STATUS OF INSECTICIDE RESISTANCE, 2La CHROMOSOMAL INVERSION AND *PLASMODIUM FALCIPARUM* INFECTION RATE IN MALARIA VECTORS IN KWALE COUNTY, COASTAL KENYA

BY

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2016

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DEDICATION

I dedicate this thesis to my parents, James Edward Kiuru and Joyce Kiuru for their love, financial and moral support; and to my fiancé Michael Waweru for the moral support during the entire study period.

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ABSTRACT

There has been increased effort globally to reduce malaria related morbidity and mortality. World Health Organization (WHO) recommends the use of long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) for the control of malaria vectors. Despite both methods contributing greatly to the decline in malaria transmission they both rely on insecticides particularly pyrethroids. The success of malaria control is threatened by several challenges among them being insecticide resistance. This study sought to determine the level of pyrethroid resistance in Kwale County, Coastal Kenya and the effects of the resistance on resting behavior and sporozoite infection rates in malaria vectors. Samples collection was done from Kidomaya and Marigiza villages in Kwale County. Adult mosquitoes were collected from both indoors and outdoors using CDC light traps and prokopack aspirator. The mosquitoes were identified using morphological and molecular techniques. They were tested for the presence of *Plasmodium falciparum* sporozoites by ELISA and karyotyping of 2La chromosomal conformation was detected by PCR. Mosquito larvae were collected, raised to F1 and tested for phenotypic resistance, genotypic resistance and chromosomal inversion. A total of 1101 *Anopheles* mosquitoes were collected, 694 as adults and 407 as larvae. Of these, 64.40% belonged to the *Anopheles funestus* complex and 33.97% to *Anopheles gambiae* complex. For the *Anopheles funestus* complex *Anopheles funestus* s.s was the dominant sub-species while for the *Anopheles gambiae* complex, *Anopheles arabiensis* was dominant. *Anopheles arabiensis* showed resistance to deltamethrin (60.44%) and permethrin (70.42%) while *Anopheles gambiae* s.s showed resistance to deltamethrin (75%) and susceptibility to permethrin. *Anopheles funestus* s.l showed 100% susceptibility to both deltamethrin and permethrin. Of 659 mosquitoes tested, 30 anophelines (28 *Anopheles funestus* and 2 *Anopheles gambiae)*, tested positive for *Plasmodium*

falciparum antigen. Despite *Anopheles funestus* being 100% susceptible they showed the highest infection rate. This could be as a result of low sample size or as a result of minimized contact with insecticides due to early biting times. The allele frequency for the 2La inversion showed a deviation from the Hardy-Weinberg expectations indicative of non-random mating. The 2La inversion frequency was significantly higher in Kidomaya (72.22%) compared to Marigiza (5.56%). There was no association between phenotypic resistance and 2La inversion while it was impossible to test for association between phenotypic resistance and sporozoite infection due to the small sample size. The presence of phenotypic resistance to pyrethroids reported in this study poses a major challenge to malaria control. This highlights the need to intensify resistance management and the search for pyrethroids alternatives. There is need to consider new methods to control outdoor malaria transmission which is on the rise due to behavioral resistance. Although there was no association between insecticide resistance and 2La inversion, evidence of non-random mating in *Anopheles gambiae* suggests some form of selection which favor individuals with the inversion.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

CDC	Centre for disease control and prevention
CBRD	Centre for Biotechnology Research and Development
DNA	Deoxyribonucleic acid
DOMC	Division of Malaria Control
F1	First Filial
ELISA	Enzyme Linked Immunosorbent Assay
GABA	gamma amino-butyric acid
HWE	Hardy Weinberg Equilibrium
IRS	Indoor residual spraying
KEMRI	Kenya Medical Research Institute
LLINs	long-lasting insecticidal nets
NMCP	National Malaria Control Program
PCR	Polymerase Chain Reaction
STATA	Statistics and Data
SL	Sensu Lato
SS	Sensu Strictu

WHO	World Health Organization
2La/a	inversion arrangement on left arm of chromosome 2
2L+^{a}/+^{a}	Standard arrangement on left arm of chromosome 2
2La/+^{a}	Heterozygous arrangement on left arm of chromosome 2
X^2	chi square
F	Fixation index

CHAPTER ONE: INTRODUCTION

1.1 Introduction

Malaria is a disease of major public health concern in Africa. The presence of highly efficient vectors and virulent malaria parasite (*Plasmodium falciparum*) in the region contributes greatly to the disease burden. The principal malaria vectors along the Kenyan coast are *Anopheles gambiae* and *Anopheles funestus,* both of which are complex species (Mbogo *et al.*, 2003). Control of the disease takes an integrated approach combining control of the parasite in the human host with control of the vector. Parasite control entails prompt treatment upon diagnosis and prevention of new infections in populations that are regarded to be at risk. The main vector control strategies are long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) both of which are insecticide dependent (WHO, 2014). The long term effectiveness of both strategies may be compromised due to development of insecticide resistance.

The effect of insecticide resistance on malaria epidemiology has so far shown varied results with some studies showing a positive correlation between the spread of insecticide resistance and rebound of malaria (Trape *et al.*, 2011). However, a recent multi-country study in Kenya, Benin,

Cameroon and Sudan does not show a direct link between insecticide resistance and effectiveness of vector control interventions (Kleinschmidt *et al.*, 2015). Already rise in malaria prevalence has been reported in the Kenyan Coast even with increased coverage of LLINs (Snow *et al.*, 2015). There are efforts being made to understand the forces (insecticide resistance, drug resistance and climate change) driving the rebound of malaria.

Different forms of resistance in malaria vectors have been reported in malaria endemic areas in Kenya, both physiological and behavioral resistance (Mathias *et al.*, 2011). Physiological resistance is tolerance of toxic effects of the insecticide by altering biological function. Two mechanism are involved in physiological resistance 1) increased metabolic detoxification of the insecticide and 2) reduction in sensitivity of the target proteins due to mutation which causes knockdown resistance (kdr) (Hemingway *et al.*, 2004). Behavioral resistance is modification of behavior to minimize contact with insecticides. Unlike physiological resistance, behavioral resistance has to be monitored over a period of time to be able to detect changes in behavior. The reported modifications are shifts from endophilic to exophilic, endophagic to exophagic, anthropophagic to zoophagic and changes in feeding time (Mutuku *et al.*,

2011;Mwangangi *et al.*, 2013). The changes in behavior occur as a means of environmental adaptation due to insecticide pressure.

Chromosomal inversions have been associated with several biological factors affecting adaptability of *Anopheles gambiae* to changes in the environment (Ayala *et al.*, 2014). The inversions are precipitated in adverse microclimatic conditions and their effect is on intrinsic factors in the mosquitoes that enhance survival of the mosquito (Ayala *et al*., 2014). 2La chromosomal inversion is one of the chromosomal inversion reported to enhance adaptability of *Anopheles gambiae*, when present it confers desiccation resistance (Gray *et al.*, 2009). Desiccation resistance is the ability to tolerate extreme dry conditions by increasing total body water content or by reducing the rate of body water loss or by tolerating a large proportion of water loss. This desiccation resistance is achieved physiologically and by behavior adjustments such as changing the resting behavior. Insecticide pressure could act as a force fueling the inversion and further causing behavior changes. These changes in behavior could be an adaptation for the mosquito to survive through the insecticide pressure. This study sought to explore insecticide pressure as an extrinsic factor fueling chromosomal inversion and the effects of the inversion on two intrinsic factors 1) resting behavior and 2) sporozoite infection rates in malaria vectors in the Kenyan Coast. This work was part of a main project

KEMRI/SERU/CBRD/134/3085, with this part focusing on the Coastal region while others focused on other malaria endemic places in Kenya.

1.2 Problem statement and justification

The main vector control method LLINs, is implemented mainly inside the houses. Female mosquitoes mainly rest indoors especially after a blood meal. 2La chromosomal inversion is associated with indoor resting mosquitoes (Ayala *et al*., 2014). These mosquitoes are thus subjected to insecticide pressure from the vector control tools implemented indoor. The insecticides could affect the resting behavior of mosquitoes thus impact the 2La chromosomal conformation to either avoid the insecticide pressure or survive through it (Mutuku *et al*., 2011). Also, with development of insecticide resistance vector resting behavior and parasite infection rates could be affected. Therefore, understanding the relationship of insecticide resistance with resting behavior of *Anopheles* mosquitoes is important as this could provide information on whether 2La can be used as a marker of behavioral resistance. This study sought to determine mosquito resting behavior with the reported resistance by comparing outdoor and indoor proportions and the frequency of 2La inversion indoor and outdoor in a natural population of *Anopheles gambiae* in Kwale. The study further correlated resistance with sporozoite infection rates to determine the effect of resistance on malaria transmission. Since most mosquito control

measures at the Kenyan coast rely on the use of insecticides establishing the level of resistance documents the efficacy and efficiency of these measures in malaria control. The frequency of 2La chromosomal inversion in *Anopheles* is an indicator of the effect of the mosquito control measures on mosquito resting behavior as a form of adaptation to the insecticides pressure. Further, understanding how these factors (insecticide resistance and frequency of 2La chromosomal inversion) relate to sporozoite infection rates is an indicator of the effects of the current vector control measures on malaria transmission.

1.3 Main objective

To investigate the relationship between insecticide resistance, 2La chromosomal inversion and *Plasmodium* infection rates in malaria vectors in Kwale County, Coastal Kenya.

1.3.1 Specific objectives:

1. To assess the distribution, feeding and resting preferences of malaria vectors in Kwale County, in the Kenyan Coast
2. To determine susceptibility status of malaria vectors in Kwale County to deltamethrin and permethrin.
3. To determine the frequency of the 2La chromosomal inversion in indoor and outdoor collected *Anopheles gambiae*.

4. To determine the *Plasmodium falciparum* sporozoite infection rate in malaria vectors.
5. To determine the correlation between insecticide resistance, 2La chromosomal inversion and sporozoite infection rate in *Anopheles gambiae* in Kwale County.

1.4 Research questions

1. Which malaria vectors occur in Kwale County?
2. Where do the vectors feed and rest?
3. What is the level of phenotypic resistance to pyrethroids in malaria vectors in Kwale County?
4. What is the frequency of the 2La chromosomal inversion among indoor resting and outdoor resting *Anopheles gambiae*?
5. What is the *Plasmodium falciparum* infection prevalence in malaria vectors?
6. How does the presence of pyrethroid resistance among *Anopheles gambiae* and *Anopheles funestus* affect their *Plasmodium falciparum* infection prevalence?

7 Does 2La chromosomal inversion frequencies correlate with insecticide resistance and *Plasmodium falciparum* infection prevalence in *Anopheles gambiae*?

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria epidemiology

Malaria poses a serious public health challenge, 124–283 million cases and 584 000 deaths are reported annually (Murray *et al.*, 2012;WHO, 2014). Ninety percent of these deaths occur in Africa, 78% of them affecting children below the age of five years (WHO, 2014). Human malaria is driven by 5 different parasites belonging to the genus *Plasmodium; Plasmodium ovale, Plasmodium vivax, Plasmodium falciparum, Plasmodium malariae* and *Plasmodium knowlesi* with *Plasmodium falciparum* being the most prevalent in Kenya and Africa (WHO, 2014). Kenya has four malaria epidemiologic zones: 1) malaria endemic areas which include the Kenyan Coast and the lake region in Western Kenya, 2) area of seasonal malaria transmission which include Northern and South Eastern parts of Kenya 3) malaria epidemic zones which include the highlands of Western Kenya and 4) low risk malaria areas which include highlands of Central Kenya and Nairobi (DOMC, 2011). A shrink in malaria epedemic areas and an expansion of low transmission areas has been reported recently (DOMC, 2011). Despite other areas in the Kenyan coast experiencing a reduction in malaria prevalence, malaria prevalence remains high in Kwale County (Figure 1).

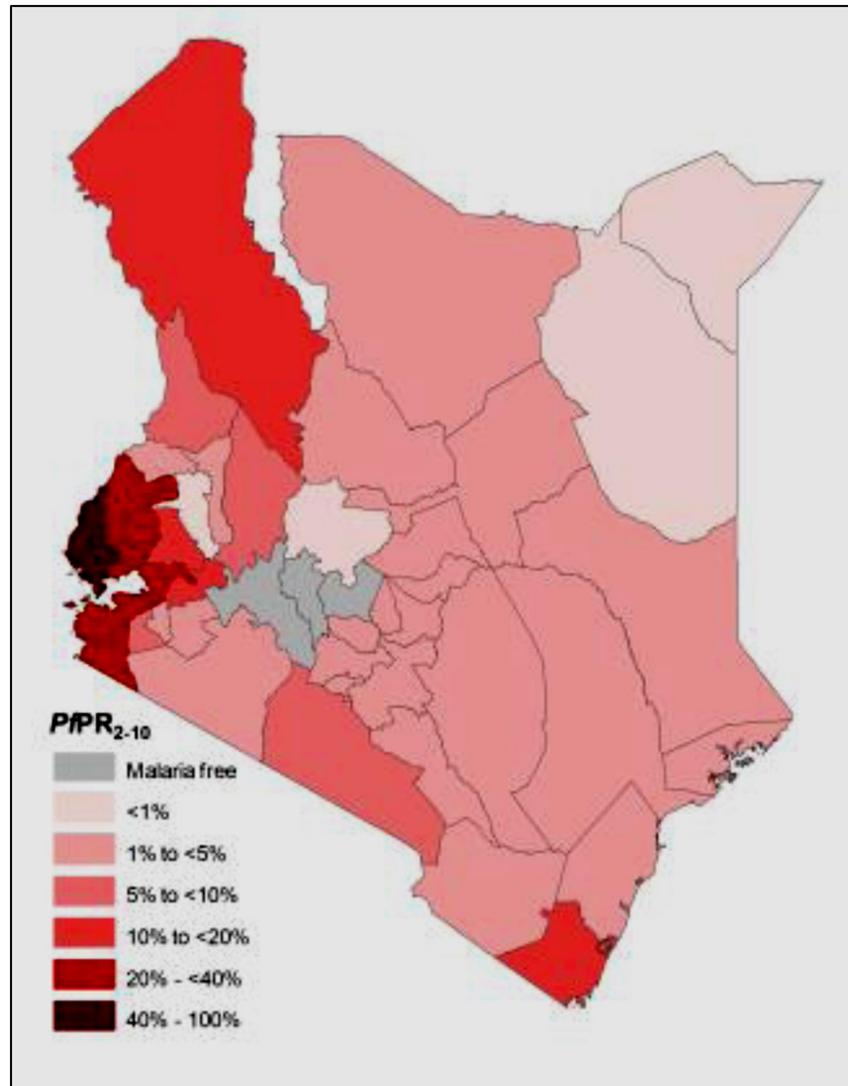

Figure 1: Map of Kenya showing *Plasmodium falciparum* prevalence by counties for the year 2010. (Map adapted from Presidents Malaria Initiative 2016 Report).

2.2 Malaria vectors

Malaria transmission is through a bite of an infected female *Anopheles* mosquito. About 400 species of *Anopheles* have been identified, of these 30 are of major importance as malaria vectors (WHO, 2014). Among these, members of *Anopheles gambiae* complex and *Anopheles funestus* complex are the main vectors in sub- Saharan Africa more so Kenya (Sinka *et al.*, 2010). The *Anopheles gambiae* complex is made up of 7 sibling species, 5 of which are vectors (*Anopheles gambiae* sensu strictu Gilles, *Anopheles arabiensis* Patton, *Anopheles merus* Donitz, *Anopheles melas* Theobald and *Anopheles bwambae* White) and 2 non-vectors (*Anopheles quadriannulatus* Theobald and *Anopheles quadriannulatus* species B). Recently two new members sibling species, *Anopheles coluzzii* and *Anopheles amharicus* have been added (Coetzee *et al.*, 2013). Among these, *Anopheles gambiae* sensu strictu, *Anopheles arabiensis* and *Anopheles merus* are found along the Kenyan Coast (Figure 2). The *Anopheles funestus* complex is made of nine sibling species: *Anopheles funestus*, *Anopheles vaneedeni* Gillies and Coetzee, *Anopheles leesoni* Evans, *Anopheles rivulorum* Leeson, *Anopheles parensis* Gillies, *Anopheles fuscivenosus* Leeson, *Anopheles aruni* Sobti, *Anopheles brucei* Service, and *Anopheles confuses*. Of these, *Anopheles funestus* plays a major role in malaria transmission by virtue of being highly

anthropophagic and endophagic (Sinka *et al*., 2010). Several factors affect the importance of the vectors in malaria transmission; time of biting, host preference, adult behavior particularly feeding and resting habits and susceptibility or resistance to insecticides.

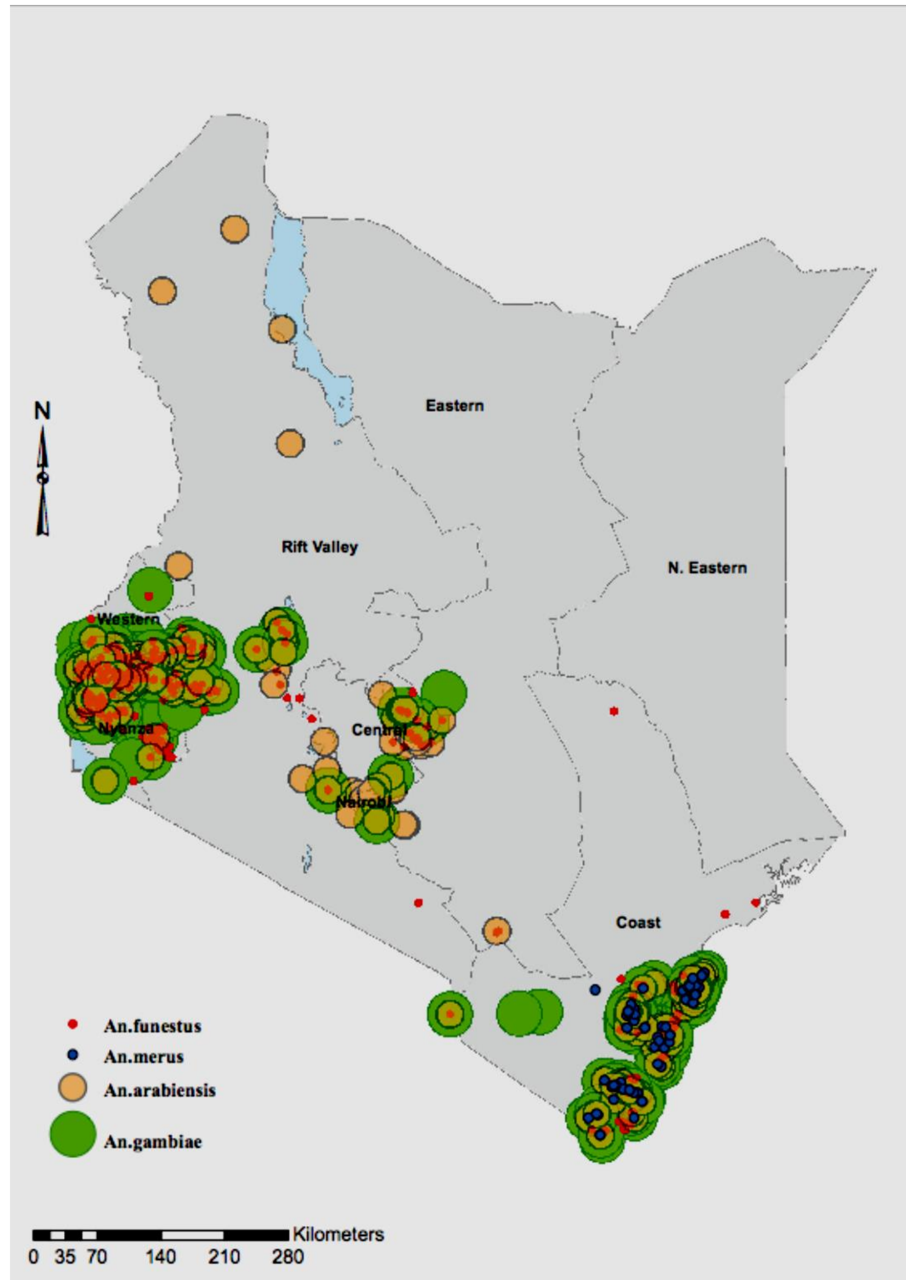

Figure 2: Map showing distribution and relative abundance of major malaria vectors in Kenya in the year 2010. (Map adapted from NMCP, 2014).

2.3 Malaria transmission dynamics

The malaria parasite is transmitted horizontally from one vector to another through host sharing. This parasite has its life cycle in both the vector and the human host thus transmission is determined by vector factors and human factors. Vector factors entail vectorial capacity, which is the ability of the mosquito to serve as a disease vector. Vectorial capacity is influenced by vector density, vector feeding behavior, vector longevity and vector competence which is the ability of the vector to transmit the pathogen (Beerntsen *et al.*, 2000). Vector feeding behavior is important as it determines the vector-human contact and could enhance or block transmission. Anthropophagy and anthropophily behavior enhance vector-human contact favoring malaria transmission (Cohuet *et al.*, 2010). Vector longevity is significant for the completion of the parasite's intrinsic incubation period which is the time required for infective malaria stages to be generated in the mosquito (Cohuet *et al*., 2010). Vector competence is dependent on susceptibility of the vector to *Plasmodium* parasite. This is influenced by intrinsic factors such as the mosquito immune system (Beerntsen *et al*., 2000). The objective of malaria control strategies is to offset the vectorial capacity to reduce malaria transmission.

2.4 Malaria control

Malaria control takes an integrated approach combining timely and effective diagnosis, and treatment with preventive measures like vector control and chemoprophylaxis. LLINs, IRS and larval source management are the recommended methods for vector control (RollBackMalaria, 2005;WHO, 2014). In Kenya, multiple strategies under the National Malaria Strategy are in place with the aim of maintaining the reduced rate of malaria related morbidity and mortality. The objectives of the National Malaria Strategy are: 1) to have 80% of people in malaria risk areas using appropriate prevention by 2018, 2) to have all clinical malaria cases managed as per the National Malaria Treatment Guidelines, 3) to have all areas experiencing malaria epidemics or under seasonal malaria transmission ready and capable of responding promptly, 4) to ensure all malaria indicators are routinely monitored in all counties, 5) to increase utilization of all malaria control interventions and 6) to improve coordination, leadership, governance and resource mobilization in malaria programs (NMCP, 2014). Vector control is paramount of the protective strategies being implemented (NMCP, 2014). Globally mosquito control relies heavily on insecticides particularly pyrethroids, which are recommended for impregnating bed nets. Since the scale up of malaria control and prevention through the roll back malaria programme and the

National Malaria Strategies a decline in malaria related mortality and morbidity has been reported (Enayati and Hemingway, 2010;WHO, 2014). In the Kenyan Coast a decline in paediatric hospital admissions due to malaria has also been reported (Okiro *et al.*, 2007). However, the gains could be jeopardized by development of drug and insecticide resistance among other challenges. Already, a rise in malaria prevalence has been reported in the Kenyan Coast even with the current malaria control strategies in place (Snow *et al*., 2015). One of the forces driving the rise of malaria in areas where malaria prevalence had declined could be insecticide resistance (Trape *et al*., 2011). Insecticide resistance is a form of adaptation due to insecticide pressure and has been associated with polymorphic chromosomal inversions (Brooke *et al.*, 2002).

2.5 Chromosomal karyotype

Chromosomal inversion is the re-arrangement of a block of genes in a chromosome. It occurs as a result of breaking of the chromosome and the re-insertion of the chromosome fragment in a reverse order during repair (Figure 3). In a population, three different karyotypes are formed as a result of the inversion; 1) standard karyotype where the inversion is absent, 2) Inverted karyotype where an inversion is present and 3) heterozygous karyotype which is a hybrid of standard and inverted karyotypes (White *et al.*, 2007a). Inversions are maintained by selection through a mechanism

that reduces recombination in the heterozygotes (White *et al*., 2007a). Studying inversion in *Anopheles* is of great interest in understanding the epidemiology and control of malaria as it affects the vectorial capacity of the vector and vector ecological habitation. In addition, insertions have been used to study different aspects of the vector for example; identification of the sibling species in complex species since the inversions are fixed among species but polymorphic between species (Coluzzi *et al.*, 2002) and phylogenetic analysis in the study of vector evolution (Xia *et al.*, 2008). By increasing adaptability of the vector, chromosomal inversions enhance survival of the vector, longevity and capability of the vector to explore new habitats and as a consequence increase the vectorial capacity (Coluzzi *et al*., 2002).

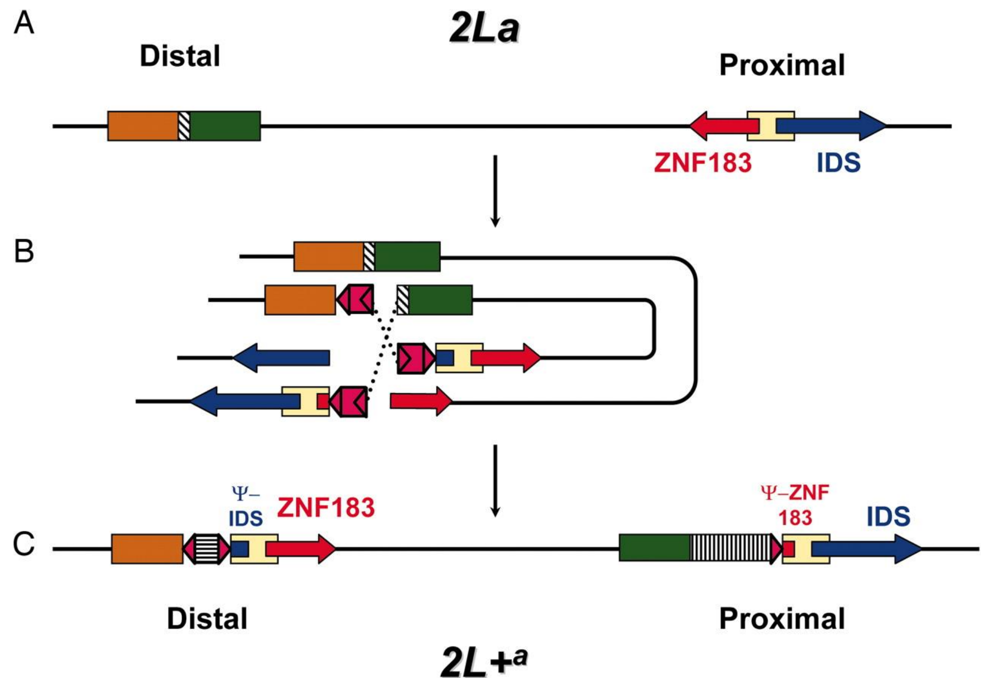

Figure 3: Image showing a model of 2L+[a] inversion generation from a 2La standard arrangement (adapted from Sharakhov *et al.,* 2006). (A) 2La standard arrangement. (B) Chromosomal break in two regions and pairing up of different sections with different orientation. (C) 2La inverted arrangement

2.5.1 Chromosomal arrangements in Anopheles

The success of *Anopheles* in attaining a wide ecological distribution has been associated with polymorphic chromosomal inversions. Inversions confer ecological adaptation in varying environmental conditions thus

increasing chances of survival even as the environment changes (Ayala *et al*., 2014). The distribution of the inversions is non-random and the pattern of distribution is shaped by environmental and geographic clines implying that inversions contribute to adaptation to the local environment (Costantini *et al.*, 2009). Different inversions have been identified in *Anopheles* with the type, number and combinations of inversion differing with species. The different inversions are associated with different phenotypic traits exhibited by the mosquito. In *Anopheles gambiae* the common inversions occur on chromosome 2, five on the right arm (2Rj, 2Rb, 2Rc, 2Rd and 2Ru) and one on the left arm (2La). In *Anopheles funestus* the common inversions occur in both chromosome 2 and 3, with inversion in the left chromosome occurring in chromosome 3 (3La) (Ayala *et al*., 2014). These inversions have been correlated with different adaptations and behavior changes (Ayala *et al*., 2014). Of interest to this study is 2La chromosomal inversion which has been associated with desiccation resistance and indoor resting behavior in *Anopheles gambiae (Coluzzi et al., 1977)*. While 2La inversion is polymorphic in *Anopheles gambiae* it is fixed in *Anopheles funestus*. In the *Anopheles gambiae* complex the conformation of the 2La inversion differs among the members of the complex. The inversion is only polymorphic in *Anopheles gambiae* sensu stricto (White *et al.*, 2007b). *Anopheles arabiensis* and *Anopheles*

merus are fixed for the inversion while *Anopheles melas, Anopheles quadrianulatus* and *Anopheles bwambae* are fixed for the standard (2L+^{a}/+^{a}) arrangement (White *et al*., 2007b).

2.5.2 2La chromosomal inversion and behavior

2La chromosomal inversion has been associated to tolerance to adverse environmental conditions e.g. aridity. Previous studies have suggested that *Anopheles gambiae* s.s which was originally a rainforest species acquired 2La and 2Rb inversions from *Anopheles arabiensis* which is an arid species by introgressive hybridization (della Torre *et al.*, 1997). This has enabled *Anopheles gambiae* s.s to colonize new dry habitats like the savannas. Carriers of the 2La inversion have been found to be thermo-tolerant and desiccation resistant (Gray *et al*., 2009;Rocca *et al.*, 2009). Physiologically this has been associated with cuticle properties like thickness and hydrocarbon composition (Reidenbach *et al.*, 2014).

Some behavioral traits have been linked to chromosomal inversion. Such behaviors include; host preference, choice of breeding place and resting behavior (Ayala *et al*., 2014). Resting behavior has direct impact on the efficiency of the current control methods since the methods target indoor resting and indoor feeding mosquitoes. The frequency of chromosomal inversion has been reported to be high in endophagic and endophilic

Anopheles gambiae (Coluzzi et al., 1977). This has been associated with microclimatic adaptations. Variants adapted to dry conditions or are desiccation resistant have a higher probability of feeding and resting indoors due to the higher nocturnal saturation deficit occurring indoors compared to outdoors (Coluzzi *et al*., 1977). In some way behavioral adaptation occurs as a result of physiological and genetic adaptation. Coluzzi *et al*. (1977) links 2R inversions to endophagy and endophily and with the recent establishment of a correlation between 2La and desiccation resistance, 2La could also be linked to resting behavior (Coluzzi *et al*., 1977).

Other important traits to malaria transmission and control associated with 2La inversion are *Plasmodium* infection and insecticide resistance. 2La inversions have been associated with low *Plasmodium falciparum* infection rates (Petrarca and Beier, 1992). Different studies have linked insecticide resistance to chromosomal inversion (Brooke *et al*., 2002;Brooke *et al.*, 2006). Unlike other traits where the trait is as a result of the inversion, insecticide resistance triggers the inversion (White, 1974). Insecticide resistance is considered as an extrinsic factor fueling chromosomal inversion, it does so by selecting against mosquitoes without the inversion (Brooke *et al*., 2002).

2.6 Insecticide resistance

Insecticides form a major component of malaria control; therefore, insecticide resistance has a great negative impact on the current vector control strategies which are LLINs and IRS. According to WHO, insecticide resistance is the ability of an insect to withstand the effects of an insecticide by becoming tolerant to its toxic effects or by avoiding contact with the insecticide (WHO, 2012). For vector control methods using insecticides, WHO recommends the use of carbamates, organophosphates and pyrethroids for IRS and use of pyrethroids only for LLINS. With the increased coverage of LLINS since the inception of rollback malaria programme, pyrethroid resistance affecting major malaria vectors has been reported in Africa (WHO, 2012). This resistance is not only fueled by the vector control practices but also by agricultural activities using chemical pesticides (Nkya *et al.*, 2014).

2.6.1 Mechanisms of resistance

Different mechanisms of resistance have been reported; physiological and behavioral resistance. Physiological resistance has been described as ability to withstand insecticide toxicity while behavioral resistance is the ability to evade possible contact with insecticides (WHO, 2012). Two forms of physiological resistance have been reported in malaria vectors; metabolic

resistance and target site modification and have been shown to sometimes occur concurrently in a population (Kawada *et al.*, 2011). Metabolic resistance entails increased metabolic detoxification of insecticide because of increased production of enzymes. Cytochrome P450 monooxygenases and esterases are the enzymes associated with pyrethroid resistance (Hemingway *et al*., 2004). Target site modification is where specific proteins are altered by point mutation rendering them less sensitive to insecticides bringing about knockdown resistance (Hemingway *et al*., 2004).

Different forms of behavioral modification have been reported for *Anopheles* due to insecticide selection pressure. The increase in LLINs coverage has been shown to cause shifts in behavior from endophagy to exophagy, endophiliy to exophiliy and anthropophagy to zoophagy (Mutuku *et al*., 2011;Russell *et al.*, 2011;Mwangangi *et al*., 2013). Other changes reported include change in feeding time to an earlier time before people are under the protection of the nets and change in species composition (Kawada *et al.*, 2012). Change in sibling species composition has also been reported for the *Anopheles gambiae* s.l (Bayoh *et al.*, 2010;Mwangangi *et al*., 2013).

There exist different ways of detecting insecticide resistance in malaria vectors. One of the WHO recommended methods is testing for phenotypic

resistance through WHO susceptibility test which is regarded as a more direct method of evaluating the adult vector control methods (WHO, 2013). This entails the exposure of adult mosquitoes to papers impregnated with diagnostic concentration of insecticides and thereafter accessing mortality twenty-four hours post exposure. To detect the mechanisms of resistance, enzyme assays are used for metabolic resistance while for resistance by target site modification molecular assays are used to test for the presence of the target site mutation (WHO, 2013).

2.6.2 Insecticide resistance and chromosomal inversion

Chromosomal inversion has been associated directly and indirectly with insecticide resistance. Direct association occurs when insecticide resistant gene is on the same loci as that of chromosomal inversion (Brooke *et al*., 2002). It has been hypothesized that the inversion may have a direct effect on the phenotypic expression of genes linked to the inversion (White, 1974). This association has been shown for phenotypic expression of dieldrin resistance gene and 2La inversion. Alanine 296 to glycine point mutation in the GABA (gamma amino-butyric acid) receptor occurs in a chromosomal position within 2La inversion (Brooke *et al*., 2006). It is for this reason that inversions have been implicated in insecticide resistance occurring in the absence of insecticide selection pressure in laboratory

strains. Since 2La inversions are stable, phenotypes associated with them are also maintained as the inversion polymorphism is maintained.

Indirect association occurs when the chromosomal inversions are associated with changes in behavior that lead to evasion of insecticides (Ayala *et al*., 2014). This happens as a form of behavioral resistance and behaviors like resting outdoors, feeding outdoors and feeding on non-human host have been associated with chromosomal inversions (Ayala *et al*., 2014).

2.6.3 Insecticide resistance and sporozoite rate

Extrinsic factors such as insecticides affect vector parasite interactions thus affecting the vectorial capacity of the vector or the vector competence. It has been postulated that insecticide resistance can directly affect *Plasmodium* transmission by affecting survival of the vectors. Resistant vectors have been shown to have a longer life span and as a result increasing their vectorial capacity (Molineaux *et al.*, 1979). Resistance has also been shown to affect susceptibility of malaria vectors to *Plasmodium* by influencing vector immunity or parasite development in the vector (James and Xu, 2012). Indirectly, insecticide exposure as an environmental factor can affect vector competence by interfering with traits like body size, blood feeding behavior and longevity (Alout *et al.*, 2013;Lefevre *et*

al., 2013). Resistance of mosquitoes to malaria parasite is determined by the mosquito genes and these genes could consequently be affected by insecticide resistance genes (Felix *et al.*, 2010). The effects of different mechanisms of resistance on vector competence have been investigated. Both metabolic detoxification and point mutation resistance have been shown to increase infection prevalence (Alout *et al*., 2013). Insecticide resistance directly impacts transmission by maintaining high vector densities that are resistant and as a result increases malaria transmission.

CHAPTER THREE: MATERIALS AND METHODS

3.1 The Study area

The study was conducted in south coast Kenya in Kwale County (Figure 4). This study was part of a main project KEMRI/SERU/CBRD/134/3085, with this part focusing on the Coastal region while others focused on other malaria endemic places in Kenya.

The area is hot and humid with a mean temperature of 24.2^0C and a relative humidity of 70%. The rains are bimodal, with long rains falling in April to June and short rains from October to December. The area has two dry seasons July to September, which is cool and dry, and January to March, which is hot and dry. Annual precipitation ranges from 400 to 1500mm/year. Drainage in the area is characterized by rivers (Ramisi and Uba), permanent and seasonal streams. Kwale County is populated by the Mijikenda ethnic group predominantly Digo and Duruma. The inhabitants of this area are subsistence farmers growing cassava, maize and coconut palms. They also keep livestock such as goats, chickens and cattle. They live in traditional Mijikenda houses characterized by wooden frame, mud wall and thatched roof.

Anopheles funestus and *Anopheles gambiae* are the main malaria vectors in the area. They occur all year round with their peak season occurring during

the rainy season (Mbogo *et al*., 2003). Sampling was done in two villages that are about 50 kilometers apart. The two villages, Marigiza and Kidomaya represent the Coastal plain and Coastal estuarine habitats respectively. For Kidomaya, the history of bednets dates back in 1998 where all households were provided with bed nets as part of a randomized clinical trial (Bogh *et al.*, 1998). After this, the distribution of bednets has been the same in the two villages through a mass bednets distribution in 2006 and 2012 (MOH, 2001;NMCP, 2014).

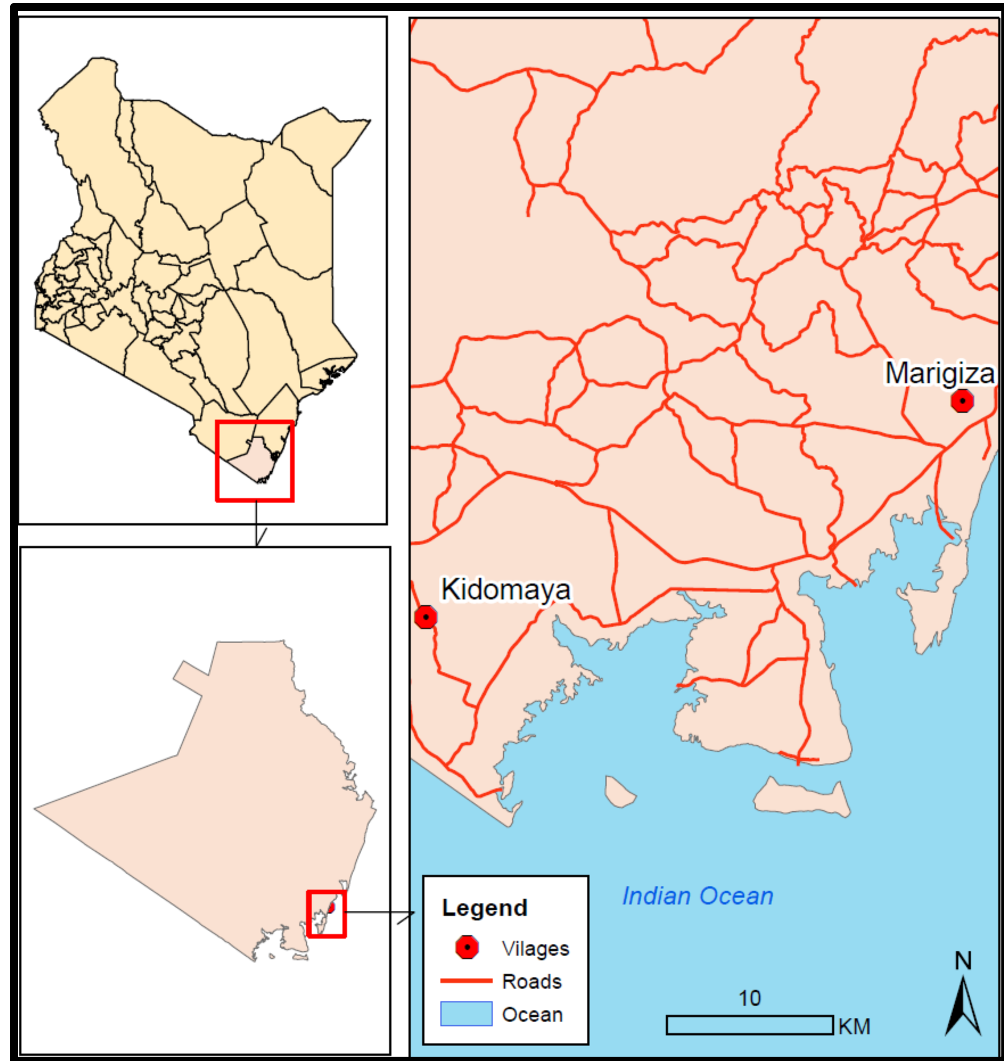

Figure 4: Map of Kenya showing the study area and the study villages: Kidomaya and Marigiza (developed using ArcGIS).

3.2 Sample size

For the determination of insecticide resistance WHO recommends the use of a minimum of 100 mosquitoes with replicates of 20-25 mosquitoes (WHO, 1998). However, this was not achieved for some replicates due to high larval mortality in the insectary. For the determination of the frequency of 2La chromosome inversion and sporozoite rate Fisher's formula (Fisher, 1954) was used to calculate the sample size.

$$n = \left\{ \frac{Z^2 \times p \times q}{d^2} \right\}$$

Equation (1)

Where n = Minimum sample size required

Z = Normal standard deviate for a 95% confidence interval (1.96)

p =Prevalence of the marker of interest (2La inversion) in the mosquito population. Since this was unknown, p=50% was used

q = (1-p)

d = significance level at 95% confidence interval (0.05)

therefore $n = \left\{ \frac{(1.96)^2 \times 0.5 \times 0.5}{(0.05)^2} \right\} = 384.16$

This sample size was hard to attain with Kwale being an *Anopheles funestus* belt and also due to the reported shifts in *Anopheles gambiae* complex sibling species composition (Mwangangi *et al*., 2013).

3.3 Mosquito sampling

Both mosquito larvae and adults were collected during the intermittent period between the end of the rainy season and beginning of the dry season (June-August) in view of capturing both seasons. Adults were collected using CDC light traps and aspirators while larvae were collected using the standard dipping method. In order to obtain live mosquitoes from the light traps, the light trap cups were emptied after every 2 hours in the night. Aspirators were replaced with a battery powered prokopack aspirator to be able to capture *Anopheles* since the densities were very low. Sampling was done once every week in the two villages. Larvae sampling was synchronized with adult sampling in both villages. Both larvae and eggs from blood fed adults collected were reared for their F1 generation that was used to test for phenotypic resistance.

3.3.1 Adult mosquito sampling

Light traps were used for both indoor and outdoor sampling. In each trapping night the light traps were set up between 1800-0600hrs. The indoor light traps was set up at the foot side of the bed 1 Meter off the

ground and approximately 1.5 Meters from the place of sleep (Mboera, 2005). The outdoor light trap was setup outside about 5 meters from the indoor light trap. The traps were removed the following morning between 0600-0700hrs and the collected mosquitoes transferred into paper cups.

Aspirations were done in the same houses and other new houses in the morning between 0700-0900hrs using prokopack aspirator (Figure 5). Live mosquitoes were provided with 6% sucrose and stored in a cool box and transported to the Msambweni Hospital Research laboratory for further processing.

Figure 5: Image showing collection of indoor resting mosquitoes using a battery powered prokopack aspirator

3.3.2 Larval sampling

Initially larvae were collected in potential larval habitats using the standard dipping method (WHO, 2013). Ten dips were made per potential larval habitat using a standard dipper (350 ml dipper) (Figure 6). This was adjusted to maximum number of larvae per habitat since the densities of *Anopheles* larvae were low and so was the number of habitats. The

adjustment was in accordance to WHO recommendations that larval collections be made from a number of different breeding habitat to avoid collecting larvae from single egg batches(WHO, 2013).

Figure 6: Larval collection using a standard 350ml dipper from a rice paddle

Individual larvae were picked from the dipper using a pipette and placed in whirl pak bags for transportation to Msambweni Hospital Research laboratory. Samples from the same larval site were pooled together and sorted by species and instar stages, first and second instar together and third and fourth together. The larvae were reared in 25 by 12 larval trays.

3.4 Mosquito rearing

Adult mosquitoes collected were sorted according to their abdominal status; gravid, half-gravid, blood fed and unfed. Live mosquitoes that were gravid, half-gravid and blood fed were kept in paper cups in the Msambweni District Hospital Research Insectary. They were maintained on 6% sucrose for 3 days before being transferred to individual egg laying tubes. The egg laying tubes were perforated eppendorf tubes lined with a moistened strip of filter paper. Eggs were collected the following morning and submerged in water for hatching. Larvae were maintained using Tetramin® fish food and were fed twice daily, 0700hrs and 1900hrs. Pupae were collected and placed in netted pupa cups for emergence. Emerged adults were transferred to mosquito cages labelled with collection site, date and time of emergence. Adult mosquitoes were maintained on 6% sucrose solution awaiting bioassays.

3.5 Insecticide bioassays

For the WHO bioassays 2-5 day old non-bloodfed female mosquitoes were used. They were subjected to WHO bioassays at temperatures of 25+(2^0C) and 70-80% relative humidity as described in WHO (2013). Mosquitoes in batches of 18-25 were placed in holding tubes for 1 hour after which any damaged mosquito was removed before being transferred to exposure tubes lined with pyrethroid impregnated papers (Figure 7). The diagnostic dose used for permethrin was 0.75% while that of deltamethrin was 0.05%. The tubes were held in vertical position and the knockdown rate recorded at intervals of 10, 15, 20. 30. 40. 50 and 60 minutes. After 60 minutes the mosquitoes were transferred to holding tubes, maintained on 6% sucrose and mortality rate determined 24 hours post exposure. Mortality rate was calculated by obtaining the total number of dead mosquitoes from all replicates for an individual insecticide and expressing this as a percentage of the total exposed.

$$\% \text{ mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total number exposed}} \text{ X100}$$

Equation 2

For the control tubes mosquitoes were exposed to papers treated with silicone oil. Control mortality was used to correct the mortality rate using Abbotts formula (WHO, 2013):

$$\frac{\%\ \text{test mortality} - \%\text{control mortality}}{100 - \%\ \text{control mortality}} \times 100$$

Equation 3

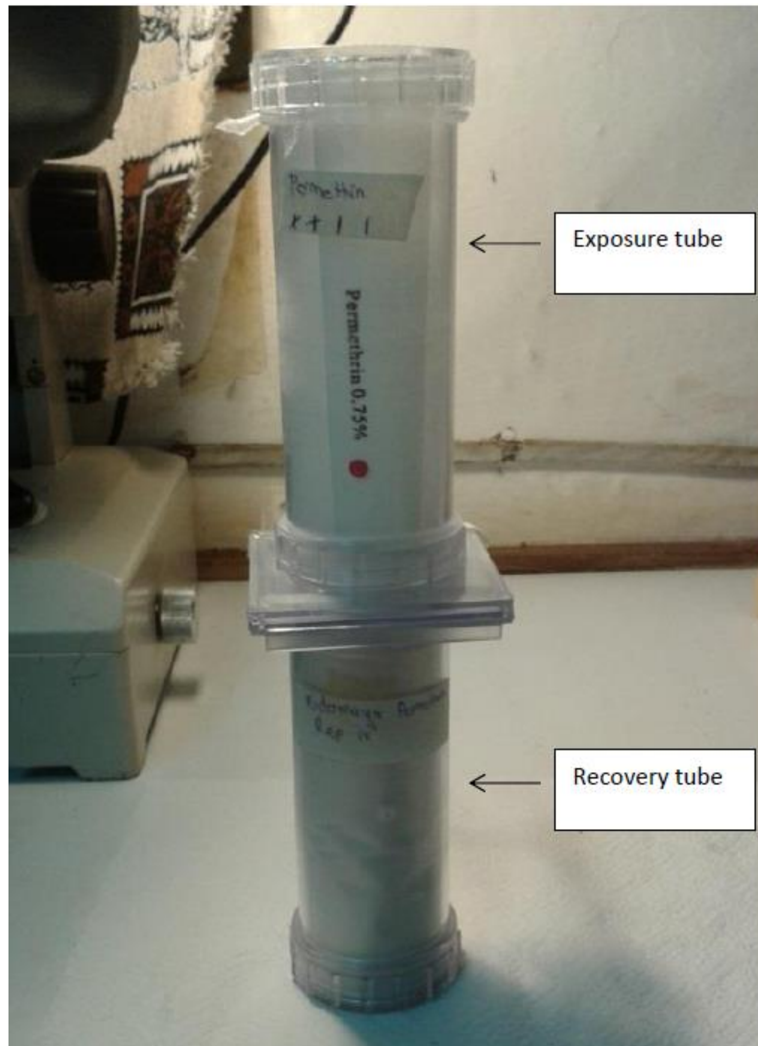

Figure 7: WHO bioassay setup showing exposure tube marked with a red dot and recovery or holding tube.

3.6 Mosquito processing

All mosquitoes collected from the field and from the bioassays were killed by briefly freezing at -20^{0}C and identified morphologically to species as described by Gillies (1987). They were then preserved by drying over silica gel granules at room temperature. All *Anopheles* mosquitoes identified morphologically were given a unique identification number. They were then cut into three portions; 1) head and thorax, 2) legs and wings and 3) abdomen, which were placed into individual eppendorf tubes and labeled with mosquito identification number. All portions were stored at room temperature awaiting further processing at the Malaria Entomology Laboratory at the Centre for Biotechnology Research and Development, KEMRI, Nairobi.

3.6.1 Sibling species identification

Genomic DNA was extracted from the legs and wings of *Anopheles* mosquitoes using alcohol precipitation method as described by Collins *et al.* (1987). The legs and wings were ground in grinding buffer made of both homogenizing and lysis buffer and placed in a 65^{0}C water bath to denature nucleases. Potassium acetate was added to purify the DNA by precipitating the proteins. This was centrifuged and absolute alcohol added

to the supernatant to precipitate the DNA. The DNA was used for sibling species identification, determination of kdr genotype and 2La karyotyping.

3.6.1.1 Anopheles gambiae s.l sibling species identification

For the *Anopheles gambiae* complex, sibling species identification was done by conventional polymerase chain reaction (PCR) as described by Scott *et al.* (1993) a reaction volume of 15µl was obtained by mixing 5.86µl of PCR water, 1.8µl of Magnesium Chloride, 3.0µl of 5x flexi buffer, 0.3µl of dNTP's, 1.2µl of Bovine Serum Albumin (BSA), 0.06µl of *taq* DNA polymerase and 0.26µl of each of the species specific primers and 2µl of the sample DNA template. This was loaded on a PCR microtiter plate and placed in a thermocycler for amplification. The amplification process was preceded by a heat activation period of 5 minutes at 94^0C. Thirty cycles were run at a denaturation temperature of 94^0C for 30 seconds, annealing at 50^0C for 30 seconds and extension at 72^0C for 30 seconds. This was followed by final elongation period of 10minutes at 72^0C. The universal primer anneals to the same position on the ribosomal DNA (rDNA) of each of the sibling species while the species specific primers which serve as reverse primers anneal at species specific templates, the size of the amplified products differs with the sibling

species. After running a 3% agarose gel electrophoresis the different size fragments characteristic to each species were observed against controls.

Table 1: *Anopheles gambiae* oligonucleotide primers, their sequences and expected post amplification fragment sizes

Primer name	Sequence (5' – 3')	Fragment size (base pairs)	Source
Universal	GTG TGC CCC TTC CTC GAT GT	-	Scott *et al.,* 1993
Anopheles arabiensis	AAG TGT CCT TCT CCA TCC TA	315	Scott *et al.,* 1993
Anopheles gambiae	CTG GTT TGG TCG GCA CGT TT	390	Scott *et al.,* 1993
Anopheles merus	TGA CCA ACC CAC TCC CTT GA	466	Scott *et al.,* 1993

3.6.1.2 Anopheles funestus s.l sibling species identification

This was also done by conventional polymerase chain reaction (PCR) as described by Koekemoer *et al.* (2002). A 15µl reaction volume was obtained by mixing 5.35µl of PCR water, 1.2µl of Magnesium Chloride, 3.0µl of 5x flexi buffer, 1.25µl of dNTP's, 0.3µl of Bovine Serum Albumin (BSA), 0.1µl of taq DNA polymerase and 0.3µl of each of the species specific primers (Table 3.2) and 2µl of the sample DNA template. This was

loaded on a PCR microtiter plate and placed in a thermocycler for amplification. The amplification process was preceded by a preheating at 94^0C for 3 minutes thirty cycles were run at a denaturation temperature of 94^0C for 30 seconds, annealing at 45^0C for 30 seconds and extension at 72^0C for 40 seconds. This was followed by post amplification extension at 72^0C for 10minutes. The sample was allowed to cool to 4^0C. The products of amplification were visualized after running a 3% agarose gel electrophoresis and observed against controls.

Table.2: *Anopheles funestus* primers, their sequences and post amplification fragment sizes.

Primer name	Sequence (5'- 3')	Fragment size (base pairs)	Source
universal	TGT GAA CTG CAG GAC ACA T	-	Koekemoer *et al.2002*
Anopheles vaneedeni	TGT CGA CTT GGT AGC CGA AC	587	Koekemoer *et al.2002*
Anopheles funestus	GCA TCG ATG GGT TAA TCA TG	505	Koekemoer *et al.2002*
Anopheles rivulorum	CAA GCC GTT CGA CCC TGA TT	411	Koekemoer *et al.2002*
Anopheles parensis	TGC GGT CCC AAG CTA GGT TC	252	Koekemoer *et al.2002*
Anopheles leesoni	TAC ACG GGC GCC ATG TAG TT	146	Koekemoer *et al.2002*

3.6.2 Sporozoite infection analysis

This was tested for *Plasmodium falciparum* circumsporozoite antigens using sandwich enzyme linked immunosorbent assay (ELISA) technique as described by Wirtz *et al.* (1987). Whereby the head and thorax of each mosquito were separately ground in 50 µl blocking buffer containing Nonidet P-40 and topped up with 200 µl of blocking buffer after grinding. Briefly, monoclonal antibodies produced against *Plasmodium falciparum*sporozoites were adsorbed on the microtiter plates by incubating for 30 minutes. The aliquots to be tested were added and if the antigen was present they formed an antigen-antibody complex that was visualized by adding a peroxidase linked monoclonal antibody which produced a green colour in the presence of its substrate. The results were read visually and compared with the positive and negative controls. Sporozoite rate was determined by calculating the percentage of positive samples from the total tested.

3.6.3 2La chromosomal genotyping

Presence of 2La karyotype was determined by PCR assay with primers designed for proximal breakpoints of the 2La and $2La+^a$ chromosomal conformation as described by White *et al*. (2007b) . One universal primer and two specific primers for the 2La inversion and for the standard was

used (Table 3). A reaction volume of 25 µl containing: 5.75 µl of PCR water, 2.0µl of Magnesium Chloride, 5.0µl of 5x flexi buffer, 2.0µl of dNTP's, 2.0µl of Bovine Serum Albumin (BSA), 0.25µl of *taq* DNA polymerase, 2.0µl of each of the species specific primers, 1µl of the universal primers and 3µl of the sample DNA template. This was loaded in PCR tubes and placed in a thermocycler. An initializing step of heat activation at 94^0C for 2 minutes preceded the amplification. For the amplification 40 cycles were made at 94^0C for 30 seconds, annealing at 58^0C for 45 seconds and extension at 72^0C for 60 seconds. The final elongation was at 72^0C for 10 minutes. The products of the amplification were separated by gel electrophoresis and visualized using a gel-reader after staining with ethidium bromide. The products were observed against a genomic marker and compared with controls.

Table 3: 2La karyotyping PCR assay primers, their sequences and the expected post amplification fragment sizes.

Primer	Target	Sequence (5'-3')	Fragment size (base pairs)	Source
23A2	Universal	CTCGAAGGGACAGC GAATTA	-	White *et al.*, 2007
27A2	2La/a	ACACATGCTCCTTGT GAACG	492	White *et al.*, 2007
DPCross5	$2L+^a/+^a$	GGTATTTCTGGTCAC TCTGTTGG	207	White *et al.*, 2007

3.7 Data analysis

Data was entered using Microsoft Excel 2010 and analyzed using STATA version 14. Resistance was determined using the WHO classification of mortality rate where 98-100% mortality indicated susceptibility, 80-97% suggested possible resistance while <80% mortality suggested resistance. For 2La chromosomal inversion conformity to Hardy-Weinberg expectations was tested in STATA using the GENHW command as described by Cleves (1999).The Hardy-Weinberg equilibrium was tested using chi-square (χ^2) (Yates' correction for 2 x 2 contingency tables) and Wright's F statistics where $F= 1 - (H_{OBS} / H_{EXP})$ where H_{OBS} is the observed

heterozygosity and H_{EXP} is the expected heterozygosity .When the absolute value of $F > 1.96/\sqrt{N}$

where N=total samples tested and P<0.05 there is a significant departure from expected values. Negative F values indicate excess heterozygosity while positive values indicate deficient heterozygosity.

Test for association between phenotypic resistance and 2La chromosomal inversion and the association between phenotypic resistance and sporozoite infection in collected *Anopheles* mosquitoes were based on Pearson chi-squared statistics.

3.8 Ethical considerations

Verbal consent was obtained from household heads or their representatives before mosquito collection. There was no risk to humans associated with setting light traps both indoor and outdoor. While there were no direct benefits to members of participating households, data obtained from this study would be useful in evaluating and guiding current and future insecticide based control strategies. The proposal was reviewed and ethical approval granted by KEMRI Scientific Ethics Research Unit, Proposal Number: CBRD/PROP/137.

CHAPTER FOUR: RESULTS

4.1 Mosquito collections

A total of 1101 *Anopheles* mosquitoes was collected in the two villages, 591 in Kidomaya and 510 in Marigiza. Of the *Anopheles* mosquitoes collected, 64.40% (n=709) were *Anopheles funestus*, 33.97% (n=374) were *Anopheles gambiae* and 1.63% (n=18) were secondary malaria vectors that included; *Anopheles coustani*, *Anopheles squamosus*, *Anopheles pretoriensis* and *Anopheles pharoensis* (Table 4.1). The species composition differed significantly by village (χ^2 =20.45, P= 0.0001). The proportion of mosquitoes collected as adults was 63.03% (n=694) while those collected as larvae was 36.97% (n=407). From the adults collected 154 were bloodfed and oviposited, the eggs hatched but did not survive past the 2nd larval instar. Of those collected as adults 4.90% (n=34) were males while 95.10% (n=660) were females.

Table 4: Number of *Anopheles* species collected as adults or larvae at Kidomaya and Marigiza villages in Kwale County in June-August 2015.

Species	Kidomaya		Marigiza		Total
	Adult	Larvae	Adult	Larvae	
Anopheles funestus	309	46	290	64	709
Anopheles gambiae	78	144	2	150	374
Anopheles squamosus	4	3	0	0	7
Anopheles coustani	2	0	3	0	5
Anopheles pharoensis	5	0	0	0	5
Anopheles pretoriensis	0	0	1	0	1
Grand Total	398	193	296	214	1101

4.2 Sibling species identification

A total of 374 *Anopheles gambiae* that were identified morphologically were further subjected to sub-species identification (Figure 8). The sub-species composition was: 88.24% (n=330) *Anopheles arabiensis*, 4.81% (n=18) *Anopheles gambiae* s.s while 6.95% (n=26) did not amplify. The 26 that did not amplify could imply the presence of other species as only *Anopheles arabiensis, Anopheles gambiae* and *Anopheles merus* were tested for. The difference in *Anopheles gambiae* sibling species composition in the two villages was found to be significant (χ^2 =37.74, $p<0.001$). *Anopheles arabiensis* was dominant in both villages with

44.55% (n=147) collected in Marigiza and 55.45% (n=183) collected in Kidomaya. For *Anopheles gambiae* s.s 88.89% (n=16) were collected in Kidomaya and 11.11% (n=2) collected in Marigiza (Table 5).

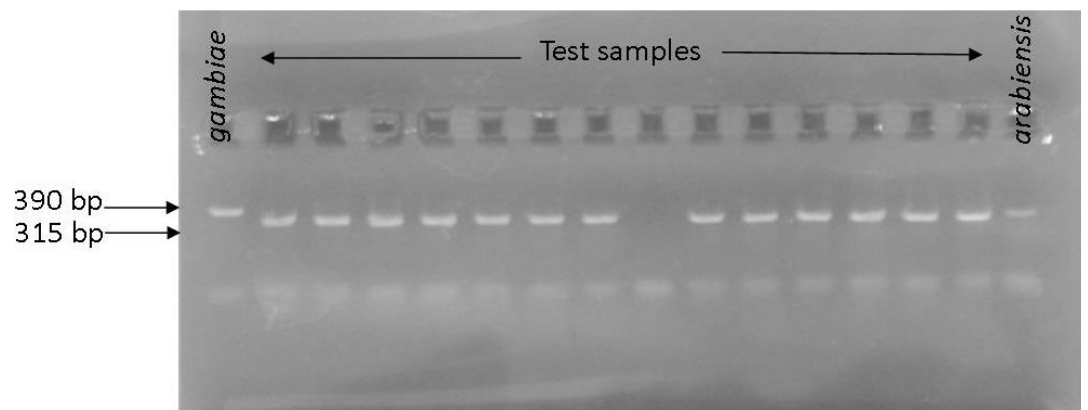

Figure 8: Gel image showing different characteristic fragments of *Anopheles gambiae* complex, *Anopheles gambiae* s.s with an amplicon size of 390 base pairs and *Anopheles arabiensis* with an amplicon size of 315 base pairs. arabiensis =*Anopheles arabiensis* control, gambiae= *Anopheles gambiae* control.

The 709 morphologically identified *Anopheles funestus* were genotyped into sub-species by PCR (Figure 9). The sub-species composition was 76.02% (n=539) *Anopheles funestus*, 3.53% (n=25) *Anopheles leesoni*, 2.96% (n=21) *Anopheles parensis*, 1.41% (n=10) *Anopheles rivulorum*, 0.85% (n=6) *Anopheles vaneedeni,* 0.85% (n=6) hybrids while 14.39% (n=102) did not amplify. The composition of the hybrids was: one

Anopheles parensis/Anopheles leesoni, one *Anopheles funestus/ Anopheles parensis* and four *Anopheles vaneedeni/ Anopheles parensis*. There was a significant difference in Anopheles funestus sub-species composition between the two villages (χ^2 =17.72, p<0.001). The dominant *Anopheles funestus* sibling species was *Anopheles funestus* in both villages (Table 5).

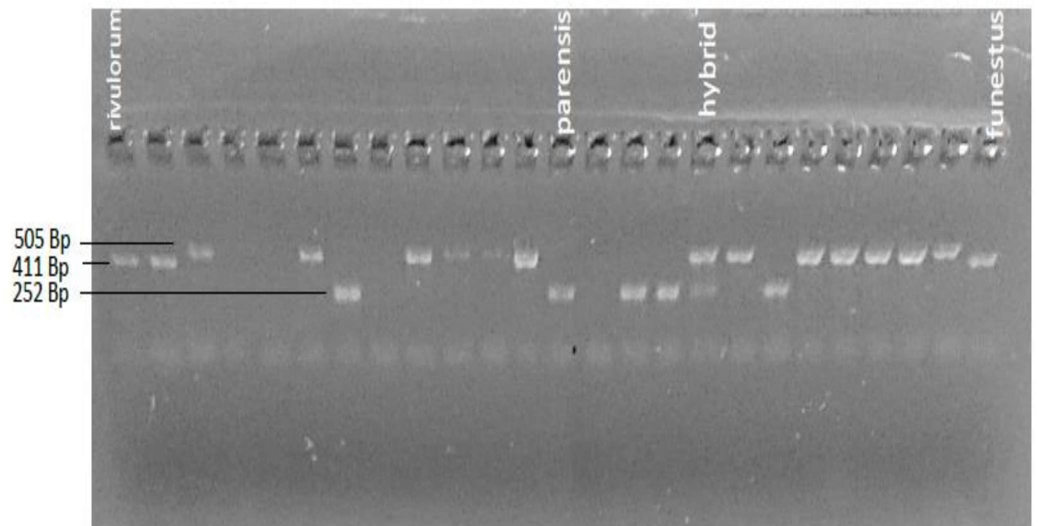

Figure 9: Gel image showing separation of characteristic fragments of *Anopheles funestus* sub-species. *Anopheles funestus* s.s with an amplicon size of 505 base pairs and *Anopheles rivulorum* with an amplicon size of 411 base pairs and *Anopheles parensis* with an amplicon size of 252 base pairs. Rivulorum=*Anopheles rivulorum* control, parensis=*Anopheles parensis* control and funestus=*Anopheles funestus* control.

Table 5: Sub species composition of *Anopheles gambiae* s.l and *Anopheles funestus* s.l collected in Marigiza and Kidomaya villages in Kwale County in June-August 2015.

Species	Sibling species ID	Kidomaya	Marigiza	Total
An. gambiae	*An. arabiensis*	177	145	322
	An. gambiae s.s.	14	2	16
	Total	**199**	**149**	**348**
An. funestus	*An. funestus* s.s.	250	289	539
	An. leesoni	24	1	25
	An. parensis	13	8	21
	An. rivulorum	1	9	10
	An. vaneedeni	1	5	6
	Hybrids	3	3	6
	Total	**289**	**312**	**607**

4.3 Outdoor and indoor collections

A significantly higher number of mosquitoes was collected outdoor 53.31% (n=370) compared to indoor 46.69% (n=324) (χ^2 =23.51, p<0.001). For *Anopheles funestus* 50.08% were collected indoor while 49.92% outdoor. For *Anopheles gambiae* 71.25% were collected outdoor

while 28.75% indoor. For the secondary vectors of malaria 93.33% were collected outdoor and 6.67%indoor (Table 6). By sibling species, a higher proportion of *Anopheles arabiensis* (70%) was collected outdoor while for *Anopheles gambiae* s.l outdoor proportions were equal to indoor proportions. For *Anopheles funestus* complex sibling species, *Anopheles funestus* s.s and *Anopheles parensis* did not show a difference in outdoor proportions compared to indoor proportions. Higher proportions were collected indoor for *Anopheles rivulorum* (90%) and *Anopheles vaneedeni* (60%) while for *Anopheles leesoni* higher proportions were collected outdoor (80%).

Table 6: Proportion of *Anopheles* mosquitoes collected indoor using Light trap and Prokopack aspirator and outdoor using light traps in Kidomaya and Marigiza in Kwale County

Species	Sibling species	Total collected	Indoor (%)	Outdoor (%)
An. funestus	-	599	50.08	49.92
	An. funestus	439	50.34	49.66
	An. leesoni	25	20.00	80.00
	An. parensis	21	52.38	47.62
	An. rivulorum	10	90.00	10.00
	An. vaneedeni	5	60.00	40.00
	Hybrids	6	50.00	50.00

An. gambiae	-	80	28.75	71.25
	An. arabiensis	60	30.00	70.00
	An. gambiae	2	50.00	50.00
An. coustani	-	5	0.00	100
An. pharoensis	-	5	0.00	100
An. squamosus	-	4	25.00	75.00
An. pretoriensis	-	1	0.00	100

4.4 Phenotypic resistance

A total of 407 F1 3-5 days old adults raised from larvae collected from Kidomaya and Marigiza were used to test for phenotypic resistance. The species composition of the 407 *Anopheles* mosquitoes used was; 72.24% (n=294) *Anopheles gambiae*, 27.03% (n=110) *Anopheles funestus* and 0.74% (n= 3) *Anopheles squamosus*. Of these 356 were exposed to pyrethroid insecticides; permethrin and deltamethrin while 51 were exposed to the control papers. Overall, the susceptibility status of malaria vectors upon exposure to pyrethroids was 76.97% while for the specific insecticides was 75.48% for deltamethrin and 78.11% for permethrin. The difference in susceptibility between the two insecticides did not differ significantly (χ^2=0.3403, p=0.5609). By village susceptibility to pyrethroids was 74.69% for Kidomaya and 78.87% for Marigiza with no significant difference ((χ^2=0.8678, p=0.352). For *Anopheles gambiae* s.l

overall susceptibility to pyrethroids was 68.58% with susceptibility to deltamethrin and permethrin being 62.38% and 72.50% respectively with no significant difference observed between the two insecticides (χ^2 =2.9451, p = 0.086). *Anopheles funestus* showed 100% susceptibility for both deltamethrin and permethrin. *Anopheles gambiae* from Kidomaya exhibited 69.57% and 67.07% susceptibility to deltamethrin and permethrin respectively. For *Anopheles gambiae* from Marigiza susceptibility to deltamethrin (56.36%) was significantly lower than that of permethrin (78.21%) (p=0.0070). *Anopheles funestus* showed 100.00% susceptibility to deltamethrin and permethrin in both villages (Figure 10).

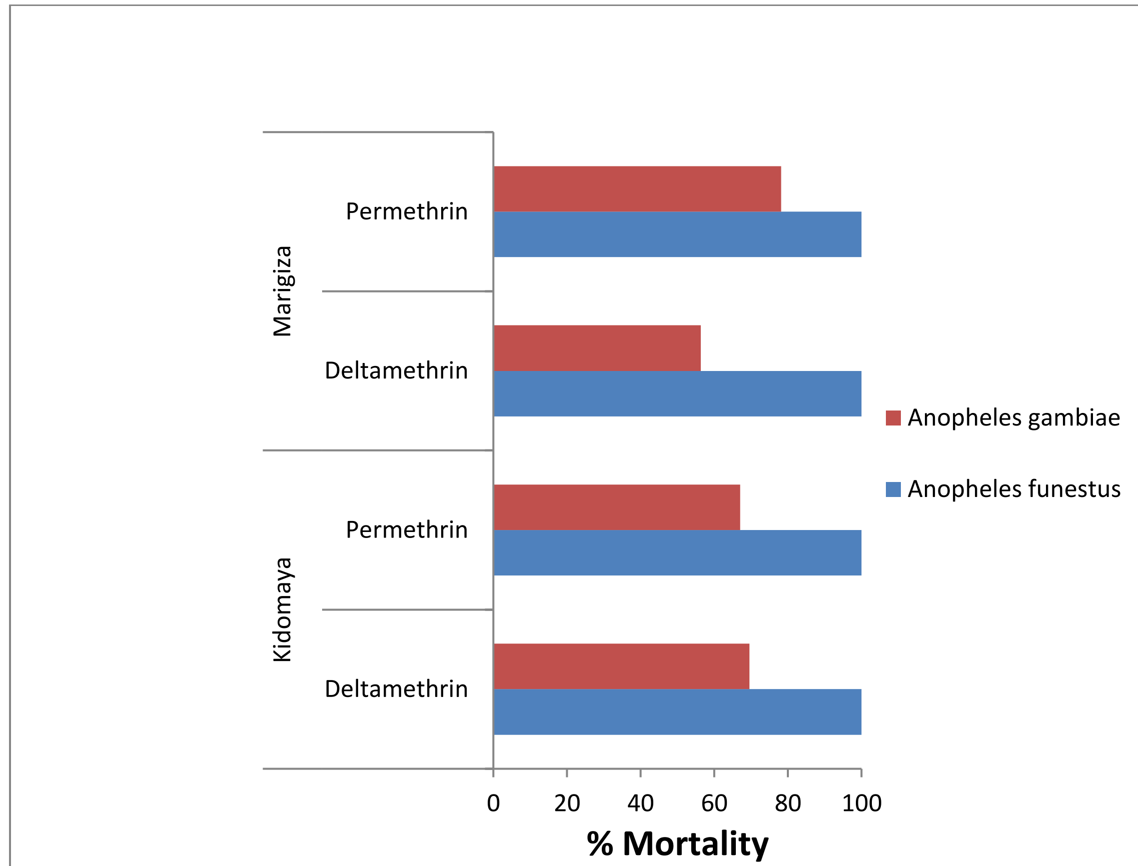

Figure 10*: Anopheles gambiae*s s.s and *Anopheles funestus* s,s overall susceptibility response to WHO diagnostic dose of deltamethrin and permethrin. % Mortality represents percentage 24 hours mean mortality after one hour of exposure to insecticide.

Two hundred and sixty-one *Anopheles gambiae* s.l were exposed to pyrethroid insecticides. Of these 61.30% (n=160) were used for the permethrin bioassay while 38.70% (n=101) were used for the deltamethrin bioassay. The sub-species composition for the *Anopheles gambiae* s.l was

94.33% (n=233) for *Anopheles arabiensis* and 5.67% (n=14) for *Anopheles gambiae* s.s. For *Anopheles arabiensis* overall susceptibility to pyrethroids was 66.52% with a non-significant difference (χ^2 =2.0159, p = 0.156). in susceptibility to permethrin (70.42%) and deltamethrin (60.44%). By village susceptibility of *Anopheles arabiensis* was 68.42% and 61.19% for deltamethrin and permethrin respectively in Kidomaya while in Marigiza a significantly higher susceptibility (χ^2 =7.562, p = 0.006) to permethrin (78.67%) compared to deltamethrin (54.72%) was observed (Figure 11). For *Anopheles gambiae* s.s overall susceptibility was 92.86%, similar to *Anopheles arabiensis,* susceptibility to permethrin (100%) was higher compared to deltamethrin (75%) with no significant difference (χ^2 =3.2, p = 0.074). By village susceptibility of *Anopheles gambiae* s.s was 75% and 100% for deltamethrin and permethrin respectively in Kidomaya with 100% susceptibility to permethrin in Marigiza. No *Anopheles gambiae* s.s from Marigiza was used for the deltamethrin bioassay. This is because sub-species used for susceptibility test were only identified after the insecticide exposure test by PCR.

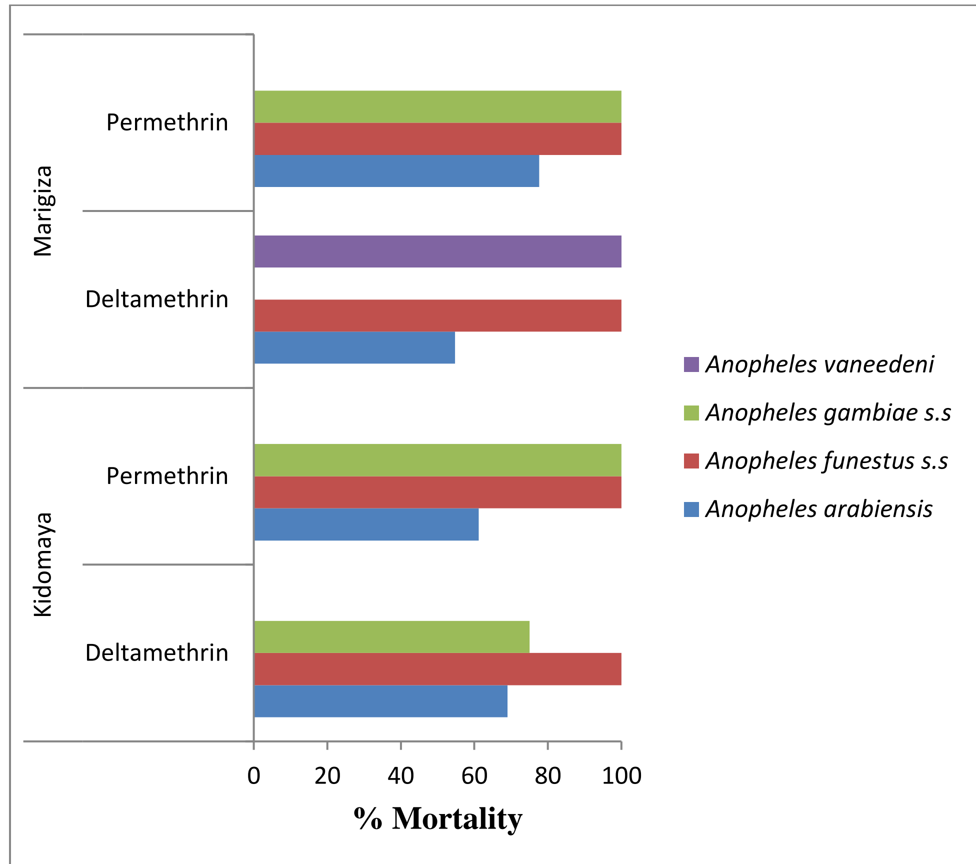

Figure 11: *Anopheles gambiae* and *Anopheles funestus* sibling species response to WHO diagnostic dose of deltamethrin and permethrin. % Mortality represents percentage 24 hours mean mortality after one hour of exposure to insecticide.

For the *Anopheles funestus* complex 95 were exposed to pyrethroid insecticides, 54 of these were exposed to deltamethrin while 36 were exposed to permethrin. The sibling species composition was 91.58%

(n=87) for *Anopheles funestus* s.s, 1.05% (n=1) *Anopheles vaneedeni while* 7.37% (n=7) did not amplify. For both deltamethrin and permethrin 100% susceptibility was observed in both villages.

4.5 Infection rate

Six hundred and fifty-nine mosquitoes were tested for the presence of *Plasmodium falciparum* circumsporozoite using sandwich ELISA. Thirty tested positive by producing a green colour after incubation with the substrate giving an overall infection rate of 4.55% (Figure 12). Of the 659 mosquitoes tested 539 belonged to the *Anopheles funestus* complex, 75 belonged to *Anopheles gambiae* complex while 15 belonged to the other *Anopheles*. Although the infection rate was higher in *Anopheles funestus* 4.94% (n=28) compared to *Anopheles gambiae* 2.60% (n=2), these did not differ significantly (χ^2 =0.8364, p=0.9039). For the other *Anopheles* the infection rate was 0.00%. Of the mosquitoes tested for *Plasmodium* sporozoite infection 368 were collected outdoor while 291 were collected indoor. This yielded an outdoor infection rate of 4.35% (n=16) and an indoor infection rate of 4.81% (n=14) with no significant difference in infection rate outdoor and indoor (χ^2 =0.034, p=0.7774). From the indoor collected mosquitoes only *Anopheles funestus* (5.20%) were infected while for outdoor collected mosquitoes both *Anopheles funestus* and *Anopheles*

gambiae were infected with an infection rate of 4.70% (n=14) and 3.57% (n=2) respectively. By village the overall infection rate 3.88% and 5.51% for Kidomaya and Marigiza respectively with no significant difference (χ^2 =0.9872, p=0.3212). For *Anopheles gambiae* complex the infection rate was 2.63% for Kidomaya while no adult *Anopheles gambiae* was collected in Marigiza. By sub-species only *Anopheles arabiensis* were infected with an infection rate of 3.51%. For *Anopheles funestus* complex the infection rate was 5.62% and 4.33% for Marigiza and Kidomaya respectively. The difference in infection rate between the two villages was not significant (χ^2 =0.4966, p = 0.481). The sub-species infection rate was 5.00% for *Anopheles parensis*, 4.84% for *Anopheles funestus* s.s and 4.00% for *Anopheles leesoni*, the rest of the sibling species were not infected (Table 7).

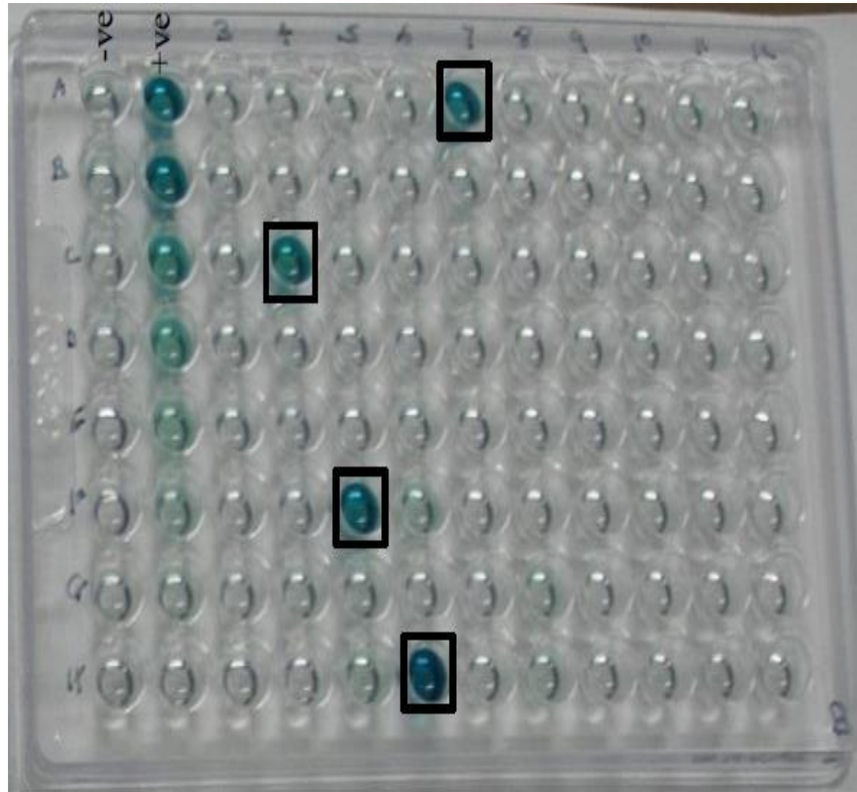

Figure 12: Image of an ELISA plate with positive samples highlighted. –ve = column of negative *Plasmodium falciparum* control and +ve = column of positive *Plasmodium falciparum* control in serial dilution.

Table 7: *Plasmodium falciparum* sporozoite infection rates in *Anopheles* mosquitoes collected in Marigiza and Kidomaya in Kwale County in June-August 2015.

Species	Sibling species	Marigiza	Kidomaya	Overall infection
An. gambiae	*An. arabiensis*	(0)	3.51 (57)	3.51 (57)
	An. gambiae	(0)	0.00 (2)	0.00 (2)
An. funestus	*An. funestus*	2.54 (208)	1.94 (205)	4.84 (413)
	An. leesoni	2.91 (1)	4.00 (24)	4.00 (25)
	An. parensis	0.00 (8)	5.00 (12)	5.00 (20)
	An. rivulorum	0.00 (8)	0.00 (1)	0.00 (9)
	An. vaneedeni	0.00 (4)	0.00 (1)	0.00 (5)
An. coustani	*	0.00 (3)	0.00 (2)	0.00 (5)
An. pharoensis	*	(0)	0.00 (5)	0.00 (5)
An. squamosus	*	(0)	0.00 (4)	0.00 (4)
An. pretoriensis	*	0.00 (1)	(0)	0.00 (1)

Number outside parenthesis is the infection rate in %. Number inside parenthesis indicate the total number tested. *Indicates species has no sub-species (not a complex species).

4.6 2La molecular karyotype

All *Anopheles gambiae* (n=18) were subjected to the 2La molecular karyotyping assay and showed polymorphism for the 2La inversion (Figure

13). Of these 88.89% (n=16) were homokaryotypes and 11.11% (n=2) were heterokaryotypes ($2La/+^{a}$). Of the homokaryotypes 81.25% (n=13) were homozygous for the 2La inversion arrangement (2La/a) while 18.75% (n=3) were for the standard arrangement ($2L+^{a}/+^{a}$) (Table 8). The two heterokaryotypes were collected from marigiza while all homokaryotypes were from Kidomaya. A significantly higher inversion arrangement (2La/a) was observed in Kidomaya compared to Marigiza (p=0.0000). The 2La inversion karyotype frequencies showed a departure from the expected frequencies according to the Hardy-Weinberg equilibrium (χ^2 =8.2882, degrees of freedom=1 and p=0.004). A deficiency in heterozygotes was observed in the population (F=0.6774 and $1.96/\sqrt{N}$=0.4754).

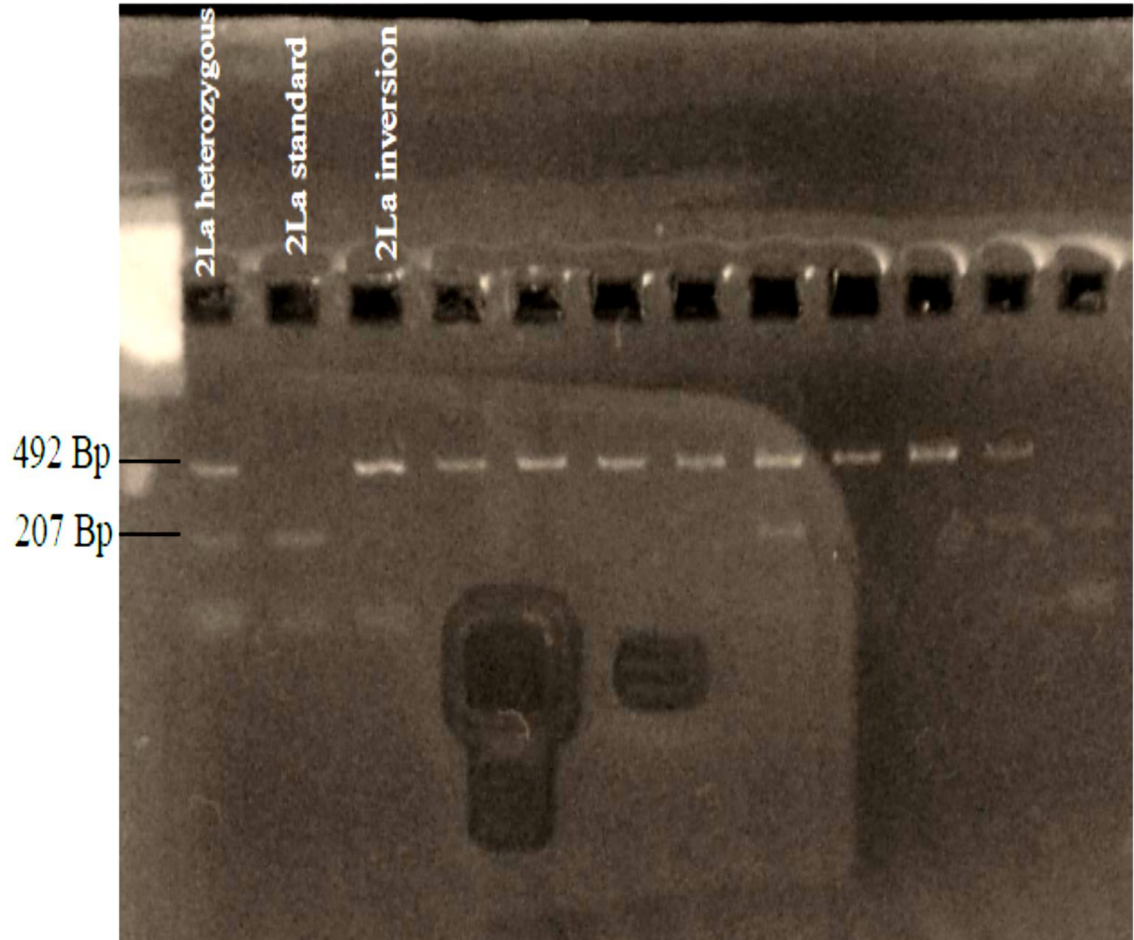

Figure 13: Gel image showing different characteristic fragments of the inversion, standard and heterozygous arrangement, inversion arrangement with an amplicon size of 492 base pairs, standard arrangement with an amplicon size of 207 base pairs and heterozygous arrangement with both bands.

Table 8:2La chromosomal inversion allele frequencies in *Anopheles gambiae* s.s collected in Kidomaya and Marigiza in Kwale County

Karyotype	N	Observed frequency	Expected frequency	p allele frequency	Q allele frequency
2La/a	13	72.22%	60.49%	0.07	0.93
2La/+^{a}	2	11.11%	34.57%	0.32	0.68
2L+^{a}/+^{a}	3	16.67%	4.94%	0.75	0.25

2La/a = inversion, 2L+^{a}/+^{a} = wild type, 2La/+^{a} = heterozygous, N= total number tested, p allele frequency is the expected frequency at HWE equilibrium if the other two groups are assumed to be correct, q allele frequency is the frequency of the alternative allele.

From the field collected adults two *Anopheles gambiae* s.s were tested for the 2La karyotype and sporozoite infection. One was a heterozygous collected outdoor while the other was a homozygous with a standard arrangement collected indoor, none tested positive for *plasmodium falciparum* antigen. Due to the limited sample size (n-2) it was impossible to carry our correlation analysis for 2La arrangement and sporozoite infection rate and for 2La and resting behavior.

From the bioassays 16 *Anopheles gambiae* s.s were obtained and subjected to the 2La molecular karyotyping assay. Of these 93.75% (n= 15) were susceptible while 6.25% (n= 1) was resistant. The resistant was

homozygous for the inversion arrangement. The 2La arrangement for the susceptible mosquitoes was; 73.33% (n=11) homozygous with 2La/a, 13.33% (n=2) homozygous with $2L+^{a}/+^{a}$ and 13.33% (n=2) heterozygous ($2La/+^{a}$. The frequency of 2La/awas higher in the resistant *Anopheles gambiae* compared to the susceptible (Table 9). This was not statistically significant (Pearson χ^2 = 0.3556, d.f =2, P = 0.837). The resistant population was composed of the live mosquitoes after the bioassay while the susceptible population was composed of those that were dead after the bioassay. Fixation index statistics showed a deficiency in heterozygosity in the resistant population. Regression analysis did not show any association between phenotypic resistance and 2La inversion (R^2=0.0000).

Table 9: Allele frequencies for 2La inversion and standard arrangement in resistant and susceptible *Anopheles gambiae* s.s

Susceptability	N	2La/a frequency	$2L+^a/+^a$ frequency	F
Resistant	1	2(100%)	0(0%)	-
Susceptible	15	24 (80%)	6 (20%)	0.58

N=total samples tested, F= Interindividual fixation index: 1 - (H_{OBS} / H_{EXP}) where H_{OBS} is the observed heterozygosity and H_{EXP} is the expected heterozygosity.

Thirteen *Anopheles arabiensis* were tested for the 2La karyotype and they all showed fixation for the 2La/a arrangement.

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Successful insecticide resistance management requires routine monitoring of insecticide resistance. WHO recommends inclusion of vector distribution, biting and resting preferences and insecticide susceptibility tests during resistance monitoring (WHO, 2012). Despite the Kenyan Coast being endemic for malaria and an area where malaria vectors are primarily controlled using LLIN the status of insecticide resistance remains unknown. This study documents the species distribution, their indoor and outdoor proportions as well as resistance to pyrethroids in malaria vectors in the Kenyan coast.

5.1.1 Species distribution

This current study records occurrence of higher densities of *Anopheles funestus* s.l compared to *Anopheles gambiae* s.l. These findings support previous studies that have reported changes in species composition with a relative increase in *Anopheles funestus* s.l compared to *Anopheles gambiae* s.l (Mutuku *et al*., 2011;Mwangangi *et al*., 2013). These changes are alluded to insecticide pressure from the current vector control methods (McCann *et al.*, 2014;Lwetoijera *et al.*, 2014). Although the overall density

of *Anopheles funestus* was high, the proportion of *Anopheles funestus* s.l reared from field-collected larvae was low compared to the proportion of *Anopheles gambiae* s.l. This could have occurred as result of high mortality rate for the *Anopheles funestus* larvae in the insectary during rearing due to the difficulty associated with rearing *Anopheles funestus*.

For the sibling species composition of *Anopheles gambiae* complex, the present study reports *Anopheles arabiensis* as the dominant sub-species. Previously in the Kenyan Coast and other regions in Kenya *Anopheles gambiae* s.s was the dominant subspecies while *Anopheles arabiensis* was regarded as a secondary vector (Mbogo *et al*., 2003). However, since the up-scaling of vector control a reverse in the trends has been reported with a relative increase in *Anopheles arabiensis* which is regarded as a more flexible species relative to *Anopheles gambiae* s.s (Bayoh *et al*., 2010;Mwangangi *et al*., 2013).

For *Anopheles funestus* sub-species composition this study reveals a complex composition compared to previous studies in the area where only three subspecies were identified (Kamau *et al.*, 2003). Five sub-species were identified: *Anopheles funestus* s.s, *Anopheles leesoni*, *Anopheles parensis*, *Anopheles rivulorum*, *Anopheles vaneedeni* and six hybrids. *Anopheles funestus* s.s dominated the *Anopheles funestus* population with a

proportion of 76.02%. This correlates with findings from other studies in the Kenyan Coast and other regions in Kenya where *Anopheles funestus* has been reported as the dominant sub-species (Kweka *et al.*, 2013;Kamau *et al.*, 2002;Kamau *et al*., 2003). Unlike *Anopheles gambiae* s.l where more exophagic and exophilic species have taken over, this study shows that *Anopheles funestus* s.s. which is regarded as highly anthropophilic and endophagic remains the dominant sub-species. There is little information on *Anopheles funestus* sub-species composition in the Kenyan Coast. In most of the previous studies *Anopheles funestus* has only been identified morphologically thus it is impossible to tell if there has been a change in sub-species composition over time. However, the increased complexity reported in this study could be an indicator of possible changes in species composition as a result of the current vector control strategies. As it was the case with *Anopheles gambiae*, reports of change in sub-species composition in the Kenyan Coast as form of behavioral resistance came before other forms of resistance could be detected suggesting that behavioral resistance could be used as an early indicator for upcoming resistance. The occurrence of more *Anopheles funestus* sub-species in the current study could be a possible indicator of possible resistance to pyrethroids in *Anopheles funestus* in the near future.

5.1.2 Indoor and outdoor preferences

A significantly higher proportion of malaria vectors was collected outdoor compared to indoor. This is in consistence with other studies that have reported changes in resting and feeding behaviors as a mode of behavioral resistance (Bayoh *et al*., 2010;Mutuku *et al*., 2011). While *Anopheles arabiensis* exhibited exophily and exophagy, *Anopheles gambiae* s.s in the current study showed equal proportions indoor and outdoor. Previously, *Anopheles gambiae* s.s was regarded as the main malaria vector in Africa due to its endophilic, exophilic and anthropophilic behavior. However, following the increased upscaling of vector control in many countries in Africa, a switch in behavior has been reported in different studies (Bayoh *et al*., 2010; Reddy *et al.*, 2011). With the main vector control methods targeting endophilic, exophilic and anthropophilic these behavioral adjustments pose a big thereat to malaria control.

Similarly, *Anopheles funestus* s.s did not show any difference in outdoor and indoor proportions this indicates a possible change in resting and feeding behavior since *Anopheles funestus* s.s was previously considered to be more anthropophagic, endophilic and endophagic (Coetzee and Fontenille, 2004). The other sub-species of *Anopheles funestus* are regarded as more exophagic and exophilic, however the results here show only *Anopheles vaneedeni* had higher proportions outdoor compared to

indoor. *Anopheles parensis* showed a 50:50 split which has also been the case in earlier studies too (Kamau *et al*., 2003). Higher proportions of *Anopheles rivulorum* have been found outdoors in earlier studies in the Kenyan Coast (Kamau *et al*., 2003) but in higher proportions indoor in other areas (Kawada *et al*., 2012). For the current study, higher proportions of *Anopheles rivulorum* were found indoors this could have been as a result of shifts in behavior but also could be due to biasness of the collection methods used. Light traps and aspirators were used for indoor collections while for outdoor collections only light traps were used. Overall, the comparison of indoor and outdoor proportions highlights the need to consider control of malaria vectors outdoor.

5.1.3 Plasmodium falciparum sporozoite infection rate

Contrary to recent studies in the area, the current study reports a high overall *Plasmodium falciparum* infection rate of 4.55% (Mutuku *et al*., 2011;Onyango *et al*., 2013). There are several plausible explanations for the high infection rate. First, increased insecticidal interventions over time might have led to reduced susceptibility of malaria vectors to insecticides used to treat nets. This means the nets become less effective in repelling, deterring and killing malaria vectors. Reduced efficaciousness of bednets translates to increased human vector contact leading to high infection rate in mosquitoes (Gimnig *et al*., 2003). Second, differences in sampling

season could lead to differences in infection rates. Higher infection rates have been reported in drier seasons compared to wet seasons (Mwangangi *et al*., 2013). For this study mosquitoes were collected during the intermediate of the dry and rain season, June to August. Third, the difference in sampling method could lead to differences in infection rates. This study used light traps and prokopack aspirator. Light traps have been reported to increase the proportion of infected mosquitoes 2-3 times fold (Mbogo *et al.*, 1993). Results from this study show that the rate of malaria transmission outdoor was the same as that of indoor. This is in consistent with other studies that have reported increased malaria transmission outdoor with increased bed net coverage (Russell *et al*., 2011). There lacks possible knowledge to explain outdoor infection rates since it is still not known whether the outdoor infection is as a result of the vectors resting outdoor after an indoor blood meal or they actually feed outdoor. Nevertheless, this highlights the need to consider control of outdoor malaria transmission. Though the difference in infection rate was not significantly different between species, a higher infection rate was observed in *Anopheles funestus* compared to *Anopheles gambiae*, which was consistent with other studies (Mutuku *et al*., 2011) . This could be as result of the current vector control methods, which target anthropophagic and endophagic mosquitoes, thus the change in infection rate between the

species with time. The high infection rate in *Anopheles funestus* can be attributed to their high density in the area and their presence in large proportions outdoor.

5.1.4 Resistance to pyrethroids

This study documents a mortality rate of 75.48% and 78.11% for deltamethrin and permethrin respectively in malaria vectors in Kwale County. For the assessment of phenotypic resistance, WHO classifies a population into three categories based on their percentage mortality or susceptibility: A population with 100%-98% mortality is regarded susceptible, 97%-90% mortality indicates possible resistance that needs confirmation either using more bioassays or assessing the level of resistant genes, <90% indicates resistance (WHO, 2013). Based on this classification, this study reveals presence of phenotypic resistance to pyrethroids in malaria vectors. The population used for the bioassays was mainly composed of *Anopheles gambiae* s.l thus from the results of this study we can report resistance of *Anopheles gambiae* s.l to both deltamethrin and permethrin. For the specific sub-species of the *Anopheles gambiae* complex, *Anopheles arabiensis* exhibited resistance to both insecticides in two villages. For *Anopheles gambiae* s.s, the population from Kidomaya was resistant to deltamethrin while that from Marigiza was 100% susceptible to both insecticides. This difference in susceptibility of

Anopheles gambiae s.s between the villages could be attributed to earlier introduction of pyrethroids in Kidomaya earlier than Marigiza, as part of a randomized clinical trial (Bogh *et al*., 1998). The population of *Anopheles funestus* in both villages exhibited susceptibility to both deltamethrin and permethrin. By sub-species, both *Anopheles funestus* s.s and *Anopheles vaneedeni* were 100% susceptible to both deltamethrin and permethrin. Though phenotypic resistance has not been documented in the Kenyan Coast findings from this study support previous studies in the Coast that that have reported occurrence of behavioral resistance encompassing changes in species composition with a relative increase in *Anopheles funestus* s.l compared to *Anopheles gambiae* s.l (Mutuku *et al*., 2011;Mwangangi *et al*., 2013). To our knowledge, this is the first report of phenotypic resistance in *Anopheles gambiae* s.l in the Kenyan Coast. Previous insecticide resistance studies in the Kenyan Coast focused on genotypic resistance which entails assessing the level of knock down resistance gene; L1014S-*kdr* allele. So far, this allele has not been reported in the Kenyan coast but has been reported in other areas in Kenya (Stump *et al.*, 2004;Chen *et al.*, 2008).

5.1.5 2La chromosomal inversion

Anopheles gambiae s.s was polymorphic for the inversion a on the 2Lchromosome with frequencies of 77.78% and 22.22% for the inverted

and standard arrangement respectively. The observed frequency for the 2La inversion in *Anopheles gambiae* was high compared to that observed in Kisumu (Petrarca and Beier, 1992). The observed 2La karyotype frequencies showed a deviation from the Hardy-Weinberg proportions, indicative of non-random mating. The deviations occurred as a result of deficiency in heterozygotes. These findings are contrary to findings of other studies in the Kenyan Coast where the 2La inversion did not show deviation from Hardy-Weinberg proportions (O'Loughlin *et al.*, 2014).

In this study a significantly higher frequency of the 2La inversion was recorded in Kidomaya compared to Marigiza. Interestingly, *Anopheles gambiae* s.s population in Kidomaya had shown a lower susceptibility rate to pyrethroids. This suggests a possible association between insecticide resistance and the 2La inversion. The introduction of bednets earlier in Kidomaya could also explain the high frequency of the inversion in relation to resistance.

It was not possible to test for associations between sporozoite infection rates and 2La inversion because of the low numbers of *Anopheles gambiae* s.s field adults collected (n=2). This also made it impossible to test for association between resting behavior and 2La inversion.

No association was observed between phenotypic resistance and 2La inversion. This might have been because of the small sample size (only one out of sixteen mosquitoes was phenotypically resistant to pyrethroids).

5.2: Conclusion

The dominant malaria vectors belonged to *Anopheles gambiae* complex and *Anopheles funestus* complex. *Anopheles arabiensis* and *Anopheles funestus* s.s are the dominant sub-species from each of the complex. A higher proportion of *Anopheles arabiensis* and other secondary malaria vectors are exophagic and exophillic while for *Anopheles funestus* the level of endophagy and endophilliy is similar to exophagy and exophilliy.

Results from this study show phenotypic resistance to pyrethroids in malaria vectors in Kwale County. *Anopheles gambiae* s.l population in was resistant to pyrethroids while *Anopheles funestus* s.l was susceptible. The *Anopheles gambiae* population in Kidomaya was less susceptible to pyrethroids compared to that in Marigiza.

Anopheles funestus was the predominant malaria vector for transmission of *Plasmodium falciparum* with a higher infection rate compared to *Anopheles gambiae* with malaria transmission occurring both indoor and outdoor.

There was evidence of non-random mating in *Anopheles gambiae* through inversion frequencies indicative of selection and adaptation to ecological variabilities. There was no association between phenotypic resistance and

2La inversion while it was impossible to test for association between phenotypic resistance and sporozoite infection.

5.3 Recommendations

1. Other modes of resistance like presence of knock down resistance allele and metabolic resistance need to be evaluated in the region as part of resistance management.
2. With equal indoor and outdoor infection rate there is need to develop and adopt outdoor control methods for malaria vectors.
3. There is need to assess the level of genotypic resistance which will enable the testing of association between resistance and *Plasmodium falciparum* infection rates.
4. Due to the low densities of *Anopheles gambiae* s.s it was impossible to determine the possibility of using 2La inversion as a genomic marker for behavioral resistance. However, this can be pursued using archived samples collected after the increased coverage of bednets or from archived samples used to report behavioral resistance.

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APPENDICES

Informed consent form

INFORMED CONSENT FORM FOR HOUSEHOLD HEADS FOR COLLECTION OF MOSQUITOES

Protocol Title: Effects of insecticide resistance on 2La chromosomal inversion and *plasmodium falciparum* infection rates in malaria vectors in kwale county, coastal kenya

Principal investigator: Caroline Kiuru-University of Nairobi

Supervisors:

Prof. Florence Oyieke-University of Nairobi

Prof. Richard Mukabana- University of Nairobi

Dr. Damaris Matoke- Kenya Medical Research Institute, Nairobi

Dr. Joseph Mwangangi-Kenya Medical Research Institute, Kilifi

Why is this study being done?

Various methods are used to control mosquitoes in the Kenyan coast, among them being use of treated bednets and house sparaying. These methods use chemicals called insecticides and could be a problem if mosquitoes cannot be controlled using them. The purpose of this study is to find out if the current insectisides are able to control mosquitoes and the effect of the insecticides on malaria transmission.

What is involved in this study?

This study involves collection of mosquitoes from inside and outside of your houses. The collections will be made using a CDC light trap and an aspirator. A CDC light trap is a trap that emits light which attracts the mosquitoes at night. An aspirator is a tube made of glass and rubber that is used to suck resting mosquitoes from the walls in the morning.

What are we requesting from you?

We are requesting you to allow us to enter your house and collect mosquitoes at least once in the month of August and October 2015. Collections will be done in the rooms where people sleep. The CDC light trap will be set up in the evening between 1730-1800hrs and removed the following morning at around 0600hrs. After removing the light traps the aspirators will be used to collect those mosquitoes that were not captured by CDC light traps. Aspiration will take about 5 minutes per house.

What are the risks of the study?

Allowing us to collect mosquitoes inside and outside your house will not expose you to any risk. However, there could be minor inconveniences and loss of privacy due to staff entering your house.

Are there benefits to taking part in the study?

There may be no direct benefit to you for being in this study but the information obtained from this study will help in development of new mosquito control methods and in resistance management.

What about confidentiality?

We will keep the information we collect confidential. Your name will not appear in any report generated from the study.

What other options are there?

You do not have to be in this study. Your participation is voluntary and if you agree to participate then you will sign below showing that you have understood the instructions provided above. If you agree to participate in this study and change your mind later, you can withdraw from the study at any time without any problems.

Can I stop being in the study?

Yes, you can withdraw from the study at any time without any problems.

Do you have any questions about the study?

If so you can ask them now.

Whom do I call if I have any questions?

Caroline Wanjiku Kiuru

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Signature

Signing below indicates that you have read and been informed about the research study in which you voluntarily agree to participate ; that you have asked any questions about the study that you may have; and that the information given to you has permitted you to make a fully informed and free decision about your participation in the study.

Name of the household head: ___________________________________

Signature/ Thumbprint:__________________ Date: _________________

In the presence of a witness

Witness

I observed the consenting process. The household head read and was informed of the contents of this form and given a chance to ask questions.

This was satisfactory to him and he voluntarily accepted and signed to take part in the study.

Name of the witness:

____

Signature/ Thumbprint: ____________________ Date: _______________