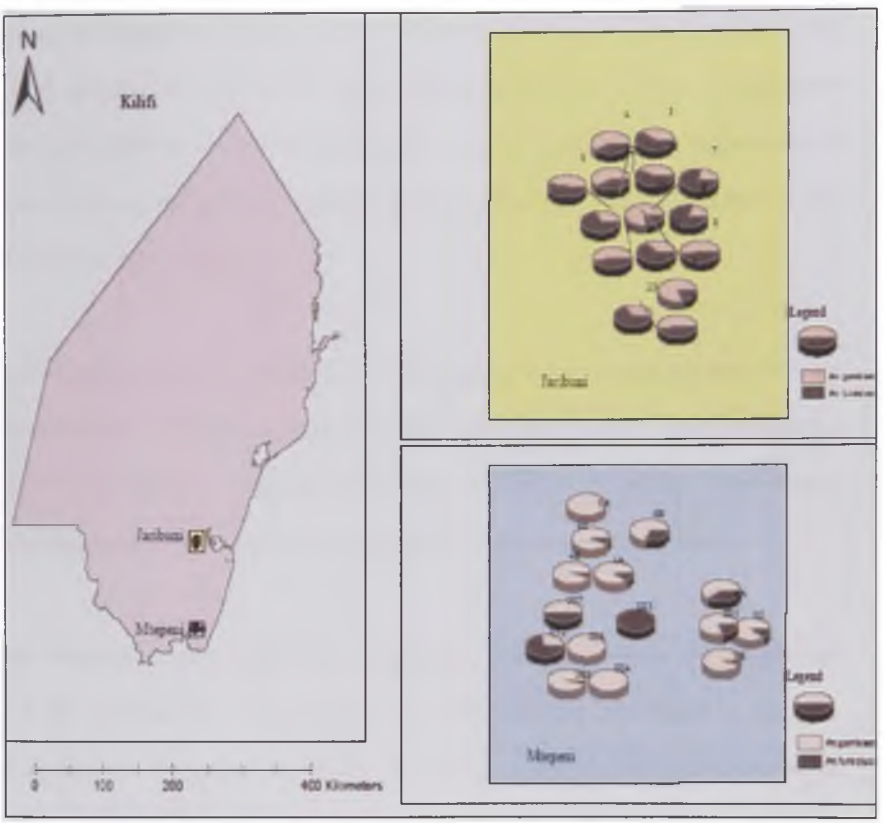

Figure 4.6: Spatial distribution of *Anopheles gambiae* and *An. funestus* species at Jaribuni and Mtepeni, Kilifi District in coastal Kenya. 003, 004, 5C, 4B, etc denote the compounds from which were sampled for *Anopheles* mosquitoes. Each compound comprised of 5-8 households.

4.5 Discussion

Entomological studies on the *Anopheles* species in Kilifi District have recognized the occurrence of five *Anopheles* species, mainly *Anopheles gambiae s.s., An. arabiensis, An. funestus, An. coustani, An. nili* and *An. squamosus.* (Mbogo *et al.,* 1995). Among these species, only *An. gambiae s.s.* and *An. funestus* have been implicated in the transmission of *P. falciparum* malaria and are thus regarded as the predominant vectors of malaria in the area (Mbogo *et al.,* 1995; 2003).

The abundance and distribution of these vectors is dependent on ecological and climatic factors (which partly determine the availability of suitable larval habitats for breeding), the proximity of larval habitats to humans and the type and design of housing. These factors also greatly determine the spatial and temporal patterns of disease on a local scale.

The results obtained by this study, indicate variability in the abundance and distribution patterns of *An. gambiae* and *An. funestus*. *Anopheles funestus* was found to be more abundant in Jaribuni compared to Mtepeni, where *An. gambiae* was the major vector species. This variability in species abundance at the two sites may be attributed to local ecological differences. In Jaribuni, larval habitats are pools of water created by a stream which is perennial, with clear water, and is covered with vegetation most of the year. Such habitats are mostly preferred by *An. funestus.* The Jaribuni River is perennial, flowing all year round, with levels of water fluctuating during the dry and rainy seasons. As a result, small pools of water are present by the riverside during the rainy season, which dry as the amount of rainfall decreases. It is however important to note that variability in mosquito

abundance between 2001 and 2002 might have been due to differences in mosquito collection methodology applied. In 2001, only the PSC method was applied in mosquito collection whereas in 2002 light traps, DRI, HBO and HBI were all combined. During 2002, the Mark release recapture study was ongoing, and required the maximization of mosquito collection efforts to ensure optimum recovery of released mosquitoes.

The observation of more abundant *An. gambiae* populations at the start of the rainy season follows their preference to breed in fresh pools of water that are open and sunlit (Gillies, 1968) whereas the distribution of *An. funestus* is strongly influenced by the availability of more permanent waters. Hence, the results obtained that, *An. funestus* exhibited very little seasonal variation in densities throughout the study period could be explained by the permanent presence of the river that ensured the availability of suitable breeding habitats. Succession of larval habitats by *An. funestus* towards the drier period suggests the variability in habitat preferences for the two species, an important aspect of eco-phenotypic adaptations already described for anophelines in West Africa (Coluzzi *et al.,* 1979).

Naturally, it is expected that climatic factors, namely rainfall, temperature and relative humidity will have a great influence on the abundance of mosquitoes. Results from this study indicate that daily rainfall and relative humidity were strongly associated with the abundance of *An. gambiae* in 2001, while daily rainfall, relative humidity, minimum and maximum temperature were strongly associated with *An. funestus* abundance in Jaribuni during the same year. In Mtepeni, only maximum and minimum temperatures were strongly associated with *An. funestus* abundance and daily rainfall strongly influenced the numbers of

An. gambiae present in Mtepeni. In 2002, no significant associations between climatic factors and species abundance were recorded in both Jaribuni and Mtepeni. The absence of a strong correlation between the seasonal rainfall patterns and *An. funestus* has also been reported by Mbogo *et al.* (2003). Studies by Minakawa *et al.* (2002b) in western Kenya revealed that the abundance of *An. funestus* was much lower than *An. gambiae* or *An. arabiensis* in the rainy season, but it increased dramatically in the dry season, to exceed those of *An. gambiae* and *An. arabiensis* at some sites. Together, these observations suggest that even though climatic factors determine the availability of larval habitats, ecological factors do also play a critical role in the suitability of larval habitats for anopheline mosquito breeding. While the strong association between *An. gambiae* and rainfall restricts the activity of *An. gambiae* to a relatively short period when breeding conditions were suitable, these observations could also be attributed to the complex nature of interactions between climatic and ecologic variables, which may have specific time lags that have not been captured by the present analyses.

Although this study did not detect any strong statistically significant associations between the environmental variables and mosquito occurrence and abundance, significant spatial heterogeneity in the *Anopheles* species composition was observed at the two sites. The spatial and temporal heterogeneity in species occurrence observed at these two sites is an important factor for the dynamics of malaria transmission in Kilifi, and is important for the planning of vector control interventions especially those aimed at reducing larval productivity. Such control interventions can be planned sohat insecticide application is done during the dry season before larval populations build up.

CHAPTER FIVE

ESTIMATION OF POPULATION SIZE, DISPERSAL AND SURVIVAL OF *ANOPHELES GAMBIAE* AND *ANOPHELES FUNESTUS* ALONG THE KENYAN COAST USING MARK-RELEASE-RECAPTURE METHODS

5.1 Introduction

Information on the population size, survival probability, and dispersal of malaria vectors is of paramount importance in understanding the epidemiology and ecology of vector-borne diseases, for operational vector control activities and planning of future interventions. The dispersal of mosquitoes to find mating partners, nectar sources, resting sites, oviposition sites and sources of blood meal is an important factor underlying the spatial distribution of mosquitoes, and plays a major role in shaping their population structure (Service, 1997). Information on mosquito survivorship and dispersal ability is also critical for understanding disease transmission risk (Carter *et al.*, 2000) and planning vector control measures (Killeen *et al.*, 2003). Species with greater dispersal abilities are usually able to transmit pathogens over large areas and can therefore have a great impact on the efficacy of any disease control interventions. In the case of *Anopheles* mosquito species, studies on their dispersal have reported their ability to disperse over various distances ranging from between <1 to 1.5 km from the point of release (Thomson *et al.*, 1995; Costantini *et al.*, 1996). The maximum distance a vector flies is important in understanding population genetics, species distribution patterns and the spread of pathogens to new geographic areas.

The importance of *Anopheles* dispersal in the epidemiology of malaria is based on their ability to continuously achieve population exchange between neighbouring villages, or households over their lifetime, and in the process, maintain the transmission of malaria parasites between them. Apart from its possible role in homogenizing populations genetically, *Anopheles* movement between villages reportedly has an impact on malaria vector control efforts. Specifically, control trials of insecticide treated bednets (ITN's) usage have indicated that movement of mosquitoes between neighbouring villages seriously affects the entomological evaluation of the efficacy of bednet trials (Thomson *et al.,* 1995). Furthermore, the model developed by Killeen *et al.* (2003) indicates that emigration of mosquitoes from areas covered with control measures such as bednets and their continual replacement by immigrants from nearby uncontrolled areas can result in substantial attenuation of local impact. In effect, vector dispersal dilutes the impact of vector control efforts and practically compromises the benefits of village-based control especially in cases where the neighbouring village might have a larger vector population size.

Large vector population sizes have, in some cases been associated with increased malaria incidence. In most malaria-endemic areas, the number of malaria cases usually increases sharply after the rainy season. This coincides with a period when numerous possible larval habitats are available, facilitating the breeding of mosquitoes and after about three to four weeks, the vector population increases drastically, leading to an increase in disease transmission if the conditions are favourable. Lindblade *et al.* (2000) describes a situation in northern Uganda, where malaria transmission is unstable and the population has little or no immunity, making the highlands prone to explosive outbreaks of malaria when densities of

Anopheles exceed critical levels and conditions favour transmission. It is therefore expedient to include *Anopheles* population density as a monitoring and warning tool for malaria epidemics in the highland areas of Africa, which is feasible as demonstrated by Lindblade *et al.* (2000). In malaria-endemic areas where transmission is stable, an increase in vector density might lead to an increase in malaria incidence but not necessarily an epidemic since the population has developed immunity over time. However, this is largely debatable and is dependent on several factors, for example the quality of health care, treatment-seeking behaviour, etc. An increase in vector abundance or distribution does not automatically cause an increase in disease incidence, and an increase in incidence does not result in an equal increase in morbidity and /or mortality.

Transmission requires that the reservoir host, a competent arthropod vector and the pathogen be present in an area at the same time and in adequate numbers to maintain transmission. When conditions for vector breeding are favourable, then the population density is very likely to increase. However, for the vector to achieve its success in disease transmission, it must be fit to survive long enough to sustain parasite development and therefore successful transmission to the host. A vector that survives for a long time also maximizes on its chances of achieving human-vector contact and disease transmission. For *Anopheles* vectors of malaria, the daily survival probability and frequency of human host contact are the two most sensitive components of their roles in pathogen transmission (Charlwood *et al.*, 1997; Buonaccorsi *et al.*, 2003). Vector survival through successful parasite transmission is also an important selective agent in shaping malaria population genetics and the evolution of phenotypes such as pathogen virulence (Ferguson *et al.*, 2003). The probability of vector

survival is also influenced by weather and parasite factors. It has been shown that mosquito survival rates become progressively lower at temperatures below 18^0C and as temperatures rise above 22^0C (Le Sueur, 1991). Anderson *et al.* (2000) have reported that *Plasmodium* infection in mosquitoes reduces both vector fecundity and longevity of mosquitoes, while Taylor and Hurd (2001) have reported that several properties of infected blood such as anaemia and antibodies influence mosquito fitness. Mosquitoes feeding on infected hosts are exposed to these factors.

One of the methods most commonly used to estimate mosquito populations is the mark-release-recapture (MRR) technique (Service, 1993b). The MRR technique is a field-based methodology in which either insectary reared or field captured adult mosquitoes are coloured with fluorescent dye(s) or radioactive labels and released into the environment to disperse as they would under natural conditions. The dispersing adult mosquitoes are then recaptured in subsequent days using known standard field mosquito collection methods such as indoor resting catches, human landing catches and light traps. Data derived from MRR experiments have been useful in reporting various population parameters of disease vectors such as their dispersal ability, longevity, distribution patterns and population density estimates, all important in describing vector-borne disease epidemiology. One way to estimate the population size is to capture and mark individuals from the population and then resample to see what fraction of the recovered individuals carry marks. The strength of mark recapture studies is that they can provide information on birth, death and movement rates in addition to information on absolute abundance.

Naturally, most populations constantly change in size because of births, deaths, immigration and emigration. When marked samples are taken on more than one occasion, this procedure becomes useful for obtaining such estimates from an open population. The critical assumption of mark recapture studies is that individuals are equally 'catchable' so that marked individuals at any given sampling time have the same chances of capture as unmarked individuals. However, this assumption is often violated in natural populations given the variable response of individual dispersers to the effects of ecological and environmental factors, differences in behaviour and dispersability.

Several MRR experiments have been conducted with populations of *Anopheles* and *Aedes* mosquitoes. 150 such studies have been recorded and among these are those on the female flight range of anopheline species in the 1900s e.g, Thompson *et al.* (1995); Costantini *et al.* (1996) and Toure *et al.* (1997) in West Africa, Charlwood *et al.* (1997) and Takken *et al.* (1998) in East Africa, and on *Aedes aegypti aegypti* in coastal Kenya (Trpis and Hausermann 1986). A more recent study by Harrington *et al.* (2001) compares the dispersal rates of two cohorts of *Aedes aegypti* of different ages from a central release point.

Along the Kenyan coast, the major vectors of malaria *Anopheles gambiae* s.l. and *Anopheles funestus* occur in sympatry, and knowledge of their population characteristics, such as dispersal and population sizes of these mosquitoes is of paramount importance in understanding the epidemiology and ecology of malaria in this area. To obtain this information for *Anopheles* species along the Kenyan Coast, MRR experiments with emergent females reared from larval stages in the field were conducted. As opposed to the

release of field caught adult females as has been done in most previous MRR studies, 3 day old female *Anopheles* mosquitoes were released. The objective was to determine their actual dispersal capability from their larval habitats to either find a source of blood meal or to seek a mate. With this approach any possible effect of gonotrophic state on dispersal capability was eliminated. Estimates of the population size and survival probabilities of *An. gambiae* and *An. funestus* at two sites were obtained using this approach.

5.2 Materials and Methods

5.2.1 The study sites

The study sites have been described in detail in Chapter 3. Briefly, the MRR experiments were conducted at two of the described study sites, namely Jaribuni and Mtepeni. More detailed description and information on rainfall pattern and temperature prevailing for the two sites are also given in Chapter 3 of this thesis.

5.2.2 Mapping of study compounds and larval habitats

The study sites were surveyed on foot to identify all potential larval habitats. The latitude and longitude of the productive larval habitats and households were obtained using a hand held geographic positioning system (GPS), [Garmin International Inc., Olathe, KS] as described in Chapter 3. Geo-referenced layers of roads and major landmarks were overlaid onto that of the larval habitats and households distribution using ArcView version 3.2a®. Figure 5.1 shows a map of the larval habitats where larval sampling was conducted and the compounds (which contain the houses) where adult collections were done. A total of 11 compounds were mapped in Jaribuni, 10 compounds in Mtepeni. Two larval habitats in Jaribuni and three in Mtepeni were sampled. The point distance command in ArcInfo was used to calculate distances between larval habitats and the households.

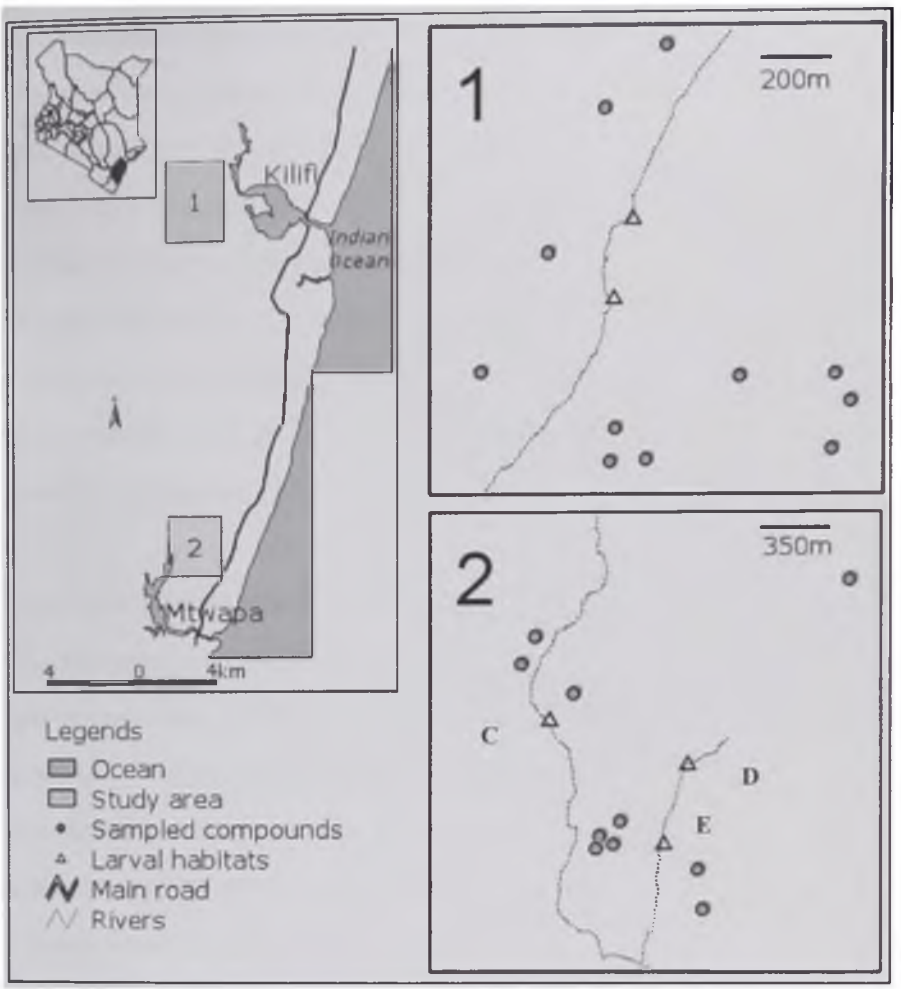

Figure 5.1: Map of the study area, showing Kilifi District and the two study sites along the Kenyan Coast. Site 1: Jaribuni Site 2: Mtepeni. Box 1 and 2 on the right show the spatial location of the studied larval habitats (A, B, C, D & E) and the compounds where MRR adult collections were made.

5.2.3 Mosquito rearing and mark-release-recapture experiments

The study was conducted from February to April 2002 at Jaribuni and from May to July 2002 at Mtepeni. The level of the river Jaribuni was low (Figure 5.2); thus only two productive larval habitats were identified and selected for sampling during the study period. At Mtepeni, three larval habitats were identified being swampy areas that had retained water after the rainy season. At each study site, temporary field insectaries (Figure 5.3) were constructed and this simulated field conditions for mosquito rearing. The field insectary was constructed using wooden poles on the walls and roof. The wall on one side of the roof was covered with cellophane paper to allow some light into the insectary during day time.

Larvae were collected from the larval habitats using the standard dipping method. They were then sorted into different basins according to the larval stages and any predators or unknown organisms seen were excluded. The basins containing larvae were then marked with details of habitat of collection and larval stage. Water used for the rearing was obtained from the habitats and grass infusions were included in the basins with some soil on the roots to try and mimic the natural habitat as much as possible. Larvae were fed on Tetramin baby fish food (TetraWerke®, Germany). When the mosquitoes reached the pupal stage, they were immediately transferred from the open basins into smaller containers within a netted cage so as to prevent any emerging adults from escaping.

Every batch of emergent adults was held for 3 days before release during which time they were fed on 6% sugar solution. To control for the effect of age and gonotropic state on the dispersal behaviour and survival of the released *Anopheles* mosquitoes, young emergent 3-4

day old adult females were used for the experiments. Before release, 0.5-litre paper cups were dusted with fluorescent powder and group(s) of adults were then manually aspirated and counted into the paper cups. The powders for dusting were day glo fluorescent pigments (Day-Glo Color Co. Cleveland, Ohio, U.S.A). Different dye colours were used for each release. To obtain four additional colour pigments, different colour mixtures of the five original colours from Day-Glo were mixed making a total of nine pigments available for all the releases.

All the marked adult female mosquitoes were returned to the original larval habitat and the lid of the paper cup removed to allow the marked mosquitoes to freely fly out. The mosquitoes that appeared exhausted (moribund) and did not fly out of the cups were counted and their numbers subtracted from the total marked and released. In order to minimize the effects of heat on the released mosquitoes due to high daytime temperatures, all the releases were made from 1500 to 1600hrs. A total of nine separate releases were made at Jaribuni, four releases from the first habitat and five from the second. At Mtepeni, five releases were made, two each from the first two habitats and one release from the third habitat. Given that the rate of adult productivity was variable for each batch of larvae from the habitats, each of the releases consisted of different numbers of females. At both sites, there was an interval of at least eight days between releases.

Figure 5.2: A section of the Jaribuni River during the study period

Figure 5.3: The temporary field insectary that was constructed at Jaribuni for rearing Anopheline mosquitoes in the field.

5.2.4 Recapture

Mosquito recaptures from houses within the compounds selected for the study began one day after the day of release and continued for 14 consecutive days. Recapture efforts were concentrated within a radius of 1.5 kilometres within the study area and conducted sampling in 11 compounds and a total of 21 houses in Jaribuni, and 10 compounds in Mtepeni, and a total of 19 houses. Three mosquito collection methods were used; daytime catches of indoor resting mosquitoes, human landing catches both indoors and outdoors and light traps. These methods have been described in Section 3.3 above. The frequency for DRI collections was once daily in all the houses, and a total of 16–21 DRI was done for each house. DRI collections were conducted from 8.00am to 11.00am while HBO and HBI were conducted from 7.00PM till 12.00 midnight. It was not possible to conduct HBO & HBI in all the houses each night, and so a sequential sampling scheme was generated for use during the night collections, whereby a total of 8 to 10 compounds selected on either side of the release points were sampled each night. To determine which compounds to sample daily, the compounds were randomly selected then assigned a night of collection. Collections of mosquitoes in paper cups were placed in coolers and transported to the laboratory. The sampling strategy and selection of houses are described earlier (see Chapter 3).

In the laboratory, all the mosquitoes were counted and examined at 40X using a fluorescent compound microscope (Olympus B201, Japan) to detect colour marked individuals. Mosquitoes were classified to gonotrophic state based on abdominal appearance as unfed, fed, half gravid or gravid. This was followed by morphological identification using the taxonomic keys of Gillies and DeMeillon (1968). Further identification of species in the *An.*

gambiae complex and the *An. funestus* was done using the PCR methods of Scott *et al.* (1993) and Koekemoer *et al.* (2002) respectively. Sporozoite ELISA tests were conducted on the blood-fed mosquitoes recaptured 10 days after release to determine sporozoite infection as this would provide information on the activity of the released mosquitoes in malaria transmission.

5.2.5 Data analysis

Recapture data for mosquitoes from nine releases at the two habitats in Jaribuni and data from the five releases at three habitats in Mtepeni were analysed to estimate the population size and daily survival probability of *An. gambiae* and *An. funestus* mosquitoes. Only recaptures obtained during the first day of recapture were included in the population ize estimates. Recapture rates were calculated as the proportion of the total number of marked mosquitoes recaptured over the total number originally marked and released.

To estimate the population density (P) of *Anopheles* mosquitoes, the Lincoln Index method (Blower *et al.*, 1981) was used:

$$P = an/r, \text{ ----------Equation 1, when } r \geq 20.$$

Where *a* denotes the number of mosquitoes originally marked and released,

n denotes the total number of mosquitoes captured on a subsequent occasion,

r denotes the number recorded as marked out of the total number captured.

When $r<20$ then:

$$P = a(n+1)/(r+1) \text{ ----------Equation 2.}$$

The variance of P (Var P) is given by :

Var (P) = $a^2n(n-r)/r^3$ ----------Equation 3 when r≥20

and Var (P)=a^2 (n+1)(n-1)/$(r+1)^2(r+2)$ ---------Equation 4 when r<20.

The daily survival probability of *An. gambiae* and *An. funestus* mosquitoes was estimated using the exponential model developed by Gillies (1961). In this model, the loss of marked recaptures is described by the function: $N=Asp^d$,

where N = number of marked females recaptured,

A= total numbers marked and released,

s = recapture rate,

p =survival rate and

d = number of days after release.

Using this model, a plot of the logarithm of the (number of recaptures +1) or *log (N+1)* over days after release *(d)*, allows the estimation of p as the antilogarithm of the slope of the fitted regression line. Using the grouped data formula developed by Chiang *et al.* (1991), the mean dispersal from the point of release for each species at each site was estimated:

$$\bar{d} = \frac{\sum_{i=1}^{n} r_i d_i}{\sum_{i=1}^{n} r_i}$$

(Where r_i = number recaptured at each point (i), d_i = distance in meters of point i from release site and n = total number of recapture points), and also estimated the maximum distance

traveled from the habitat of release to the recapture compound. The distances between the release habitats and each compound were calculated by the point distance command in Arc Info using the longitude and latitude records for all compounds and habitats obtained with the GPS.

5.2.6 Ethical clearance and informed consent. The mark-release-recapture studies were reviewed and approved by the Institutional Review Board of the Kenya Medical Research Institute, Nairobi, Kenya. Consent was obtained from the heads of households to permit mosquito collection from their houses and from individuals who conducted the human landing catches after the study was explained in the local language. Examples of the Informed Consent forms are attached as Appendix I.

5.3 Results

5.3.1. Recapture rates

At Jaribuni, 182 *Anopheles* mosquitoes were recaptured from a total of 739 released corresponding to a recapture rate of 24.6% (95% CI: 21.6-27.9). Of the total recaptured, a significantly higher proportion of *An. funestus s.s.* (73.6%), were collected compared to 26.4% of *An. gambiae s.s.* (χ^2= 7.16; df = 1, p =0.007). At Mtepeni, a total of 1,246 *Anopheles* were released and out of these, 54 were recaptured, corresponding to a recapture rate of 4.33% (95% CI 3.27-5.62). Significantly fewer *An. funestus s.s.* (3.7%) compared to (96.3%) *An. gambiae s.s.* were recaptured (χ^2=8.32, df =1, p = 0.004).

Sporozoite ELISA analysis revealed a 4.40% (8/182) *P. falciparum* sporozoite infection rate for the recaptured anophelines from Jaribuni. Of these, 2.75% were *An. gambiae s.s.* and 1.65% were *An. funestus s.s.* None of the mosquitoes collected from Mtepeni were sporozoite positive.

5.3.2 Estimates of population size

The details of the results obtained are given in Tables 5.1 and 5.2. The estimates of population size (P) for all *Anopheles* at Jaribuni ranged from 5,157 to 55,484. Estimated mean population size was 23, 351. At the Mtepeni site, the estimates were lower, ranging from 658 to 4,193 and the estimated mean population size was 2,697. It is important to note that during recapture, only *An. gambiae s.s.* and *An. funestuss.s.* were recaptured during indoor collections by indoor resting catches, and by human landing catches, both indoors and outdoors. No *An. arabiensis* and *An. merus* were caught during recapture hence the

calculations for population size estimates are based on recaptures of *An. gambiae s.s.* and *An. funestus s.s.* The estimated mean population size of *An. gambiae s.s.* was 17,279 (74%) and 1,578 (26%) for *An. funestus* as in Jaribuni, while in Mtepeni, the population size of *An. gambiae* was 2,597(96.3%) and *An. funestus* was 99.7 (3.7%).

Table 5.1: Numbers of *Anopheles* mosquitoes released (a), captured (n) and recaptured (r), and population size (P) estimates for *Anopheles* mosquitoes at Jaribuni.

Release site	Release number	Number released (a)	Number captured (n)	Number of marked mosquitoes recaptured (r)	Estimated population size (P)	SE	95% Confidence interval
A	1	88	1,260	1	55,484	32,008	-7252 -118,220
	2	184	2,540	22	21,243	4,148	12,405- 30,082
	3	52	2,348	8	13,572	4,290	5,164 – 21,980
	4	75	1,029	3	19,312	8,628	2,401- 36,224
B	1	97	726	12	5,425	1,448	2,587 – 8,262
	2	80	2,384	8	21,200	6,701	8,066 - 34,334
	3	96	1,059	4	20,352	8,300	4,082 - 36,622
	4	47	1,029	0	48,410	34,198	-18,618-115,438
	5	20	1,546	5	5,157	1,948	1,339- 8,974
Total					210,155		
Mean					23,351		

Table 5.2: Numbers of *Anopheles* mosquitoes released (a), captured (n) and recaptured (r), and population size (P) estimates for *Anopheles* mosquitoes at Mtepeni

Release site	Release number	Number released (a)	Number captured (n)	Number of marked mosquitoes recaptured (r)	Estimated population size (P)	SE	95% confidence interval
C	1	94	13	1	658	352	-31 - 1,347
	2	398	40	3	4,080	1,779	591-7,567
D	1	234	45	2	3,588	1,754	149 - 7,027
	2	185	67	2	4,193	2,066	144 - 8,242
E	3	335	25	8	968	294	391 – 1,543
Total					13,487		
Mean					2,697		

5.3.3 Survival

The survival probability was estimated for all the recaptured mosquitoes, which were mainly *An. gambiae s.s.* and *An. funestus s.s.* Figure 5.4 shows the scatter plots of the logarithm of the number of marked recaptures log (n+1) of *An. gambiae* and *An. funestus* respectively plotted against the number of days after release with regression lines fitted to the data. At the Jaribuni site, estimated daily survival probability was 0.95 (95% CI = 0.88 - 1.00) for *An. gambiae* and 0.96 (95% CI= 0.89 - 1.00) for *An. funestus.*

At Mtepeni, estimates of daily survival probability by using the same method were 0.95 (95% CI, 0.87 - 1.03) for *An. gambiae* and 0.83 (95% CI, 0.79 - 0.86) for *An. funestus.*

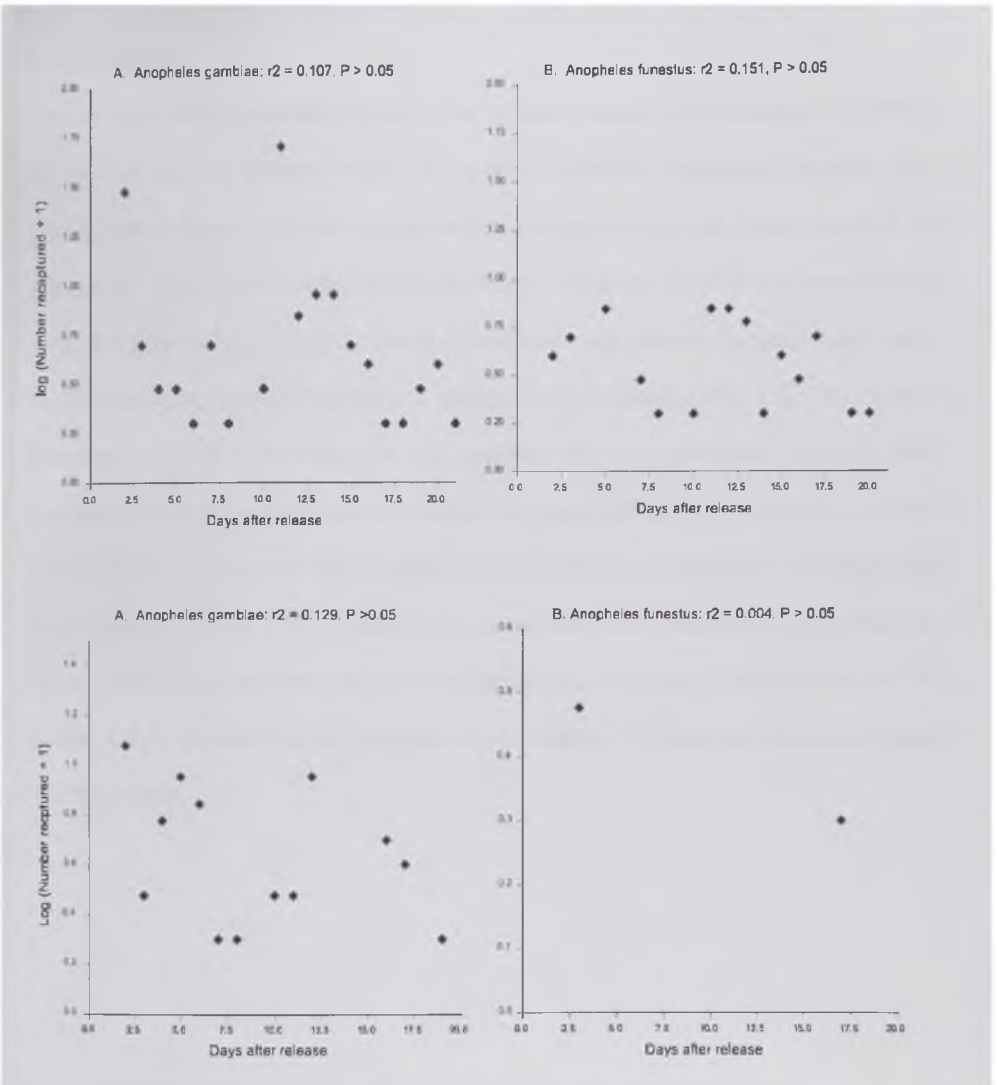

Figure 5.4: Regression of the log transformation of the number of marked female mosquitoes recaptured against the days after release, A: *Anopheles gambiae* at Jaribuni; B: *An. funestus* at Jaribuni; C: *An. gambiae* at Mtepeni; and D: *An. funestus* at Mtepeni.

5.3.4 Dispersal

At both study sites, mosquitoes released from different habitats were recaptured together in the same house or in different houses and on some occasions, recaptures were made from houses located further from the release habitat, although there were houses nearer to the release site. There was no direct relationship between distance travelled and the number of days after release (Fig. 5.3). But a cluster of recaptures was observed between days 1 and 5 and between days 10 and 15 for both *An. gambiae* and *An. funestus* (Fig. 5.4). This number decreases at day 20. On average, both *An. gambiae* and *An.funestus* from both sites were recaptured 11± 6 days after the day of release. Maximum recoveries of marked *An. gambiae* and *An. funestus* were made from compounds located between 200 and 400 metres from the release habitats (Table 5.3). Although some mosquitoes were recaptured further from the release habitat, these numbers dropped with an increase in distance. Between 650 and 750 metres, < 5 *An. gambiae* and *An. funestus* were recaptured. The maximum distance recorded was 661 metres.

Table 5.3: The total number of *Anopheles gambiae* and *Anopheles funestus* mosquitoes recaptured at various distances from the release points in Jaribuni and Mtepeni.

	Jaribuni		Mtepeni	
Distance from release point (meters)	Number recaptured *An. gambiae*	Number recaptured *An. funestus*	Number recaptured *An. gambiae*	Number recaptured *An. funestus*
≤ 200	13	7	12	1
250	4	3	16	0
300	17	1	0	0
350	27	16	4	0
400	22	6	15	0
450	2	0	1	1
500	2	3	0	0
550	11	1	2	0
600	33	7	0	0
≥601	3	4	2	0

5.4 Discussion

The main objective of this study was to obtain information on the population sizes of *An. gambiae* and *An. funestus*, and to establish *Anopheles* mosquito dispersal and distribution. Recapture rates of 24.6% and 4.33% were recorded at the Jaribuni and Mtepeni study areas respectively. The observed difference in recapture rates suggests possible effects of local variability in topographical and ecological features, since experimental procedures and recovery efforts were similar at both sites. It is however expected that the rate of mosquito movement will be different in the two study sites, given that the landscape at Mtepeni is hilly with thick vegetation as compared to Jaribuni, which is characterized by a less hilly terrain. This being the first MRR study with *Anopheles* along the Kenyan coast, the information gathered provides important insights on how local geography and ecological differences can influence disease transmission and epidemiology.

In this MRR study, emergent *Anopheles* mosquitoes were released at the larval habitat as opposed to the release of females of unknown age, which have undergone a part of their gonotropic cycle. The results obtained suggest that apart from physical and ecological factors, female age and the proximity of the human dwellings to the larval habitats are important factors affecting mosquito dispersal. The recapture rates obtained in this study suggest the possibility that field collected and reared mosquitoes dispersed more than wild caught adults. This has been shown with *An. culicifacies* whereby reared mosquitoes dispersed wider than wild caught specimens (Reisne *et al.*, 1980; Rawlings *et al.*, 1981) and with experiments in Puerto Rico and Thailand, in which two cohorts of young 3 day and 13-day-old adult *Aedes aegypti* females were released and a higher recapture rate was obtained

for the younger cohort (35%) compared to 16% for the older cohort (Harrington *et al.*, 2001). These results imply that the release of young mosquitoes may be a better approach to improving the recovery rates in MRR experiments since young females must disperse to find food and sources of blood meal. Even though younger female mosquitoes are likely to survive longer than the older ones, this cannot rule out the fact that older females are epidemiologically more important in terms of disease transmission. A study comparing the relative dispersal of both young and older adults is recommended.

More than 500 male *Anopheles* mosquitoes were released and only two were recaptured from indoor collections. Also no *An. arabiensis* or *An. merus* were recaptured, even though mosquito collection methods employed targeted both anthropophilic and endophilic vectors. Previous studies have however indicated that *An. merus* and *An. arabiensis* can occur at very low densities at these sites compared to *An. gambiae s.s.* (Mbogo *et al.*, 2003; Mwangangi *et al.*, 2004).

The daily survival probability obtained for both *An. gambiae* and *An. funestus* in this study was generally high, up to 95% and the observed differences were not significant between them. White (1982) reports that in order for female *Anopheles* to have vector potential, their daily survival probability must be at least 60%, usually 80-90%, and if the daily mortality of an *Anopheles* population averages 50%, then less than 1% of the *Anopheles* females are likely to survive to the minimum of 10 days necessary for the extrinsic cycle of *Plasmodium falciparum*. In this study, *P falciparum* circumsporozoite protein was detected by ELISA in some of the mosquitoes recaptured in Jaribuni (2.75% *An. gambiae* and 1.65% *An. funestus*), which were recaptured 12 days after release, providing additional evidence for the ability of

the mosquitoes to survive long enough to get infected with malaria parasites which could eventually be transmitted to a human host if the mosquito remained alive and had a blood meal in the subsequent days. Mwangangi *et al.* (2004) reports the range of sporozoite infectivity rate in *An. gambiae s.s.* to be between 0.0-22.6 and 0.0-11.4 in Jaribuni and Mtepeni respectively. *Anopheles funestus* sporozoite rates ranged from 0.0-8.9 in Jaribuni and no sporozoite infections were reported for this species in Mtepeni. The high survival probability observed is an important factor in ensuring the efficiency of both *An. gambiae* and *An. funestus* as vectors of malaria in the study area.

The vector population size is also one of the major factors that contribute to the vector capacity of pathogen-transmitting insects. *Anopheles* populations have been shown to be dependent on the availability of suitable larval habitats (Minakawa *et al.,* 2002a) and as a result, are subject to a lot of fluctuations in population size. The estimates of population density obtained here do not seem high for an area such as Kilifi where malaria is endemic. However, studies have shown that at some sites in Kilifi, malaria prevalence rates of 50% occur even in the presence of very few or no mosquitoes (Mbogo *et al.,* 2003). It is however possible that the high survival probabilities of both *An. gambiae s.s.* and *An. funestus s.s.* reported here, their high preference to feed on humans (Mwangangi *et al.,* 2003) and the presence of *An. funestus s.s.* during the dry season (Mbogo *et al.,* 2003) are factors to consider as important for the continuous transmission of malaria in Kilifi.

The dispersal of *An. gambiae s.s.* and *An. funestus s.s.* was determined by estimating the distance travelled over time. Within the first 5 days after release, mosquitoes dispersed

variably, with some covering distances as short as 167 metres, and others dispersing to houses located further away (661 metres). A similar pattern of movement was also observed between 10 to 15 days after release, but this was not similar at the two sites. It is possible that mosquitoes recaptured further from the release habitat within 10 to 15 days might have been seeking their second blood meal. At Jaribuni, the numbers recaptured at days 2 to 5 was as high as those recaptured at days 10 to 15. This was not the case at Mtepeni, where the numbers recaptured at days 10 to 15 were much lower. The difference in observations suggests the non-uniformity of the factors influencing mosquito dispersal at the two sites. At both sites, no difference in dispersal was observed between *An. gambiae s.s.* and *An. funestus s.s.*, even though recapture rates were different. These results suggest distance to source of a blood meal was important in determining the destination of dispersing mosquitoes. In addition, the location or distance of the house relative to the habitat and other physical factors such as local topography and density of vegetation are important factors determining the destination of dispersers.

The variable population size estimates and high survival probability of *An. funestus* and *An. gambiae* observed along the Kenyan coast provides an explanation for the malaria transmission patterns reported, for example by Mbogo *et al.* (2003). Specific information on the population size, survival probability and dispersal capability of disease vectors is important in describing disease epidemiology. Epidemiologically, high survival probabilities in a dispersing population are very important given the epidemiological importance of older female mosquitoes in malaria transmission.

CHAPTER SIX

POPULATION GENETICS OF *ANOPHELES GAMBIAE* AND *ANOPHELES FUNESTUS* POPULATIONS ALONG THE KENYAN COAST

6.1 Introduction

Population genetics is the study of genetic variation and the factors that influence this variation in natural populations (Lanzaro and Tripet, 2000). An understanding of genetic variation in disease vector populations provides a foundation for understanding the role of the vector in disease epidemiology. Genetic variation in natural populations of a vector species is shaped by factors such as non-random mating, population size, migration, mutation and selection and in turn, these may influence variation in vectorial capacity and vector competence traits. To identify and characterize genetic variation in natural populations some of the methods commonly used include the analysis of chromosomal variants, isozymes, DNA markers and morphologic mutants (Coluzzi and Sabatinni, 1967; Mahon *et al.,* 1976). Among these, DNA markers provide an easy approach and have been applied widely in population studies, being highly suitable for the direct analysis of genetic material (Post *et al.,* 1992). The recent introduction of the polymerase chain reaction (PCR) technology has revolutionized genetic research, making it possible to genotype large numbers of individual specimen within a short time. As a result, population genetics and

molecular biology studies have been remarkably successful and productive in the last decade.

Over the years, the malaria vectorial system has evolved and become more complex, with growing resistance to some of the most commonly used insecticides e.g pyrethroids leading to the re-emergence of malaria in areas where it was previously eradicated (Krogstard, 1996), and threatening the use of some malaria control tools such as insecticide treated bednets. This situation has led to the search for new tools for malaria control, with considerable interest focussed on the development of transmission blocking vaccines and genetically-modified mosquitoes refractory to the malaria parasite to replace wild mosquito populations (Crampton *et al.,* 1994; Boete and Koella, 2002). The success of genetic control programmes however requires proper knowledge and understanding of the genetic structure of the populations and the levels of genetic exchange between populations, if any.

Through population genetics studies, it is possible to identify discrete population groups across spatial scales, determine the extent of their geographical distribution and evaluate the degree to which they may be reproductively isolated from other populations. In addition, population genetic studies are useful in estimating the rate at which genes spread within and between natural populations, and in identifying some of the factors influencing genetic exchange between populations. In the analysis of *An. gambiae* populations, microsatellite DNA analysis has been widely used to generate genetic maps of the mosquito (Dimopoulos *et al.,* 1996), to study the genetic differentiation and population genetic structure of *An. gambiae* (Lanzaro *et al.,* 1995, 1998; Donnelly *et al.,* 1999) and to identify the genes

affecting parasite development (Zheng *et al.,* 1996). Studies on the population genetics of *Anopheles gambiae* (Lehmann *et al.,* 1996; Besansky *et al.,* 1997; Lanzaro *et al.,* 1998; Donnelly *et al.,* 1999; Simard *et al.,* 2000) have contributed to a better understanding of the population biology of this species with the primary objective of these studies being to investigate the processes that lead to the spread of genes between natural populations. Inferences of gene flow are useful predictors of the likelihood of genetic exchange, such as the genes conferring insecticide resistance or *Plasmodium* refractoriness and such information is useful in the evaluation of how genes will spread in a population following the release of genetically modified organisms. Within a population, gene flow can lead to the introduction or re-introduction of genes into a population and an increase in the genetic variation of that population; whereas across populations, moving genes around can make genetically distant populations similar to one another, reducing the chance of speciation.

Knowledge of *An. gambiae* population structure has been useful for understanding the scales over which dispersal, genetic drift and selection operate (Slatkin, 1987), for identifying heterogeneity in disease transmission due to distinct vector populations and for tracking and predicting the spread of genes of interest such as those conferring insecticide resistance and antimalarial drug resistance.

Anopheles gambiae s.s. and *An. arabiensis* are the most widely genotyped *Anopheles* species. The integrated genetic and cytogenetic map of *Anopheles gambiae* developed by Zheng *et al.* (1996) of all three chromosomes based on microsatellite polymorphisms has greatly facilitated further molecular genetic studies on *An. gambiae.* Previous cytogenetic

studies on the *An. gambiae* in West Africa suggested the division of the species into partly or fully isolated chromosomal forms termed Mopti, Bamako, Bissau, Forest and Savanna (Bryan *et al.,* 1982; Coluzzi *et al.,* 1979; 1985). Molecular studies, however found high genetic similarities between the Savanna and Bamako forms (Favia *et al.,* 1997; Della Torre *et al.,* 2001; Gentille *et al.,* 2001) suggesting a higher precision of molecular markers in population studies. In East Africa, there has been no indication of reproductive isolation among populations (Kamau *et al.,* 1998; Lehmann *et al.,* 1997) but high genetic differentiation has been reported between populations separated by the Rift Valley Complex (Kamau *et al.,* 1998; Lehmann *et al.,* 1999) and low differentiation between populations from East and West Africa (Besansky *et al.,* 1997; Kamau *et al.,* 1999).

Despite having numerous population genetics studies conducted on *An. gambiae* and *An. arabiensis,* there have been much fewer reports on population genetics studies of *An. funestus,* which is also an important vector of *P. falciparum* malaria in sub Saharan Africa and the main vector in some areas (Cohuet *et al.,* 2002). In West Africa, studies on chromosomal inversion polymorphism in Burkina Faso by Costantini *et al.* (1999) reported a population substructure of two co-existing taxonomic units of *An. funestus* that differ in the degree of chromosomal polymorphism, host preference and resting habits. In Senegal Lochouarn *et al.* (1998) provide evidence for natural population heterogeneities in *An. funestus.* Kamau *et al.* (2002) reported significant chromosomal differentiation between *An. funestus* in western and coastal Kenya. Due to the lack of molecular markers for *An. funestus* this species has received very little attention, inconsistent with its major role in malaria transmission during the dry season when *An. gambiae* and *An. arabiensis* are less

active (Fontenille *et al.*, 1997). The isolation of polymorphic microsatellite loci from *An. funestus* (Sinkins *et al.*, 2000) and the development of a microsatellite map for *An. funestus* (Sharakov *et al.*, 2004) have led to the provision of a reference set of microsatellite markers, to facilitate population genetics studies of this species, and the comparison of studies conducted in different parts of Africa.

Along the Kenyan Coast, *An. gambiae* and *An. funestus* occur in sympatry, with *An. funestus* predominating over *An. gambiae* in drier areas during the dry season and continuing malaria transmission when *An. gambiae* populations decline. During larval ecology studies along the Kenyan coast, it was observed that *An. gambiae* and *An. funestus* shared larval habitats during the rainy season, but as the dry season progresses, *An. gambiae* larval and adult population densities reduce considerably, leaving *Anopheles funestus* as the predominant species through the dry season (Mbogo *et al.*, 2003). This seasonal rise and fall in *Anopheles* numbers led to the hypothesis that the severe population reduction during the dry season might have implications for the population genetic structure of *An. gambiae*, such that if the increase in population after a dry season is as a result of migrants from neighbouring sites, then new alleles/genotypes are bound to be introduced into the resident population, leading to an increase in the number of alleles within the population, and a possible increase in some allele frequencies. The greater the numbers of alleles at each variable locus and of variable loci, the greater the possibility for change in the frequency of some alleles at the expense of others if there are selection forces favouring the change of some traits and that the variation be relevant for the traits being selected.

There has been no field study conducted to establish whether habitat recolonization after the dry season is due to egg survival through the dry season, or as a result of new migrants arriving from neighbouring areas hence it is not clear whether the adult population undergoes local extinction, aestivation or employs egg survival strategies to maintain itself during the dry season. A study to examine the tolerance of *An. gambiae* egg stage to desiccation stress during the dry season by Shililu *et al.* (2004) indicated that *An. gambiae* eggs can survive up to 15 days under dry conditions, with their susceptibility to desiccation depending on soil type and limited desiccation tolerance of *An. gambiae* eggs may only contribute to short term survival in moist or dry soil. In addition, even though direct observations of individual movement through mark release recapture (MRR) data has been informative in estimating how far mosquitoes are able to move, these methods are inefficient at detecting long distance dispersal and are rarely able to demonstrate if dispersal has resulted in effective breeding. Through population genetics measurements, it is possible to determine what happens to the population during the dry season by looking at the changes in the population genetic structure of *Anopheles* species between seasons.

In this study, two *Anopheles gambiae* populations and one *Anopheles funestus* population were genotyped using 11 microsatellite loci to: a) determine if there are any changes in the population genetic structure of the *Anopheles* populations between seasons; b) determine if there is any population differentiation between *An. gambiae* populations in two study sites; c) and compare the estimates of gene flow between *Anopheles* populations as measured by direct methods (MRR experiments) and indirectly using microsatellite DNA analysis).

6.2 Material and Methods

6.2.1 The study sites

Mosquito samples for this study were collected from Jaribuni and Mtepeni. These sites have been described in Chapter Three (see Section 3.1.4 above).

6.2.2. Mosquito collection

The mosquitoes used for the population genetics study were sub-samples of those recaptured during the mark release recapture studies and those collected during the longitudinal studies which looked at the *Anopheles* species distribution patterns.

6.2.3 The extraction of mosquito genomic DNA

The various buffers and solutions used during DNA extraction and amplification were prepared as outlined in Appendix II.

DNA extraction from both *An. gambiae* and *An. funestus* was done using the method by Collins *et al.* (1997). Each mosquito sample (legs) was placed in a 1.5 ml eppendorf tube and homogenized in 100µl Bender buffer (0.1 M NaCl, 0.2M Sucrose, 0.1M Tris–HCl, 0.05M EDTA pH 8.0 and 0.5% SDS) with a sterile plastic grinder. This was then incubated at 65°C for 30 minutes followed by the addition of 15 µl of pre-chilled 8M KAc, mixed well by tapping the tube and then left on ice for 30 minutes. It was then centrifuged for 5 minutes at 14000 rpm and the supernatant transferred into a fresh tube. To the supernatant, two volumes of pre-chilled absolute ethanol was added, mixed well by tube inversion and then incubated at -40^0C for 2 hours. This was followed by centrifugation at 14000 rpm to pellet

the DNA. The supernatant was then discarded and the pellet was left to dry by evaporation after which it was re-dissolved in 25 µl of TE buffer and left to stand on ice for 1 hour. The DNA was stored at –20°C until ready for use.

6.2.4 PCR Identification of the *Anopheles* complex

6.2.4.I. Identification of the *Anopheles gambiae* complex

Five sets of primers designed from the DNA sequences of the intergenic spacer (ITS) region of *An. gambiae* complex ribosomal DNA (rDNA) were used in PCR for the member species identification (Scott *et al.*, 1993). The sequence details of these primers abbreviated UN, GA, ME, AR and QD and the expected sizes of the PCR products are given in Table 6.1. The UN primer anneals to the same position on the rDNA sequences of all five species, GA anneals specifically to *An. gambiae s.s.* ME anneals to both *An. merus* and *An. melas*, AR to *An. arabiensis* and QD to *An. quadriannulatus*.

The PCR reaction mix of 20 µl contained 1X PCR buffer supplied by the manufacturer (Sigma, USA), 200µM of each of the 4 oligonucleotide triphosphates (dNTPs), 10µM of each oligonucleotide primers and 0.125 U of *Taq* DNA polymerase enzyme (Sigma, USA). 0.5 µl of the genomic DNA was used as template for the amplification reaction. Sterile double distilled water was used to make up the volume to 20µl. The reaction mix was spun down briefly at 14,000rpm. The amplification was carried out using a PTC 100 thermal cycler (MJ Research Inc., USA)

The cycling parameters for the reaction were as follows; 94° C for 3min (initial denaturation), followed by 35 cycles of 94°C for 30s, 50°C for 30s, 72°C for 60s (annealing)

and ended by a final of cycle of 94^0 C for 30s, 50° C for 30 s and 72^0C for 10 min. For each reaction a positive control with PCR products of *Anopheles* of the same primer set and negative controls that contained no DNA template were included.

Table 6.1: DNA sequence details of the synthetic oligonucleotide primers used for the identification *An. gambiae* s.l. species and their melting temperatures (From Scott *et al.* 1993).

Name of Primer	Sequences (5' –3')	Melting Temperature T_m (^{0}C)	Expected Amplified DNA size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	58.3	468
GA	CTG GTT TGG TCG GCA CGT TT	59.3	390
ME	TGA CCA ACC CAC TCC CTT GA	57.2	464
AR	AAG TGT CCT TCT CCA TCC TA	47.4	315
QD	CAG ACC AAG ATG GTT AGT AT	42.7	153

6.2.4.2. Identification of the *Anopheles funestus* complex

Members of the *An. funestus* complex were identified using the cocktail PCR method based on the ITS2 region developed by Koekemoer *et al.* (2002). The sequence details of the primers (abbreviated UV, FUN RIV, PAR, LEES and VAN), the melting temperatures and expected diagnostic sizes of the amplified DNA products are shown in Table 6.2.

The PCR reaction mix of 25µl for each sample contained 10X reaction buffer, (500mM KCl, 100mM Tris- HCl pH 8.3), 1.5mM $MgCl_2$, 10pmol /primer of each primer, 10mM of each dNTP and 0.5 U of thermostable *Taq* DNA polymerase. DNA amplification was carried out using a PTC 100 thermal cycler, MJ Research Inc., USA. Amplification cycles were as follows: One cycle at 94^0C for 2 minutes, followed by 30 cycles at 94^0C for 30 seconds, 45^0C for 30 seconds, and 72^0C for 40 seconds and a final extension step of 72^0C for 40 seconds. PCR products were then electrophoresed in 4% agarose gel stained with Ethidium bromide. DNA standards of the species supplied by Koekemoer *et al.* were used as positive controls. A reaction mix with no template DNA was included as a negative control.

Table 6.2: DNA sequence details of the synthetic oligonucleotide primers used for the identification *An. funestus* species and their melting temperatures (From Koememoer et al., 2002).

Name of Primer	Sequences (5' –3')	Melting Temperature T_m (^{0}C)	Expected Amplified DNA size (bp)
UV	TGT GAA CTG CAG GAC ACA T	55.34	–
FUN	GCA TCG ATG GGT TAA TCA TG	52.4	505
VAN	TGT CGA CTT GGT AGC CGA AC	58.0	587
RIV	CAA GCC GTT CGA CCC TGA TT	58.8	411
PAR	TGC GGT CCC AAG CTA GGT TC	60.5	252
LEES	TAC ACG GGC GCC ATG TAG TT	60.2	146

6.2.5 Microsatellite DNA analysis

6.2.5.1 Microsatellite genotyping of *Anopheles gambiae s.s.*

Eleven microsatellite loci (Table 6.3) were selected from the published genomic map of Zheng *et al.* (1996). The position of the markers was determined from the genomic map (Zheng *et al.*, 1996), and cytogenetic maps (Coluzzi and Sabattini, 1967). To minimize the confounding genome wide patterns with locus specific effects, we selected microsatellite loci located outside of polymorphic inversions. At each locus, at least 40 specimens were genotyped per season, and 150 specimen per site (Tables 6.3 & 6.4) by PCR amplification in a PTC-100 Thermal cycler (MJ Research Water town, MA) and Hybaid thermal cycler (Hybaid Ltd, UK). The forward primer of each primer pair was DNA from each specimen, 10X Buffer, 0.2mM of each DNTP, 20pmols primer, 1.5 mM $MgCl_2$, and 0.5 units *Taq* polymerase enzyme. DNA amplification was conducted through 35 cycles as follows: An initial denaturation step of 2 minutes at 95^0C was followed by 35 cycles of 30s at 55^0C, and 45s at 72^0C. Each PCR reaction product was prepared for electrophoresis by mixing with sequencing Dye followed by denaturation for 5 minutes and electrophoresis was performed using 1.5 µl of the final mixture in a 5% acrylamide gel on a LICOR 4200 DNA sequencer using standard protocols.

Table 6.3: Microsatellite loci studied in *Anopheles gambiae*

Locus	Chromosomal location	No. of Repeats	Allele size (bp)
AGXH1D1	X	(CCA)	172
AGXH131	X	(GT)48	175
AGXH678	X	(AG)7	153
AG2H46	2	(GT)8	138
AG2H143	2	(TC)9	160
ND30	2	(GT)6	184
ND36	2	(GT)9	92
AG3H555	3	(GT)8	81
AG3H88	3	(GT)9	176
AG3H249	3	GT(15)	129
AG3H29	3	(TGA)	148

6.2.5.2 Microsatellite genotyping of *Anopheles funestus*

Eleven tri-nucleotide microsatellite markers used in this study were selected from the reference of microsatellite markers published by Sharakov *et al.* (2004). Table 6.2 shows the microsatellite markers, and their chromosomal location. The PCR procedure was similar to that applied for *An. gambiae* above, but for *An. funestus*, each 25ul reaction mix consisted of 10X PCR buffer, (10mM Tris, pH 8.0, 50mM KCl), 2.0mM $MgCl_2$, 0.3mM dNTPs, 10mM BSA, 0.5units of Taq DNA polymerase and 10pmol of each primer. The forward primer of each locus was labelled with M13, a fluorescent dye compatible with LICOR horizontal gel electrophoresis. DNA amplification was completed in a Hybaid thermal cycler, (MJ Research Water town, MA). An initial denaturation step at 95^0C for 2 min was followed by 35 cycles of 30s at 95^0C, 30s at 56^0C and 35s at 72^0C. The last step was a final extension at 72^0C for 30 min. Each PCR reaction product was prepared for electrophoresis by mixing with 5µl sequencing Dye (a mixture of 3.5 µl formamide and 1.5µl blue dextran) followed by denaturation for 5 minutes and electrophoresis was performed using 1.5 µl of the final mixture

Table 6.4: Microsatellite loci studied in *Anopheles funestus*

Locus	Chromosomal location	No of repeats	Allele size range (bp)
AFND12	X	$(GA)_6$	87-107
FUNQ	X	$(TG)_9$	84-98
AFUB3	2R	$(GGT)_{5+4}$	171-195
FUNO	2R	$(CA)_6TA(AC)_4$	110-132
AFUB11	2L	$(GCT)_5$	125-134
AFND23	2L	$(GAG)_5$	85 - 75
AFND36	2	$(CAC)_4$	128-133
AFUB10	2L	$(GT)_{6+4}$	114-146
AFND7	3R	$(GT)_8$	70-84
AFUB12	3L	$(GA)_6$	87-107
AFUB6	3	$(GT)_{7+3}$	184-212

6.2.6 Analysis of PCR products

Different methods can be applied in the analysis of PCR products. Most commonly used is gel electrophoresis, using either different types of agarose gels or polyacrylamide gel electrophoresis to separate the amplified DNA fragments based on molecular weight. Agarose gels are suitable for PCR products smaller than 600basepairs, whereas polyacrylamide gels are most suitable for separating products from polymorphic loci.

6.2.6.1 Agarose gel electrophoresis

Following the PCR for species identification, PCR products for both *Anopheles gambiae* and *An. funestus* were electrophoresed on separate 2% agarose gels stained with 0.5µg/ml EtBr to detect the presence of amplified DNA fragments. Eight microlitres of each sample was added to 1µl of (5X) bromophenol blue gel loading dye for the electrophoresis. The 2% gel was prepared and electrophoresed in 1X TAE buffer using a mini gel system (BIORAD, USA) at 100 volts for one hour and then photographed over a UV transilluminator (UPC, USA) at short wavelength using a Polaroid camera and film type 667 (Polaroid, USA).

6.2.6.2 Polyacrylamide gel electrophoresis

PCR products resulting from microsatellite DNA analysis of both *An. gambiae* and *An. funestus* were resolved on 5% denaturing polyacrylamide gels using a LICOR 4200 DNA sequencer, (Licor BioSciences Nebraska, USA) running gene scan software. Products from each microsatellite locus were run on separate gels with individual mosquito samples per lane, and a 1kb DNA marker was included for allele sizing.

6.2.6.3. Microsatellite allele sizing

Allele sizing was performed using Gene ImagIR 3.55 fragment analysis software (Licor Biosciences, Nebraska, USA). The Gene ImageIR runs gene profiler, a software package that uses automatic lane and peak finding to detect the presence of bands in a gel. Based on the standard licor ladder, the profiler calibrates the microsatellite bands for size and intensity. Using calibration markers in each gel electrophoresis run, Gene Profiler automatically calculates the molecular weight (M_r), isoelectric point (pI) and concentration values of any DNA fragment detected. The software then corrects and analyzes distorted gel images for lanes that slant, curve or smile, ensuring the highest degree of accuracy possible. Gene Profiler uses Gaussian deconvolution for accurate determination of peak areas and it also provides four curve-fitting algorithms for precise molecular weight determination. The molecular weight value determined at the highest peak is recorded as the fragment size. The DNA fragment size data are then stored in a built-in database for rapid gel-to-gel comparisons, and images stored as TIFF files.

6.2.7. Data analysis

All analyses to determine the population genetic polymorphism, genetic structure and gene flow patterns in *An. gambiae* and *An. funestus* populations were performed using the GENEPOP software, version 3.4. (Raymond and Rossuet, 1995). Genetic polymorphism was measured by the number of alleles observed per locus, observed heterozygosity per locus (A) per season in each of the study sites, and allele frequencies observed in each population sample.

Each individual locus was tested separately for departure from Hardy-Weinberg equilibrium using the Markov Chain algorithm of Guo and Thompson (1992) available in GENEPOP. The *Fis* statistic (Raymond and Rousset, 1995) was computed to check whether any distortion from the Hardy-Weinberg equilibrium during each season was due to heterozygote deficiency or excess. Heterozygote deficiency, if observed in multiple loci might suggest that samples analyzed consist of several pooled subpopulations (commonly referred to as the 'Wahlund' effect), the effects of inbreeding, or the presence of null alleles.

Pairwise genotypic linkage disequilibrium was tested with Fisher's exact test, under the null hypothesis that genotypes at one locus are independent of genotypes at another locus to confirm whether the deviations from the Hardy Weinberg equilibrium observed within samples were not due to the Wahlund effect, inbreeding or linkage with genes under selection pressure or locus specific effects such as null alleles. Microsatellite null alleles result from mutations in the region complementary with one of the oligonucleotide primers and thus cannot be amplified by PCR. The Wahlund effect or inbreeding should cause

within population linkage disequilibrium because members of the different subpopulations will have different probabilities of carrying certain combinations of alleles. However if null alleles cause the heterozygote deficits, linkage disequilibrium is not expected because all individuals are equally expected to carry a null allele and the association between alleles from different loci is not disturbed.

Population differentiation was examined using the fixation index, *F*-statistic (*Fst*) (Wright, 1978). *Fst* was calculated in GENEPOP according to Weir and Cockerham (1984) to examine the differences in allele frequencies. *Fst* is the variance among allele frequencies, standardized by the mean allele frequency among populations. *Fst* considers all alleles mutationally equidistant from each other (Infinite allele model) and is calculated based on the number of different alleles. Tests were conducted for population differentiation between the dry and wet season populations, and between the two study sites Mtepeni and Jaribuni. Genotypic permutation tests in *FST*AT (Goudet, 1995) were used to determine if *Fst* estimates differed significantly from zero.

Gene flow, or the number of migrants (Nm) per population per generation between the two study sites was estimated from the derived pairwise *Fst* values using the formula Nm = (1-*Fst*)/4*Fst* (Slatkin, 1995). Estimates for gene flow between the two sites were conducted for only *An. gambiae* because *An. funestus* was obtained only at the Jaribuni site.

The current effective population sizes of the *An. gambiae* and *An. funestus* populations in Jaribuni and Mtepeni and the effective population size of *An. funestus* in Jaribuni were

estimated based on the temporal variation in allele frequencies. The effective population size was determined based on the formula: $Ne = (1/(1-He)^2 - 1)/8m$, Where He is heterozygosity at the locus (estimated from genepop and m is the mutation rate and is set at m = 0.000205 per generation.

6.3 Results

A total of 417 adult mosquitoes (211 from Jaribuni and 206 from Mtepeni) were identified morphologically as members of the *Anopheles gambiae* complex. One hundred and ninety-one female mosquitoes were identified as *An. funestus* from Jaribuni and only two *An. funestus* from Mtepeni. *Anopheles gambiae* were further identified by PCR following the method of Scott *et al.*, (1993) to the species level. *Anopheles funestus* mosquitoes were also further identified by PCR to the species level as *An. funestus s.s.* following the method of Koekemoer *et al.* (2002). Plates 6.1 and 6.2 are gel photographs showing some of the *An. gambiae s.s.* and *An. funestus s.s.* identified by PCR.

6.3.1 Species composition

Out of 417 *An. gambiae*; 211 specimens were identified from Jaribuni and 206 from Mtepeni. From

Jaribuni, species identification was as follows: 203 *An gambiae s.s.*, four *An. merus* and four were unidentified even after repeated PCR. *Anopheles gambiae s.s.* samples identified from the dry season were 101 (N=58 for February, 43 for July) and 102 from the wet season (N=60 for April, 42 for October). For the Mtepeni population, 204 *An. gambiae s.s.* were identified by PCR, 98 for the dry season (N=52 for February, 46 for July) and 106 from the wet season (N= 49 for April, 57 for October). Two specimens were unidentified after repeated PCR. Neither *An. merus* nor *An. arabiensis* were identified from Mtepeni. Overall, 97.6% of the specimens were *An. gambiae s.s.*

All the *Anopheles funestus* populations from Jaribuni were identified by PCR, 77 for the dry season (N=33 for February and 44 for July) and 103 for the wet season (N= 49 for April, 54

for October). Only two specimens were obtained from Mtepeni and identified by PCR as *An. funestus s.s.* No other *An. funestus* species were recorded. The *An. funestus* samples from Mtepeni were excluded from microsatellite analysis, as they were not representative of the entire population.

Plate 6.1: Ethidium bromide stained 2%agarose gel electrophoresis of PCR products obtained from the amplification of *Anopheles gambiae* DNA for species identification. Lanes 1-10 = Amplified DNA from adult *An. gambiae* s.s female mosquitoes, M = 100 bp molecular weight marker, A and G are positive controls for *An. arabiensis* and *An. gambiae s.s* at 315bp and 390bp respectively

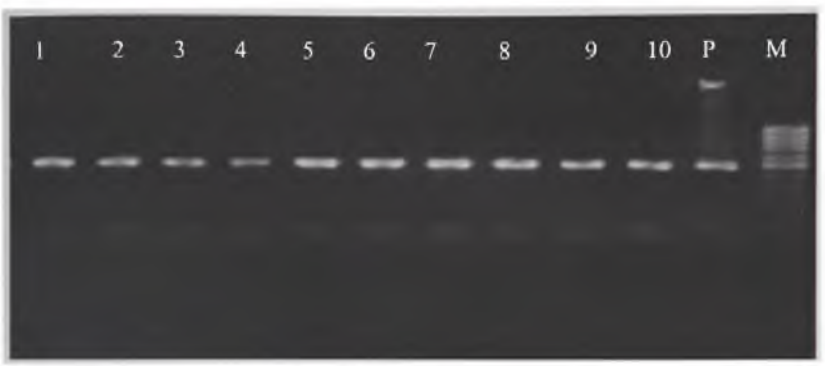

Plate 6.2: Ethidium bromide stained 2% agarose gel electrophoresis of PCR products obtained from the amplification of *Anopheles funestus s.s.* DNA for species identification. Lanes 1-10 = Amplified DNA from adult female *An. funestus s.s.* mosquitoes, M = 100 bp molecular weight marker.

6.3.2 Genetic differentiation

6.3.2.1 Genetic differentiation within *An. gambiae* populations

The population genetic diversity within *Anopheles gambiae s.s.* populations from Jaribuni and Mtepeni was quantified by the number of alleles per locus and expected heterozygosity (H_E). During the dry seasons in Jaribuni, an average of 6.5 alleles per locus were observed in February (Range = 3-11) and July (Range = 3 -10); and the average expected heterozygosity was 0.6154 for February and 0.6011 for July. During the wet seasons, an average of 8.0 (range 6–10) and 8.3 (range 5.7-10.9) alleles per locus were observed for April and October, respectively and expected heterozygosity was 0.6854 for April and 0.7264 for October (Table 6.5).

In Mtepeni, an average of 6.1 (range = 4.3-7.8) and 6.7 (range = 4.9-8.5) alleles per locus were observed during the dry seasons in February and July respectively, and during the wet season, 8.7 (Range = 6.9 -10.5) and 5.5 (Range = 4.4 -6.6) alleles per locus were observed in April and October, respectively. The average expected heterozygosity was 0.5890 for February and 0.5964 for July, and 0.7745 and 0.6009 for April and October, respectively (Table 6.6).

There were no significant differences observed in the number of alleles or heterozygosity within the Jaribuni or Mtepeni populations ($p>0.05$). However there was a significant difference between the total number of alleles recorded in Jaribuni and Mtepeni ($p=0.04$), Overall, expected heterozygosity (He) was higher that observed heterozygosity (Ho) for both the *An. gambiae* and *An. funestus* populations.

Table 6.5: Sample size (N), total number of Alleles (A_{Tot}), Heterozygosity observed under direct count (H_o), expected Nei's unbiased heterozygosity (H_e) and inbreeding coefficients (F_{is}) by locus for samples of *An. gambiae* from Jaribuni, Kilifi.

		POPULATIONS			
Locus		**February (dry) N=58**	**July (dry) N=43**	**April (wet) N=60**	**October(wet) N=42**
29	A_{Tot}	9	10	7	4
	H_e	0.76	0.77	0.64	0.72
	H_o	0.73	0.32	0.31	0.33
	P value	0.0000	0.0000	0.0000	0.0000
	F_{is}	+0.0367	+0.5941	+0.5211	+0.5437
131	A_{Tot}	4	4	7	7
	H_e	0.55	0.58	0.64	0.65
	H_o	0.44	0.42	0.43	0.51
	P value	0.0292	0.0421	0.0000	0.0050
	F_{is}	+0.2058	+0.2754	+0.3197	+0.2118
143	A_{Tot}	12	12	8	7
	H_e	0.86	0.86	0.84	0.80
	H_o	0.38	0.45	0.30	0.5
	P value	0.0000	0.0000	0.0000	0.0000
	F_{is}	+0.2986	+0.4812	+0.7155	+0.3790
1D1	A_{Tot}	5	5	7	5
	H_e	0.19	0.21	0.51	0.51
	H_o	0.20	0.19	0.25	0.52
	P value	1.0000	0.1026	0.0000	0.1892
	F_{is}	-0.0654	+0.0504	+0.4983	-0.0137
2H46	A_{Tot}	16	14	15	17
	H_e	0.86	0.89	0.81	0.91
	H_o	0.48	0.48	0.35	0.51
	P value	0.0000	0.0000	0.0000	0.0000
	F_{is}	+0.4448	+0.4591	+0.5656	+0.4380
ND30	A_{Tot}	6	4	6	5
	H_e	0.75	0.61	0.73	0.75
	H_o	0.52	0.4	0.33	0.43
	P value	0.0000	0.0000	0.0000	0.0000

	F_{is}	+0.3121	+0.3495	+0.5575	+0.4256
249	A_{Tot} H_e H_o P value F_{is}	6 0.69 0.39 0.0000 +0.4424	8 0.95 0.47 0.0000 +0.3741	12 0.82 0.54 0.0000 +0.3468	11 0.74 0.44 0.0000 +0.4072
ND36	A_{Tot} H_e H_o P value F_{is}	16 0.34 0.40 0.0251 -0.1926	3 0.38 0.18 0.0002 +0.5213	3 0.50 0.20 0.00 0.5960	3 0.48 0.29 0.0137 +0.3939
678	A_{Tot} H_e H_o P value F_{is}	17 0.85 0.68 0.0000 +0.2006	7 0.75 0.57 0.0000 +0.2525	21 0.91 0.53 0.00 +0.4167	14 0.90 0.59 0.0000 +0.3550
555	A_{Tot} H_e H_o P value F_{is}	4 0.60 0.64 0.9887 -0.0565	6 0.72 0.38 0.0000 +0.4750	8 0.76 0.29 0.00 +0.6166	8 0.69 0.42 0.0000 +0.3981
88	A_{Tot} H_e H_o P value F_{is}	8 0.71 0.31 0.0000 +0.5773	5 0.68 0.36 0.0000 +0.4735	6 0.75 0.31 0.00 +0.5902	10 0.84 0.31 0.0000 +0.6307
Mean	A_{Tot} H_e H_o	6.5 0.6154 0.4482	6.5 0.6011 0.3864	7.9 0.6854 0.3345	8.3 0.7264 0.4409

Table 6.6: Sample size (N), total number of Alleles (A_{Tot}), Heterozygosity observed under direct count (H_o), expected Nei's unbiased heterozygosity (H_e) and inbreeding coefficients (*Fis*) by locus for samples from Mtepeni, Kilifi.

		POPULATIONS			
Locus		February (dry) N=52	July (dry) N=46	April (wet) N =49	October(wet) N=57
29	A_{Tot}	2	2	8	7
	H_e	0.47	0.39	0.79	0.80
	H_o	0.49	0.53	0.64	0.66
	P value	0.7692	0.0397	0.000	0.0000
	F_{is}	-0.0504	-0.3455	+0.1907	+0.1815
131	A_{Tot}	4	4	3	3
	H_e	0.59	0.57	0.64	0.41
	H_o	0.54	0.33	0.37	0.37
	P value	0.0001	0.0019	0.000	0.6309
	F_{is}	+0.0809	+0.4220	0.4334	+0.0975
143	A_{Tot}	8	6	10	4
	H_e	0.61	0.97	0.83	0.66
	H_o	0.63	0.65	0.58	0.45
	P value	0.0385	0.4553	0.000	0.0000
	F_{is}	+0.0725	+0.0559	0.3044	+0.3251
1D1	A_{Tot}	5	5	7	8
	H_e	0.54	0.24	0.70	0.75
	H_o	0.71	0.24	0.52	0.63
	P value	0.1053	0.0521	0.000	0.0005
	F_{is}	-0.3302	-0.0136	0.2582	+0.1636
2H46	A_{Tot}	7	10	13	5
	H_e	0.57	0.74	0.87	0.53
	H_o	0.43	0.36	0.51	0.42
	P value	0.0013	0.0000	0.000	0.1288
	F_{is}	+0.2500	+0.5172	0.4116	+0.2186
ND30	A_{Tot}	3	5	6	4
	H_e	0.54	0.57	0.75	0.68
	H_o	0.53	0.50	0.5	0.92
	P value	0.0696	0.0944	0.000	0.0000
	F_{is}	+0.0125	+0.1199	0.3400	-0.3597

249	A_{Tot}	8	10	9	6
	H_e	0.75	0.78	0.77	0.25
	H_o	0.69	0.66	0.65	0.23
	P value	0.3023	0.0700	0.000	0.1154
	F_{is}	+0.0821	+0.1625	0.1549	+0.0741
ND36	A_{Tot}	3	5	8	4
	H_e	0.22	0.20	0.69	0.63
	H_o	0.004	0.07	0.38	0.60
	P value	0.0000	0.0000	0.000	0.0301
	F_{is}	0.6377	+0.6490	0.4461	0.0347
678	A_{Tot}	7	12	14	4
	H_e	0.75	0.86	0.89	0.68
	H_o	0.72	0.74	0.79	0.8
	P value	0.2526	0.0198	0.000	0.0002
	F_{is}	0.0371	+0.1338	0.1156	+0.0534
555	A_{Tot}	9	6	11	6
	H_e	0.66	0.67	0.87	0.48
	H_o	0.40	0.3	0.48	0.30
	P value	0.0000	0.0000	0.000	0.0000
	F_{is}	+0.3899	+0.5568	0.4433	+0.3721
88	A_{Tot}	11	9	7	9
	H_e	0.78	0.57	0.72	0.74
	H_o	0.55	0.16	0.3	0.55
	P value	0.0000	0.0000	0.000	0.0000
	F_{is}	+0.3029	+0.7256	0.5864	+0.2586
Mean	A_{Tot}	6.1	6.7	8.7	5.5
	H_e	0.5890	0.5964	0.7745	0.6009
	H_o	0.5209	0.4127	0.5200	0.5391

6.3.2.2 Genetic differentiation within the *An. funestus* populations

Genetic diversity within the *Anopheles funestus* populations from Jaribuni was quantified by the number of alleles per locus and expected heterozygosity (H_E). During the dry seasons an average of 3.7 alleles per locus were observed in February (Range = 2 - 6) and 3.8 in July (Range = 1-7) and the average expected heterozygosity was 0.71 for February and 0.40 for July. During the wet seasons, an average of 3.8 (range 3–9) and 4.5 (range 2-7) alleles per locus were observed for April and October, respectively and expected heterozygosity was 0.39 for April and 0.45 for October (Table 6.7).

Table 6.7: Sample size (N), total number of Alleles (A_{Tot}), Heterozygosity observed under direct count (H_o), expected Nei's unbiased heterozygosity (H_e) and inbreeding coefficients (F_{is}) by locus for *Anopheles funestus* mosquitoes from Jaribuni, Kilifi.

		POPULATIONS			
Locus		February (dry) N=33	July (dry) N=44	April (wet) N=49	October(wet) N=54
FUNQ	A_{Tot}	6	3	4	7
	H_e	0.57	0.52	0.73	0.80
	H_o	0.53	0.57	0.62	0.51
	P value	0.0204	0.0109	0.0000	0.00
	F_{is}	+0.198	-0.085	+0.146	+0.363
ND12	A_{Tot}	2	1	3	2
	H_e	0.63	0.000	0.40	0.36
	H_o	0.31	0.000	0.51	0.46
	P value	0.5575	0.0000	0.013	0.04
	F_{is}	-0.216	0	-0.278	-0.297
FUN O	A_{Tot}	5	6	3	7
	H_e	0.45	0.55	0.26	0.50
	H_o	0.68	0.53	0.30	0.49
	P value	0.0020	0.053	1.0000	0.002
	F_{is}	+0.1900	+0.031	-0.134	+0.015
FUB11	A_{Tot}	3	3	4	4
	H_e	0.88	0.22	0.18	0.14
	H_o	0.12	0.24	0.19	0.13
	P value	1.0000	1.000	1.0000	0.026
	F_{is}	-0.036	-0.113	-0.072	+0.079
FND23	A_{Tot}	2	4	3	5
	H_e	0.60	0.67	0.54	0.69
	H_o	0.53	0.33	0.58	0.28
	P value	1.0000	0.0000	0.5219	0.0000
	F_{is}	+0.273	+0.504	-0.085	+0.605

AFUB10	A_{Tot} H_e H_o P value F_{is}	6 0.78 0.33 0.028 +0.339	7 0.65 0.92 0.0000 -0.417	9 0.66 0.78 0.0024 -0.188	7 0.51 0.53 0.03 -0.034
AFND20	A_{Tot} H_e H_o P value F_{is}	2 0.94 0.06 1.0000 -0.016	2 0.05 0.05 1.0000 -0.012	3 0.04 0.04 1.0000 -0.005	2 0.04 0.04 1.0000 -0.010
AFUB3	A_{Tot} H_e H_o P value F_{is}	5 0.5 0.56 0.58 +0.111	6 0.57 0.54 0.8 +0.058	4 0.57 0.50 0.88 +0.131	6 0.61 0.62 0.01 -0.008
AFUB12	A_{Tot} H_e H_o P value F_{is}	2 0.97 0.38 0.00 +0.914	3 0.17 0.09 0.005 +0.472	2 0.25 0.08 0.0006 +0.688	3 0.44 0.1 0.0000 +0.781
AFUB6	A_{Tot} H_e H_o P value F_{is}	2 0.70 0.51 0.085 +0.415	2 0.50 0.58 0.361 -0.162	3 0.46 0.48 0.866 -0.034	3 0.52 0.52 1.0000 -0.01
AFND7	A_{Tot} H_e H_o P value F_{is}	6 0.82 0.0012 0.0000 +0.352	5 0.45 0.34 0.0059 +0.248	4 0.16 0.14 0.025 +0.096	3 0.36 0.32 0.2810 +0.084
Mean	A_{Tot} H_e H_o	3.7 0.71 0.39	3.8 0.40 0.38	3.8 0.39 0.38	4.5 0.45 0.36

6.3.3 Tests for Hardy Weinberg Equilibrium and genotypic linkage disequilibrium

Tests for Hardy Weinberg expectations were conducted both within and between populations using the Markov chain algorithm (Guo and Thompson, 1992) available in GENEPOP.

6.3.3.1 *Anopheles gambiae* populations

Within population deviations from Hardy Weinberg equilibrium were found in 88.6% of 44 tests in Jaribuni ($p<0.05$) whereas in Mtepeni, there were deviations in 68.2% of 44 tests ($p<0.05$).

In Jaribuni, analysis of samples within population as defined by season of collection revealed departures from HWE ($P<0.05$) at most loci for all the populations, which also showed significant heterozygote deficiency as indicated by positive Fis values (Table 6.5). The loci which were in HWE were 1D1 and 555 in the February population, 1D1 in the July population, and 1D1 and ND30 in the October population. These loci showed heterozygote deficits except the loci 1D1 and 555 from the February population, which had an excess of heterozygotes.

Hardy-Weinberg predictions were also significantly rejected ($P<0.05$) when all the samples were pooled and analyzed as a single population. Two loci, 1D1 and ND30 were in HWE ($P>0.05$). All the loci in deviation from HWE also showed heterozygote deficiency with positive *Fis* values. Significant heterozygote deficits ($P<0.05$) were detected for all the loci except locus 1D1.

In Mtepeni, 4 loci (29, 1D1, ND30, 249, and 678) conformed to HWE expectations in the February population, and 4 loci (143, 1D1, ND30, and 249) in the July population. For the April population, all loci deviated from HWE whilst in the October population, locus 131, 2H46, 249, 555 and 88 were in HWE. When all the populations were pooled, HWE was significantly rejected at all loci ($p<0.05$). Following departures from HWE observed at most loci in both the Jaribuni and Mtepeni populations, data from these two populations were pooled and tested for overall population heterozygote deficiency, as measured by *Fis* (the coefficient of inbreeding). High inbreeding co-efficients were recorded in the Jaribuni population compared to the Mtepeni population (Figure 5.1), suggesting either a pooling of several similar sub-populations within the Jaribuni population, or a high level of inbreeding among *An. gambiae* within the Jaribuni site.

Based on the null hypothesis that genotypes at one locus are independent of genotypes at the other locus, linkage disequilibrium analysis was performed within each season to determine whether the deviations from HWE were due to inbreeding, a Wahlund effect or the effect of locus specific events such as null alleles or linkage with genes under selection pressure.

Out of 56 pairwise tests for the Jaribuni population, 6 pairs of loci (10.7%) showed significant linkage disequilibrium in February ($P<0.05$). In the July population, none of them showed significant linkage disequilibrium ($P>0.05$). In April, 15 out of 55 pairs (27.3%) were significant and in October, only one out of 55 pairs (1.82%) were in disequilibrium. When data from all seasons were pooled and analyzed as a single population, 20 out of 55 pairs (36%) of loci showed significant linkage disequilibrium. Linkage disequilibrium

In Mtepeni, 4 loci (29, 1D1, ND30, 249, and 678) conformed to HWE expectations in the February population, and 4 loci (143, 1D1, ND30, and 249) in the July population. For the April population, all loci deviated from HWE whilst in the October population, locus 131, 2H46, 249, 555 and 88 were in HWE. When all the populations were pooled, HWE was significantly rejected at all loci ($p<0.05$). Following departures from HWE observed at most loci in both the Jaribuni and Mtepeni populations, data from these two populations were pooled and tested for overall population heterozygote deficiency, as measured by *Fis* (the coefficient of inbreeding). High inbreeding co-efficients were recorded in the Jaribuni population compared to the Mtepeni population (Figure 5.1), suggesting either a pooling of several similar sub-populations within the Jaribuni population, or a high level of inbreeding among *An. gambiae* within the Jaribuni site.

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analysis of populations from Mtepeni indicated significant linkage in 6 out of 55 (10.9%) pairs of loci in February, 2 out of 55 (3.6%) pairs in July, 30 out of 55 (54.5%) pairs in April and 2 out of 55 (3.6%) pairs in October. When data from all seasons were pooled, all pairs of loci showed significant linkage disequilibrium.

Significant linkage disequilibrium suggests the independent segregation of these loci.

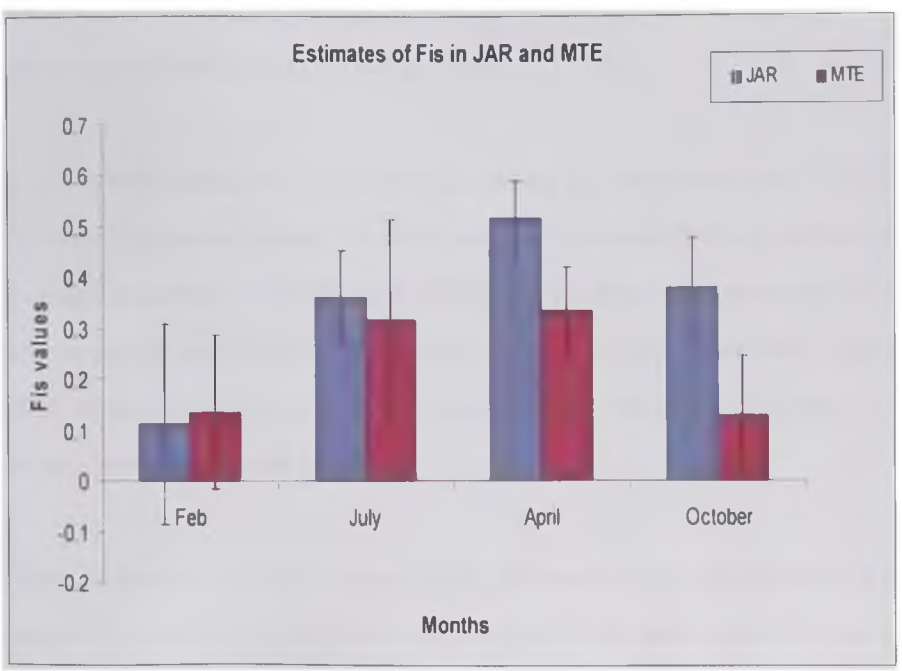

Figure 6.1: Estimates of the coefficient of inbreeding (*Fis*) in the Jaribuni and Mtepeni populations of *An gambiae*

6.3.3.2 *Anopheles funestus* population

The analysis of samples within each population as defined by season of collection revealed significant deviations from HWE within *An funestus* populations in 26 out of 44 (59.1%) tests done across the wet and dry seasons in Jaribuni ($p<0.05$). Out of these tests, significant heterozygote deficiency was observed in 17 of the tests, and these were clustered in the dry season populations. When all the samples were pooled and analyzed as a single population HWE predictions were significantly rejected in 7 out of 11 loci ($P<0.05$). The loci in H-W equilibrium were FUB11, FND20, FUB3, and FUB6.

The loci in HWE during the dry seasons (i.e. February and July) were FUB 11, ND20, FUB3, and FUB6 in both months. The ND12 and FND23 were in HWE only in February, but deviated from HWE in July. During the wet seasons, (i.e. April and October), only locus ND20 and AFUB6 were in HWE in both months. Other loci in HWE were FUN O, FUB11, FND23 and AFUB3 for the month of April and AFND 7 for the month of October. All these loci showed heterozygote deficiency.

Analysis of linkage disequilibrium was carried out based on the null hypothesis that genotypes at one locus are independent of genotypes at the other locus. This was to determine whether the deviations from HWE within seasons were due to inbreeding, a Wahlund effect or the effect of locus specific events such as null alleles or linkage with genes under selection pressure. Out of 44 pair wise tests done for each population (season), only 2 tests (2.3%) were significant for the dry seasons ($P<0.05$) and 10 tests (22.7%) for the

wet seasons (P<0.05). When data from all seasons were pooled and analyzed as a single population, 11 out of 44 pairs (25%) of loci showed significant linkage disequilibrium (P<0.05)

6.3.4 Genetic Differentiation

6.3.4.1 Genetic differentiation in *Anopheles gambiae* among seasons (populations)

Population differentiation between seasons was measured by *Fst,* as calculated using the Weir and Cockerhams method available in Genepop. *Fst* examines the differences in allele frequencies between populations, and is calculated as the variance among allele frequencies standardized by the mean allele frequency in each population. According to Wright, (1978); *Fst* ranges from 0 to 1 and the higher the value, the greater the genetic differentiation. According to Wrights guidelines, There is little differentiation when *Fst* ranges between 0-0.05, moderate between 0.05 0.15, great between 0.15 – 0.25 and very great when *Fst* >0.25. Figure 6.2 shows a plot of the *Fst* values between different seasons in Jaribuni and Mtepeni.

In Jaribuni, *Fst* values between the dry seasons (dry/dry=Feb/July) were low (*Fst* = 0.011), as was the case between the wet seasons (wet/wet=April/October) (*Fst* = 0.042). Comparison between wet and dry (wet/dry=Feb/April) indicated a higher *Fst* value (*Fst* = 0.059) and (April/July) (*Fst* = 0.045). *Fst* values were not significantly different. In Mtepeni, *Fst* values between the dry seasons (dry/dry=Feb/July) were higher (*Fst* = 0.038), as was the case between the wet seasons (wet/wet=April/October) (*Fst* = 0.181). Comparison between wet and dry (wet/dry=Feb/April) indicated a higher *Fst* value (*Fst* = 0.102) and (April/July) (*Fst* = 0.104). *Fst* values were not significantly different.

6.3.4.2 Genetic differentiation in *Anopheles gambiae* among sites

Using *Fst,* genetic differentiation between the pooled Jaribuni and Mtepeni populations was measured to determine how genetically distinct *An. gambiae* populations were between the two study areas, Mtepeni and Jaribuni are. The *Fst* value for *An. gambiae* populations between Jaribuni and Mtepeni was 0.1102.

6.3.4.3 Genetic differentiation between *An. funestus* populations from Jaribuni among seasons

Genetic differentiation between the wet and dry season populations of *An. funestus* in Jaribuni as measured by *Fst* was moderate for most population pairs. Comparisons between February and July (Dry/dry) were 0.101; and the wet/wet comparison between April/Oct was 0.059. February and April (Dry/wet) *Fst* was 0.138, and July vs April (Dry/wet) indicated *Fst* value of 0.134. Fun Q is the only locus that indicated great differentiation between all population pairs, compared to all other loci. The low to moderate level of genetic differentiation between the *An. funestus* populations indicates that the *An. funestus* population in Jaribuni is generally panmictic across seasons. Figure 6.2 shows a plot of the *Fst* values between seasons in Jaribuni and Mtepeni.

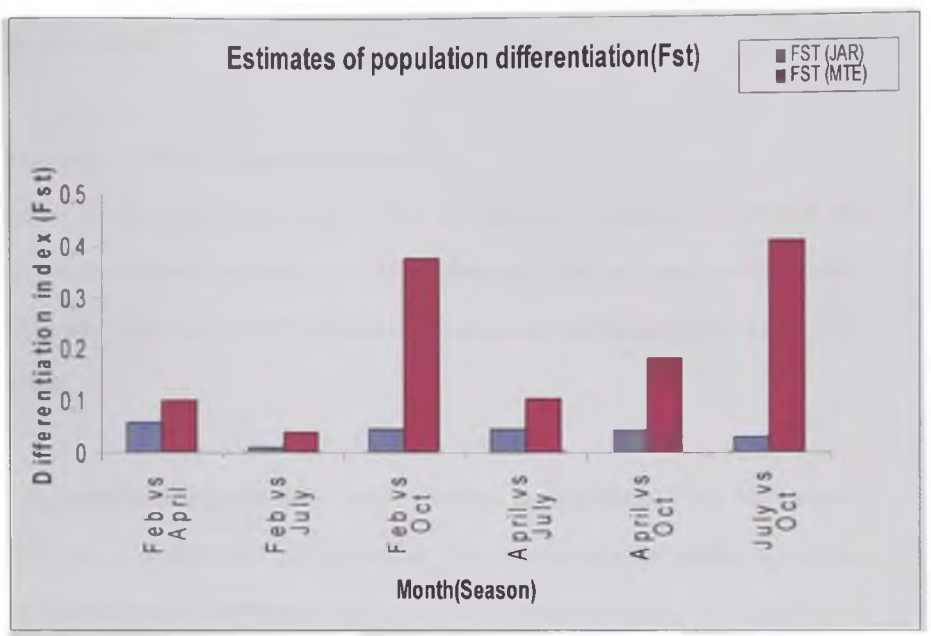

Figure 6.2: Estimates of population differentiation (Fst) between *An. gambiae s.s.* populations in Jaribuni and Mtepeni

6.3.5 Gene flow between *An. gambiae* populations in Jaribuni and Mtepeni

Estimates of gene flow (N*m*) between *An. gambiae* populations in Jaribuni and Mtepeni were derived from *Fst* using the formula *Fst*= (1+4Nm)-1 derived by Slatkin, (1995). N*m* between the Jaribuni and Mtepeni sites was 0.532 indicating a limited interchange of genes between the two sites.

6.3.6 Estimates of Effective population sizes

The current effective population sizes of the *An. gambiae* populations in Jaribuni and Mtepeni and the effective population size of *An. funestus* in Jaribuni were estimated based on the temporal variation in allele frequencies following the stepwise mutation model using the formula:

$$Ne = (1/ (1-He)^{2}-1)/8m$$

Where *He* is the heterozygosity at the locus (estimated from genepop), m is the mutation rate and is set at m=0.000205 per generation. The *An. gambiae* population in Jaribuni recorded higher effective population sizes during the wet seasons (April, *Ne* = 33,067) and October (*Ne* = 19,207.3). *Ne* during the dry season was lower (February *Ne* =11,427.43, July *Ne* =15,563.2) [Table 6.8]. In the *An. gambiae* population in Mtepeni, the *Ne* was higher during the long rainy season as expected (April *Ne* =67,515.2), and was persistent through the following dry season (July) with a reduction in October. Effective population sizes in the *An. funestus* population from Jaribuni in February were higher than the *Ne* recorded for *An. gambiae* in Jaribuni and Mtepeni.

Table 6.8 Effective population sizes of *An. gambiae* mosquitoes from Jaribuni and Mtepeni, and *Anopheles funestus* from Jaribuni, Kilifi.

		February (dry)	April (wet)	July (Dry)	October (wet)	Mean *Ne*
An. gambiae	**Jaribuni**	11,427.43	33,067.44	15,563.2	19,207.3	19,816.33
	Mtepeni	4,595.28	5,979.13	17,968.8	5,163.8	8,426.75
An. funestus	**Jaribuni**	4484.1	1,859.3	1,956.9	2,993.03	2,823.3

6.4 Discussion

In natural environments, *Anopheles* mosquito populations are subject to factors, which influence their population dynamics, population genetic structure and their efficiency as vectors. Along the Kenyan coast, *Anopheles* populations experience fluctuations in population size following variations in rainfall and temperature factors and these changes are likely to influence the population dynamics and population genetics of the vectors.

This study examined the seasonal variation in the population genetics of *Anopheles gambiae* and *An. funestus* at two sites, along the Kenyan coast, namely Jaribuni and Mtepeni. The population genetics software GENEPOP was used to determine the population genetic diversity, population differentiation, estimates of gene flow between the *An gambiae* and *An funestus* populations and the effective population size estimates of *An. gambiae s.s.* and *An funestus* during the wet and dry seasons.

Results on the genetic diversity of *An. gambiae* populations from both Jaribuni and Mtepeni indicated that the total number of alleles recorded in the wet season were higher compared to the dry season, suggesting that the observed increase in mosquito population sizes shortly after the commencement of the rainy season was accompanied with an introduction of new alleles, either due to immigration into these sites, or due to the loss or disappearance of some alleles during the dry season when natural conditions become unfavourable for vector breeding. This hypothesis is supported by studies by Lehmann *et al.* (2003) on *Anopheles* mosquitoes from various sites in Africa, which showed that the frequency of certain

inversion arrangements increased every dry season and decreased every wet season, demonstrating selective effects.

Temporal variation in environmental conditions such as temperature and rainfall distribution is likely to influence the structure of vector populations. Mosquito populations have been reported to undergo fluctuations in population size during seasonal dry spells experienced in tropical environments. These reductions in population size affect gene frequencies, which are reduced in small population sizes leading to a reduction in heterozygosity due to genetic drift. As population sizes decrease, gene frequencies fluctuate to an extent that particular genes become the only alleles consistent in the population (Wright, 1951).

In the *Anopheles gambiae s..s* populations in Jaribuni and Mtepeni, the observed increase in diversity during favourable breeding conditions might be due to increased population sizes, able to maintain greater genetic diversity. The lack of significant differences in the variation of number of alleles per season suggests that even with the temporal dynamics of population sizes and environmental factors, the populations are genetically stable over time, and temporal variations observed might be due to variations in population size, or immigration of genetically distinct individuals. Immigration between these populations was investigated directly by mark release recapture experiments described in Chapter 4, and results indicate a maximum dispersal distance of 661 metres from the larval habitats. This is comparable with the levels of gene flow estimated using the indirect approach by analyzing microsatellite DNA which in this study, indicated high levels of gene flow between seasons for both *An. gambiae* and *An. funestus*, and moderate levels of gene flow between *An. gambiae* populations from Jaribuni and Mtepeni

Departure from Hardy Weinberg expectations within populations was accompanied with heterozygote deficiency in both the Jaribuni and Mtepeni Populations. This also suggests an excess of homozygotes occurring as a result of increased inbreeding within populations, a phenomenon which is common in small populations. As in previous microsatellite studies, (Kamau *et al.,* 1998; Lehmann *et al*., 1999; Simard *et al.,* 2000), most deviations from HWE in this study were associated with positive coefficients of inbreeding (Fis). An analysis of Fis per population sampled during the wet and dry seasons revealed higher Fis values during the dry seasons (February and July), when reductions in population size are observed. During this period, inbreeding is enhanced by mating between a small number of individuals.

Site by site comparison indicates higher inbreeding at the Mtepeni site compared to the Jaribuni site, and this is explained partially by the observed population sizes, which are lower in Mtepeni, and the ecological differences that exist at the two sites. At the Jaribuni site, the river flows all year round, with variation in water levels depending on rainfall. Larval habitat availability at this site depends on river water levels, which were higher during the rainy season. The adult mosquito population size in Jaribuni does not undergo extremely drastic reductions as observed in the Mtepeni population where no larval habitats exist during the dry season encouraging inbreeding between the few existing individuals, and leading to the high Fis values. This comparison illustrates the effect of ecological variations on the population genetic structure of vector populations, and how this is likely to influence vector-borne disease transmission.

Deviation from HWE is a common finding in studies that utilize microsatellite loci. This deviation is usually attributed to null alleles (Lehmann *et al.*, 1996; Garcia de Leon *et al.*, 1997), selection (Garcia de Leon *et al.*, 1997), the pooling together of several sub populations (Wahlund effect), or the comparison of sympatric populations with very limited interchange of individuals. Linkage disequilibrium tests were conducted to evaluate the cause of heterozygote deficits. If the heterozygote deficits observed were due to inbreeding or the Wahlund effect then it should cause linkage disequilibrium because members of different sub-populations have different probability to carry certain combination of alleles (de la Chapelle & Wright, 1998). Significant linkage disequilibrium was observed between pairs of loci that also showed heterozygote deficiency. This suggests either departure from random mating, hence inbreeding and / or selection for certain genotypes following the prevailing environmental and ecological conditions. The alleles in disequilibrium occurred together in individuals with a probability higher than expected by chance alone, and this also indicated departure from HWE or selection.

Comparison of genetic differentiation between seasons was conducted using *Fst*, an index which measures the variance among allele frequencies standardized by the mean allele frequency among populations (Weir and Cockerham, 1984). Inter season comparison by *Fst* revealed lower *Fst* values, indicating low levels of population differentiation in Jaribuni probably due to the local ecological effect, given the constant availability of larval habitats at the site. This may contribute to low levels of among season population differentiation in Jaribuni since the populations do not reduce to levels similar to that observed in Mtepeni where a complete absence of larval habitats was observed during the dry season, with higher

population differentiation. Unlike Jaribuni, at the Mtepeni site there is no river or any other form of water source that ensures the continuous availability of larval habitats. Instead, the habitats are available only during the rainy seasons, and when the dry season commences, habitats are unavailable leading to a drastic reduction in *Anopheles* population, and hence a reduction in the number of alleles and genotypes in circulation

From these observations, the populations of *Anopheles gambiae* are genetically variable over seasons. Each season contains a small locally inbred population with a few similarities spread across seasons, either due to a few remnants from the previous population and migrants into the resident population when breeding conditions become favourable. Field studies by Minakawa *et al.* (2001) in Western Kenya, demonstrated two survival strategies of *An. gambiae* during the dry season, mainly continuous reproduction throughout the year and embryo dormancy in moist soil for at least several days. This suggests that anopheline mosquitoes do not necessarily suffer a severe population bottleneck during the dry season and thus maintain a fairly large effective population size (Minakawa *et al.*, 2001).

Population size is an important factor in the genetic structure of populations. In Chapter 3, the direct estimates of population size, from Mark release recapture experiments are reported. This is compared with indirect estimates of effective population size (Ne) derived from microsatellite data by adopting the methods of Waples (1989) and Pollak (1983). Under ideal natural conditions, the effective population size (Ne) is expected to be lower than the actual (direct) population size. In the current study, this was only observed for the *An. gambiae* population in Jaribuni. Estimates of current Ne were higher than direct

population size for both *An. gambiae* in Mtepeni, and *An. funestus* populations in Jaribuni. This finding suggests that a proportion of the population of these two species is either hidden (in vegetation or burrows) with respect to sampling, or the species may be maintaining large populations of adults by extensive mobility, resulting in a low density deme spread over a large geographical area. The area covered by this deme might be much larger than the area covered during the MRR experiments; hence extensive ecological studies are required to verify these observations.

CHAPTER SEVEN

GENERAL DISCUSSION AND CONCLUSION

The aim of this study was to determine the population distribution patterns, abundance, dispersal and the population genetics of *Anopheles gambiae s.s.* and *Anopheles funestus s.s* at two sites along the Kenyan coast. *Anopheles funestus s.s.* was the most abundant vector at Jaribuni, while *An. gambiae s.s.* was dominant at Mtepeni site. The observed increase in *An. gambiae s.s.* populations immediately after the rainy season, followed by a quick decline and a corresponding increase in the *An. funestus s.s.* population is an important factor in the dynamics of malaria transmission at the study area. Blood feeding preferences of both *An. gambiae s.s.* and *Anopheles funestus s.s.* were found in this study to be very high for humans thus indicating that these vectors were highly anthropophilic. The aggregated sporozoite rates for both *An. gambiae s.s.* and *Anopheles funestus s.s.* were not significantly different between the two sites, but were higher for *An. gambiae s.s.* at both sites, suggesting the importance of *An. gambiae s.s.* as a vector of malaria in the study area.

Both *An. gambiae s.s.* and *Anopheles funestus s.s.* exhibited spatial and temporal distribution patterns, with species distribution and abundance being variable and dependent on the site, and the time of the year (season). Even though analysis did not reveal any strongly significant association between species distribution or abundance and climatic variables, it was evident that peaks in population abundance were very much related to seasonal rainfall patterns at the study sites. The results obtained also indicate the importance of local ecological characteristics on the availability, suitability and productivity of larval habitats.

At Jaribuni, *Anopheles funestus s.s.* was found to be the important species during the dry season when *An. gambiae s.s.* populations were low, due to the unavailability of suitable habitats for its *An. gambiae s.s.* breeding.

This study on the population sizes, dispersal capabilities and daily survival probabilities of *An. gambiae s.s.* and *Anopheles funestus s.s.* is the first on the population characteristics of the two vectors *An. gambiae s.s.* and *Anopheles funestus s.s.* along the Kenyan coast. Findings from the current study indicate moderate vector dispersal, compared to results from studies conducted elsewhere. In Malaysia, Chiang *et al.,* (1991) reported a flight range of 0.5km for the malaria vector *Anopheles maculatus* and the longest flight was 1.6km, mean recapture rate was 11.5% and survival rates were in the range of 0.669 – 0.705%. In the Gambia, Thomson *et al.,* (1995) recorded a much lower recapture rate of 1.3%, and a dispersal range between 1 – 1.5km for *An. gambiae s.s,* whereas a study in Mali by Costantini *et al.* (1996) reported a recapture rate of 0.9%, mean dispersal distance of 350 – 650 metres and a survival estimate of 80-88%.

The dispersal of *Anopheles* vectors is confounded by several factors such as elevation, wind speed and vegetation. In this study, the effects of these factors were not considered even though the variability in the results obtained suggest the possibility of the differential effects of these factors on vector dispersal at the two sites.

Vector population size was high and exhibited a high probability of survival (Range 0.83–0.95%). Similar estimates of *Anopheles* survival have been reported elsewhere. Charlwood *et al.* (1997); Costantini *et al.* (1996) and Takken *et al.* (1998) reported daily survival probabilities of between 0.813 – 0.839%, 0.67-0.82% and 0.63 – 0.78% respectively for *An.*

gambiae s.l. in Tanzania (Ifakara); Burkina Faso and Tanzania (Namawala). No significant differences between the survival probabilities of *An. gambiae* and *An. funestus* were found. These results seem consistent within the range of 0.63 – 0.83% and this is partly explained by the almost uniform environmental conditions in the study areas. However there is little information on how these generalize to other locations, given the limited number of such studies on anopheline mosquitoes in Africa. Mosquito longevity is important, as it determines the possibility of pathogen transmission, and the longer a vector survives, the better are the pathogens chances to enter new hosts and multiply.

Variation in the rates of recapture (r) and mean population sizes (p) between Jaribuni (r =24.6%; p= 16,781) and Mtepeni (r = 4.33%; P=854) elaborate on the effect of ecological differences on species occurrence and behaviour which might have an effect on their role in malaria transmission. However, from this study, we observe no significant differences in the sporozoite rates between *An. gambiae* and *An. funestus* in Jaribuni, whereas in Mtepeni, the two species show a significant difference in sporozoite rates, with *An. gambiae* showing higher sporozoite rates.

No significant differences were observed in the dispersal capability of *An. gambiae* and *An. funestus*. The dispersal of *Anopheles* vector populations is an important determinant for both malaria transmission and control, and mosquito dispersal can promote panmixia of *P. falciparum*, even without human movement. Although the maximum distance that a mosquito flies is important in understanding population genetics, species distribution and the spread of pathogens to new geographic areas; understanding the patterns and dynamics of

vector-borne disease transmission within a biotope requires the determination of the effective flight distance for the mosquito population. Few studies have been concerned with patterns of vector dispersal in relation to the epidemiology of malaria in sub-Saharan Africa (Costantini *et al.,* 1996; Thomson *et al.,* 1995; Trpis and Hausermann, 1986). Killeen *et al.* (2003) emphasize the importance of *Anopheles* dispersal to malaria control programmes, especially those aimed at reducing human vector contact, such as the use of insecticide treated bednets, whereby the emigration of mosquitoes by dispersal from intervention areas and their replacement by immigrants from nearby uncontrolled areas can result in substantial attenuation of local impact in the control site. This might also lead to an underestimation of the measured efficacy of the intervention, whose effects are shared between villages due to exchange between mosquito subpopulations (Hii *et al.,* 2001; Takken, 2002).

Genetic exchange between vector populations is highly dependent on vector dispersal. A high rate of dispersal between different populations of the same species, leads to increased levels of gene flow between populations. Studies on gene flow between *Anopheles gambiae* populations elsewhere in Africa have reported genetic exchange between *Anopheles* populations located as far as 7000 km apart (Lehmann *et al.,* 1996). In this study, moderate levels of gene flow were recorded between anopheline populations located less than 50km apart; and with a direct dispersal estimate of 661 metres. The variability in these results suggest that a high proportion of dominant markers together with a high frequency of codominant markers with null alleles may represent two important limitations for the use of microsatellites in different studies.

This work provides basic information on the *Anopheles gambiae* and *Anopheles funestus* populations in Kilifi, along the Kenyan coast. During the planning of vector control activities such as larviciding, use of ITNs and Indoor residual spraying; these findings on the spatial variability of species composition, vector abundance, dispersal, survival and population genetic structure will provide important baseline information for guiding the implementation of these vector control activities in an integrated vector management system.

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APPENDICES

APPENDIX 1

Informed Consent forms

Information and consent form for voluntary participation in human landing catches for mosquito collection

The work of KEMRI, Kilifi

We are from the Kenya Medical research institute (KEMRI), Kilifi. KEMRI assists the MOH with the normal hospital work. The special work of KEMRI-Kilifi is to learn more about illnesses that affect children in Kenya. Investigations involving various diseases that affect children are carried out among communities and in the hospital; with learning about illnesses sometimes requiring visits to the households.

The purpose of this study

One of our current interests is to learn more about the links between where adult mosquitoes live and how much malaria there is (malaria transmission intensity). We can learn this by carrying out household studies. We are therefore conducting studies on adult mosquito distribution and the intensity of malaria transmission in certain parts of Kilifi District, namely Jaribuni and Mtepeni.

Procedures of the study

In order to determine the seasonal changes in mosquito distribution patterns and malaria transmission intensity, we need to know the number of mosquito bites that any one person is likely to get from infected mosquitoes per night. The standard method for obtaining this information is by the human landing catches method. In using this method, two individuals are required. Mosquito collection using this method will be done each night for 6 nights in a week for three months. One person will sit inside the house and the other one will sit outside at night from 7.00pm to 12.00 midnight. Using an aspirator (an instrument like this) you will collect any mosquitoes that land on your feet before they bite. We will also provide you with a paper cup into which you will transfer all the mosquitoes collected. After every hour the person outside the house will interchange with the one inside. During every night's collection, a supervisor will visit your collection point to check if you are experiencing any problems. At the end of the night's collection, you will hand over all the paper cups to the supervisor who will take the mosquitoes for processing.

Only men above 18 years of age can volunteer to do this work in Kilifi.

We are asking you if you are willing to be a volunteer in this activity. If you agree, you will collect mosquitoes via the method described above for a total of 6 nights in a week for three months with one night off per week. Before you begin, you will be given one week training on how to collect and handle mosquitoes.

Benefits associated with taking part in the study

The study will not benefit you directly but will provide information in understanding the distribution of mosquitoes, which transmit malaria in this study area, and the intensity of malaria transmission. This will help in understanding the extent to which residents in this area are at risk of getting infected with malaria. This information is important for on going national and district level malaria control activities.

Foreseeable Risks associated with the study

If you take part in this study, you stand a risk of getting infected with malaria ONLY if you let the mosquitoes bite you before you collect them. You will not get infected if you avoid mosquito bites. But in the event that you get malaria infection within the study period of three months we will provide you with appropriate malaria treatment after confirmation of the infection. This however will involve only the study participants and the not any other dependant.

Participation is voluntary

Your participation will be voluntary and if you agree to participate in the study then you will sign below showing that you have understood. If there is any part of this form, which you do not understand, be sure to ask questions about it. And if you agree to participate in this study but then later on change your mind you can withdraw at any time without any problems. Because the work is very rigorous and time consuming, you will be entitled to a

monthly fee of 6, 000 Kenya Shillings during the months that you will participate in the study.

I have given you a copy of this consent form. When you sign below, it shows that you agree to join the study. Do not sign this form until you have full answers to all your questions. Do you have any questions about the study? If so you can ask them now. (Write all the concerns and comments in the text box below)

I understand the nature, duration and purpose of the study as has been explained to me. I have been given an opportunity to ask questions which have been answered to my satisfaction. I am aware that at any point I may withdraw from the study without any penalty or loss of benefits.

Name of participant:_____

Signature of participant:_____ Date:_____

In the presence of a witness:

Witness

I observed the process of consent. The prospective participant read/was explained to the contents of this form and was given the chance to ask questions. This was satisfactory to him and he accepted and signed to take part in the study.

Name of witness: _____

Signature _____ Date _____

The study co-ordinators are Janet Midega and Dr Charles Mbogo. If you have any further questions about the study you can ask them when they visit your area as they will be coming to the study areas very often, or you can contact them at KEMRI/Wellcome Trust, Kilifi District Hospital or telephone on 041-5-22063.

Information and consent form for household heads for the night collection of mosquitoes: Study of the dispersal patterns of malaria vectors in Kilifi, Kenya.

The work of KEMRI, kilifi

We are from the Kenya Medical research institute (KEMRI), Kilifi. KEMRI assists the MOH with the normal hospital work. The special work of KEMRI-Kilifi is to learn more about illnesses that affect children in Kenya. Investigations involving various diseases that affect children are carried out among communities and in the hospital; with learning about illnesses sometimes requiring visits to the households.

The purpose for this study

One of our current interests is to learn more about the links between where adult mosquitoes live and how much malaria there is (malaria transmission intensity). We can learn this by carrying out household studies. We are therefore conducting studies on adult mosquito distribution and the intensity of malaria transmission in certain parts of Kilifi District, namely Jaribuni and Mtepeni.

Procedures of the study

In order to determine the seasonal changes in mosquito distribution patterns and malaria transmission intensity, we need to know the number of mosquito bites that any one person is likely to get from infected mosquitoes per night. The standard method for obtaining this information is by the human landing catches method. In using this method, two individuals are required. Mosquito collection using this method will be done each night for 6 nights in a

week for three months. One person will sit inside your house and the other one will sit outside at night from 7.00pm to 12.00 midnight. After every hour the person outside the house will interchange with the one inside.

What we are requesting from you

We are asking you for permission to allow us to have the two individuals collect mosquitoes from your house for a total of 6 nights in a week for three months with one night off per week. If you agree, you will show us the position in you house where you will be comfortable if the person sits at night. The people who will be collecting mosquitoes from your house at night will be males who are residents of this area and so are already familiar with your household.

Benefits associated with taking part in the study

The study will not benefit you directly but will provide information in understanding the distribution of mosquitoes, which transmit malaria in this study area, and the intensity of malaria transmission. This will help in understanding the extent to which residents in this area are at risk of getting infected with malaria. This information is important for on going national and district level malaria control activities.

Foreseeable Risks associated with the study

Taking part in this study by allowing for mosquito collection in your house at night will not expose you to any risk at all.

Participation is voluntary

Your participation will be voluntary and if you agree to participate in the study then you will sign below showing that you have understood. If there is any part of this form, which you do not understand, be sure to ask questions about it. And if you agree to participate in this study but then later on change your mind you can withdraw at any time without any problems.

I have given you a copy of this consent form. When you sign below, it shows that you agree to allow us to have two people collect mosquitoes from your house at night, with one person sitting outside the house and another one sitting inside.

Do not sign this form until you have full answers to all your questions. Do you have any questions about the study? If so you can ask them now. (Write all the concerns and comments in the text box below)

I have been given an opportunity to ask questions which have been answered to my satisfaction. I am aware that at any point I may withdraw from the study without any penalty or loss of benefits.

Name of household head: _____

Signature: _____ Date: _____

In the presence of a witness:

Witness

I observed the process of consent. The household head read/was explained to the contents of this form and was given the chance to ask questions. This was satisfactory to him and he accepted and signed to take part in the study.

Name of witness: _____

Signature _____ Date _____

The study co-ordinators are Janet Midega and Dr. Charles Mbogo. If you have any further questions about the study you can ask them when they visit your area as they will be coming to the study areas very often, or you can contact them at KEMRI/Wellcome Trust, Kilifi District Hospital or telephone on 041-5-22063.

APPENDIX II

ELISA procedures and solutions

A: Blood Meal ELISA Procedure

Bloodmeals in the mosquitoes are identified by direct enzyme-linked immunosorbent assay (ELISA) using anti-host (IgG) conjugates against human and cow, non-reacting samples are tested for goat (Beir *et al.*, 1988). Mosquitoes triturates (50 µl) are added to wells of polyvinyl-chloride, U- shaped, 96-well micro-titre plates, which are covered and incubated overnight at room temperature. Each well is then washed twice with PBS containing 0.5% Tween 20 (PBS-TW 20). This is followed by the addition of 50 µl host specific conjugate (antihuman IgG, H&L) diluted 1:2,000 (or 1:2,000 (or 1:250 for bovine) in 0.5% boiled casein containing 0.025% Tween 20. The boiled casein is prepared by dissolving 5 g casein in 100 ml 0.1 N sodium hydroxide by boiling, adding 900 ml PBS, adjusting pH to 7.4, adding 0.1g Thimerosal (sodium ethylmercurithiosalicylate) and 0.02 g phenol red, and storing at 4^0C. After 1 hour, wells are washed three times with PBS-Tween 20, and 100 µl of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) peroxidase substrate is added to each well. The dark green positive visually after 30 minutes. A second host source is determined in the same microtitre plate where mosquitoes are screened for human blood. The second conjugate, phosphatase-labelled anti-bovine IgG(1:250 dilution of 0.5 mg/ml stock solution) is added to the peroxidase-labelled antihuman IgG solution. Bloodmeals are screened first for human IgG by the addition of peroxidase substrate according to the peroxidase-labelled antihuman IgG solution conjugate, phosphatase-labelled anti-bovine IgG (1:250 dilution of 0.5mg/ml stock solution) is added to the peroxidase-labelled

antihuman IgG solution. Bloodmeals are screened first for human IgG by the addition of peroxidase substrate according to the methods described above. After reading the pladtes at 30 minutes, the wells are ished 3 times with PBS-Tween 20, and 100 µl phosphatase substrate is added to each well. Plates are read after 1 hour to determine positive cow reactions.

Non-reacting samples are then tested for goat. For each test, 1:500 dilutions of human, cow, goat, dog, cat and chicken serum are added to the conjugate solution to reduce background absorbance. Each plate contained control serum samples (1:500 dilution in PBS of human, cow, goat, dog, cat and chicken and four field-collected male Anopheles ground in PBS at the same dilution as test samples.

Bloodmeal ELISA solutions

1. Phosphate buffered saline (PBS), pH 7.4

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

2. Boiled Casein, 0.5% (BC)

	500ml	**1 litre**
Casein	2.50 g	5.00 g
0.1 N NaOH	50.0ml	100 ml
PBS, Ph 7.4	450 ml	900ml
Thimerosal	0.05 g	0.10 g
Phenol red	0.01 g	0.02 g

a) Suspend casein in 0.1 N NaOH and bring boil.

b) After casein is dissolved, slowly add PBS; allow cooling and adjusting the pH to 7.4 with HCL.

c) Add the thimerosal and phenol red.

d) Shelf life – one week.

3. Wash solution (PBS – Tween 20)

PBS plus 0.05% Tween 20. Add 0.5 ml of Tween 20 to 1 L of PBS. MIX WELL. Do not store. Make each day.

4. Enzyme Diluent (BC-Tw):

100ml + 25µl Tween 20. Do not store. Make each day.

HUMAN AND BOVINE HOST BLOODMEAL PROTOCOL FOR ELISA

A. Sample Preparation:

1. Negative controls – Grind male mosquitoes in 500 µl of PBS per mosquito.
2. Positive controls – For each host serum: To 500 µl PBS add 5 µl of host serum control.
3. Blood-meal Samples: - Dilute each mosquito abdomen sample in 1000 µl of PBS.

To ensure proper grinding, first put 100 µl PBS and grind with a grinder, then add 900 µl PBS to raise to the required volume.

B. Technique:

1. To a PVC flex plate add:

   column 1 add 50 µl/well of eight negative controls

   column 2 add 50 µl/well of eight positive controls (chicken huma, pig, cat, horse, cow, goat & dog).

   One well should be designated as a blank control and receive 50 µl of PBS alone.

   The remaining wells should receive 50 µl/well of mosquito blood meal sample.

2. Incubate overnight at room temperature.
3. Wash plate with PBS-Tween 2 times.

Enzyme – Conjugate preparation:

To 5 ml of BC-Tw (Enzyme diluent) add:

HRP phosphate anti-human ---------- 2.5 µl (1:2000 dilution)

Bovine phosphatase Conjugate ---------- 20.0 µl (1:250 dilution), and Sera of each host except the one being tested for ------ 10.0 µl (1: 500 dilution).

NOTE: Add serum from all hosts except those for which enzyme –conjugate was added. All HRP conjugates should be diluted 1: 2000. Cow which is a phosphatase conjugate should be diluted 1:250.

4. Add 50 µl/well of the prepared enzyme conjugate solution.
5. Incubate for 1 h at room temperature.
6. Wash plate with PBS-TW 3 times

Peroxidase substrate preparation:

Mix solutions A and B together, 1:1 i.e 5 ml + 5 ml per plate.

7. Add 100 5 µl of substrate to each well
8. Incubate for 30 minutes
9. Read absorbance visually or at 414 nm with ELISA reader.
10. Wash plate with PBS-TW 3 times.

Phosphatase substrate preparation:

Add 2 tablets to 8 ml of distilled water and 2 ml of diethanolamine buffer.

11. Add 100 µl of the phosphatase substrate to each well.

12. Incubate for 5 hrs and read absorbance at 414nm (you can read from 2 hrs onwards).

NOTES:

Preparation for PBS (7.4), BC 0.5% and PBS-Tween are as for sporozoite ELISA.

For goat blood meal ELISA follow same directions except for use of conjugate use HRP goat dilution as in human i.e 1:2000 (2.5 µl).

B. SPOROZOITE ELISA SOLUTIONS

1. Phosphate Buffered Saline (PBS-plain):

This reagent is used for the dilution of capture monoclonal antibody (Mab) before the plates are coated and when preparing the blocking buffer and PBS-Tween.

(i) Rinse the preparatory plastic bottle with distilled water.

(ii) Add 1 litre of distilled water into the bottle.

(iii) Pour the whole content of 1 Dulbecco's PBS BOTTLE (9.7GM) INTO THE BOTTLE AND PLACE A MAGNETIC STIRRER. Leave for 10 minutes to dissolve.

(iv) The solution is now ready for use. Store in fridge when not in use.

2. PBS-Tween 20

This is a wash solution. It is used to wash plates.

i. Put 1 litre of PBS plain in the bottle,

ii. Add 500 µl of Tween-20,

iii. Mix well using magnetic stirrer.

iv. Keep in the fridge when not in use.

NOTE: Make each day do not store.

3. Blocking Buffer or Boiled casein

To 1 litre of PBS plain add.

i. 10G bovine Serum Albumin (BSA)

ii. 5g casein

iii. 0.1 g thimerosal

iv. 0.02 g phenol red.

Or

Suspend casein on 0.1 N Sodium hydroxide and bring to boil. After casein has dissolved, slowly add PBS; allow cooling and adjusting the pH to 7.4 with Hydrochloric acid (HCI). Add thimerosal and phenol red.

Casein (0.5%)	**2.5 g**	**5.0g**
0.1 N NaOH	50 ml	100 ml
PBS (pH 7.4)	450 ml	900 ml
Thimerosal	0.05 g	0.1 g
Phenol red	0.01 g	0.02 g

Mix well using a magnetic stirrer, leave it to mix for 2 hours or more. Keep in the fridge when not in use.

NOTE: This reagent is used to block the plates and prepare the Nonidet P-40.

Shelf life is 1 week at 4^0c.

4. Nonidet P-40(NP-40)

To 40 ml of Blocking Buffer add 200 µl or NP-40, mix well and store in fridge when not in use.

5. 2A10 Monoclonal antibody (capture Mab)

This is usually in clear white bottles.

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

ii. Add 20 µl of the capture Mab.

iii. Mix well and dispense 50 µl into well of the PVC plate. The amount is adjusted according to the number of plates.

6. Peroxidase Labelled Enzyme (2A10).

This is usually in dark brown bottles

i. Put 5ml blocking buffer into a clear tube

ii. Add 10 µl of the 2A10 Peroxidase labeled enzyme (conjugate) into the tube and mix.

This is enough for only one plate.

7. Peroxidase substrate solution.

Mix equal parts 1:1 of solution Aand solution B; i.e for 1 plate mix 5ml of solution A and 5ml solution B. It should be noted that this mixing is only required when using the 2 component substrate. In a one component substrate no mixing is required. Store in the fridge.

NOTE:

Solution A (ABTS) is 2.2' azino-di(30ethyl-benzethiazoline sulphonate.

Solution B is Hydrogen peroxide.

8. Enzyme check.

Put 100 µl of substrate into a vial and add 3 µl of fresh prepared enzyme. Mix and observe colour change for colourless to blue.

9. Positive control: R32tet$_{32}$ Reconstitution

Plasmodium falciparum

(i). Dissolve lyophilized : R32tet$_{32}$ (10 µg) with 1000 µl distilled water to yield 100ng/10µl. This is normally referred to as Vial I.

(ii) Vial II stock solution

(a) Put 1000 µl Blocking Buffer into a tube.

(b) Add 10 µl (100ng) of the R32tet$_{32}$ from vial I, and mix well. This gives 1000 pg/10 µl BB

Store in a freezer. It is also used to prepare the working control vial III when loading the triturates.

(iii)Vial III working solution.

(a) Put 1000 µl BB into a vial

(b) Add 20 20 µl 200 pg) R32tet$_{32}$ from vial II, and mix well. This yield 100 pg/50 µl BB. (control I).

(a). Put 500 µl BB into a vial

(b). Add to µl (1000 pg) R32tet$_{32}$ from vial II, and mix well. This gives 100 pg/50 µl) of BB (control I)

Make 1:10 dilution to get 10 pg/50 µl blocking buffer.

e.g. Put 450 µl BB into a tube and transfer 50 µl from vial III (100 pg/50 µl) and mix well. This is control II. Keep in fridge.

10. Negative controls:

The negative controls are prepared from the head/thorax of the wild males of the same species or from the laboratory reared females.

(i) Put 50 µl of NP-40 into a vial

(ii) Cut the head/thorax and put into the vial

(iii) Leave for sometimes to soften

(iv) Grind with a pestle and adjust the volume by adding 200 µl of blocking buffer.

(v) Keep in the freezer when not in use.

11. Capture Mab (lyophilized Mab) and Peroxidase conjugate Mab Reconstitution.

Dissolve the lyophilized Mab (0.1 mg/vial) and peroxidase conjugated Mab (0.1 mg) in 0.2ml (200 µl) of diluent (1:1 distilled water and glycerine). Store at 4^0c or -20^0c.

TECHNIQUE

1. Triture preparation

(a) Label the vial with corresponding numbers as marked in the ELISA working sheet.

(b) Add 50µl of BB-Np-40 into each vial

(c) Using a sharp clean surgical blade vut the mosquitoe between the trhorax and the abdomen.

(d) Pick the head thorax with forceps and dtrasfer to the sporoxoite marked vial and the abdomen to the corresponding vial marked blood meal if the mosquito is blood fed. If not blood fed or no blood meal analysis required, dicard.

(e) Leave the head/thorax to soak in the NP-40 for 20 minutes.

(f) Use a non absorbent glass or plastic rod (pestle) to grind the mosquito in the vial.

(g) Adjust the volume by adding 200 µl of the blocking buffer.

(h) Clean the pestle and wipe it dry with gauze before grinding the next sample to avoid contamination. This is repeated until all samples are prepared. Keep in the freezer until use.

II: Plate coating

(a) Label the PVC plate with appropriate number

(b) Into each well of clean PVC add 50 µl of the diluted capture Mab.

(c) Cover the plates and incubate for 30 min at room temperature in subdued light.

III: Blocking the plate

(a) Using an eight–channelled manifold to a vaccum pump, aspirate the capture Mab from the microtitre plate.

(b) Bang the plate hard on an absorbent tissue paper or gauze to ensure complete dryness of the plate.

(c) Fill each well with blocking buffer (pH 7.4) using a manifold attached toa 60 µl syringe. Incubate for 1 hour at room temperature in subdued light.

IV: Loading the plate with triturates

(a) Aspirate the blocking buffer from the wells using the manifold attached to a vacuum pump and bang to complete dryness.

(b) Using labeled ELISA processing sheets, as a guide, put 50 µl of 100, 50, 25, 12, 6, 3, 1.5, 0 pgs positive control in the first column wells. Into the next column wells add 50 µl of the negative controls.

(c) Load the first mosquito sample (50 µl triturate) into the third column well (A3) and continue in the horizontal order up to the last well in the plate.

(d) Cover the plate and incubate for 2 hours at room temperature in subdued light.

V. Addition of conjugate (Peroxidase enzyme)

(a) After 2 hrs aspirate the truturate form the wells

(b) Wash the plate 2 times with PBS-TWEEN 20

(c) Add 50 µl of the perxidase labeled enzyme and incubate for 1 h at room temperature

VI: Adding of substrate

(a) Aspirate the enzyme from the wells and wash 3 times with PBS-Tween 20 and banging it to dryness.

(b) Using an octapete multichannel pipette add 100 µl of the substrate mixture and incubate for 30 minutes. Results are read visually or at 414 nm using an ELISA plate reader.

APPENDIX III

STANDARD SOLUTIONS FOR PCR AND ELECTROPHORESIS

The following standard solutions were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lb/sq in. for 15 minutes in an Eyela Autoclave (Rikikakki Tokyo).

DNA Extraction

Bender buffer

0.1M NaCl, 0.2M sucrose. 0.1M Tris-HCl pH 7.5, 0.05M EDTA pH 9.1, 0.5% SDS. Stored at 4^0 C

0.5 M EDTA (pH8.0)

186.1 g/l in water, ph adjusted with NaOH pellets and stored at room temperature.

EtBr (10mg/ml)

1g of EtBr was completely dissolved in 100ml sddw and stored in the dark at room temperature.

KAc (5M K 8M Acetate)

60ml of 5 M KAc and 11.5 ml glacial acetic acid in 28.5 ml distilled water

TE (pH 8.0)

10Mm Tris-HCl (pH), 1mM EDTA (pH8.0). Stored at room

temperature.

Solutions for Electrophoresis

Agarose Gels

10X TAE buffer

242 g Tris base, 57.1 ml glacial acetic acid, 100ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000 ml with sddw.

0.5 M EDTA (pH 8.0)

186g of EDTA, dissolved in 800ml ddw, pH adjusted with NaOH pellets, the volume made to 1000 ml with sddw and stored at room temperature.

Li-Cor Ger Electrophoresis Protocol

1. 40% sequencing acrylamide stock was made as follows:

(Wear a mask and double gloves while preparing this solution):

Chemical	Dry weight	Multiply by	Dry weight
Acrylamide	38.0g	6	228g
Bis-acrylamide	2.0g	6	12g
ddH_2O	Up to 100	6	Up to 600ml

The solution is then mixed thoroughly, and filtered through Whatmann paper then stored wrapped in aluminium foil at 4^0C.

2. Making a solution of 5.5% acrylamide for the 25cm gel:

Chemical	Amount	Multiply by	Amount
Urea	12.6g	5	63g
40% home-made acrylamide	4.125ml	5	20.625ml
5X TBE	3.0ml	5	30ml
ddH_2O	Up to 30ml	5	Up to 150ml

The solution is then mixed thoroughly.

This solution was make in large quantities (approx. 150ml volume) and stored as stock at 4^0c in a sealed bottle.

3. Preparation of the buffer solution.

The buffer solution in the gel is a 5X TBE buffer. The running buffer, however, is a 1X TBE buffer.

The 5X TBE buffer was prepared by adding the following to a 2L beaker.

Chemical	Dry weight
Tris	108g
Boric Acid	55g
Na_2EDTA (with $2H_2O$)	9.3g

The solution was mixed by stirring until all salts have dissolved, hen brought to a final volume of 2L with ddH_2O and divide into 2 IL bottles.

4. Preparation of the Ammonium Persulfate Solution (APS):

A 10% solution was made by adding 0.1g of APS to 1ml ddH_2O.

5. The 25cm glass plates for the sequencer are then assembled and prepared. The inside of both glass plates were rinsed with 95% ethanol to clean. (The inside of a plate is determined by the beveled corners of the plate. The beveled corners help when prying the plates apart for cleaning; therefore they should go on the inside). The alcohol is allowed to dry before placing the 0.25mm spacers along the long edges of the rear plate.

The front plate plate was placed on top of the rear plate and the spacers aligned with the outside edges of the plates, making sure the plates are aligned evenly at the bottom. The left and right rail assemblies are placed over the long edges of the plates and the knobs tightened against the glass plate until finger tight.

6. The 5.5% acrylamide gel was prepareed and poured as follows:

Take 39ml of the 5.5% acrylamide gel.

Add 20ul of TEMED.

Add 20ul of the 10% APS solution.

The solution is swirled using a 60cc syringe with a 14-gauge needle. The glass plates are then angled slightly to deliver the acrylamide evenly across the top notch

of the front plate, and the gel is let to flow at a steady, even rate until it reaches the bottom of the glass plates. At this point, the glass plates are laid flat and checked for any air bubbles, which are removed with wires if present. Preparation of the gel is followed by inserting the appropriate combs or piece of equipment for forming the wells (the trough to form the base of the wells). Any empty spaces are filled with Acrylamide.

The casting plate is then in the grooved area in the rails (which will eventually be occupied by the upper buffer chamber) and tightened until finger tight. The gel is then allowed to polymerize for about 1 hour.

Gel Loading Buffers

6x Bromophenol blue

0.25% bromophenol blue was added to 40% sucrose in water and stored at 4^0 C.

Bromophenol blue xylene cyanol : 1 volume of bromophenol blue xylene cyanol and 4 volumes of cyanide

5X orange G

20%w/v Ficoll, 25Mm EDTA, 2.5mM EDTA. 2.5 % (w/v) orange G. Stored at room temperature.

DNA Molecular weight & size markers

The 100 bp DNA molecular weight size marker obtained from Sigma were diluted according to the manufacturers recommendations and used. For the 100 bp ladder, the first band size is 100 bp, the subsequent ones are 200, 300.....1000bp.

APPENDIX 1V

Example of a score sheet for population genetics data analysis using GENEPOP

Title: *Anopheles funestus* in Jaribuni Kilifi scored for 11 loci, 4 populations

FUNQ
ND12
FUNO
FUB11
FND23
FUB10
ND20
FUB3
UB12
FUB6
FND7
Pop
BS1, 000000 088088 000000 193193 188192 196196 241241 176186 165165 146146 213213
BS2, 000000 086088 119119 193193 000000 196202 241241 186186 163163 000000 213213
BS3, 000000 088088 115119 193193 000000 196196 241241 176176 163163 000000 213213
BS4, 000000 000000 119119 193193 000000 000000 241241 000000 000000 144144 213213
BS5, 035035 088088 000000 193193 000000 196196 241241 000000 000000 000000 213213
BS6, 000000 088088 121121 193193 000000 196196 241241 176186 165165 146146 213213
BS7, 035035 088088 115121 193193 000000 202202 241241 186186 165165 144144 203209
BS8, 000000 086088 119119 193193 000000 196196 241245 186191 165165 000000 213213
BS9, 035035 088088 115121 193193 000000 196206 241241 186186 165165 000000 213213
BS10, 031035 088088 000000 193193 000000 196196 241241 186186 165165 144146 209215
BS11, 000000 088088 000000 193193 000000 196196 241241 186186 165165 144144 213213
BS12, 031035 088088 111117 193193 188188 196196 241241 186186 165165 146146 213213
BS13, 035035 086088 000000 193193 000000 196196 241245 176186 165165 000000 213213