STATUS AND MECHANISMS OF INSECTICIDE RESISTANCE IN *ANOPHELES* MOSQUITOES FROM MWEA SUB-COUNTY AND KWALE COUNTY AND THEIR MALARIA PARASITE INFECTION RATES

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DECLARATION

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

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DEDICATION

To Sylvester and Mildred Uduny …… who are not just Dad and Mum, but also the first two beautiful people I ever knew. The much you have given into this work can never be repaid in this life. All I pray is that the Lord God Almighty continues to richly bless you in whatever you do. This was one of the greatest gestures of sacrifice from you. May God bless you abundantly.

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LIST OF ABREVIATIONS AND ACRONYMS

ρg	Picogram
ρg/µl	Picogram per microlitre
ρmole	Picomole
ρmole/µl	Picomole per microlitre
µg	Micrograms
µl	Microlitre
A	Adenine
AMCA	American Mosquito Control Association
An.	*Anopheles*
AR	*Anopheles arabiensis* primer
ATCH	Acetylcholine esterase
ATP	Adeninetriphosphates
BB	Blocking buffer
BF	Blood fed
bp	Basepairs
BSA	Bovine serum albumin
C	Cytocine
CBRD	Centre for Biotechnology, Research and Development
CDC	Centre for Disease Control
cDNB	1-chloro-2,4'-dinitrobenzene
CoE	Carboxylesterase
CSP	Circumsporozoite proteins
CTP	Cytosine triphosphates
CYP4J5	Cytochrome P450
dATP	adenine triphosphates
dCTP	cytosine triphosphates
DDT	Dichlorodiphenyl trichloroethane

DEET	*N*,*N*-diethyl-3-methylbenzamide
dGTP	guanine triphosphates
DNA	Deoxyribonucleic acid
dNTPs	dinucleotides triphosphates
DTNB	Dithio-bis-2-nitrobenzoic acid
dTTP	thymine triphosphates
DVBD	Division of Vector Borne Diseases
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
F_0	Wild-caught
F_1	First filial generation
FF	Homozygous resistant (*kdr*-west)
FUN	*Anopheles funestus* s.s. primer
g	Grams
G	Guanine
GA	*Anopheles gambiae* primer
GR	Gravid
GST	Glutathione-S-transferase
GSTe2	Glutathione–S-transferase epsilon 2
GTP	Guanine triphosphates
HG	Half gravid
Inc	Incorporated
IRAC	Insecticide Resistance Action Committee
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
ITS2	internal transcribed spacer region 2
IVM	Integrated Vector Management
kdr	Knock down resistance

KEMRI	Kenya Medical Research Institute
KMIS	Kenya Malaria Indicator Survey
KPO_4	Potassium phosphate
L1014F	Leucine-phenylalanine substitution at position 1014
L1014S	Leucine-serine substitution at position 1014
LEES	*Anopheles leesoni* primer
LF	Heterozygous resistant (*kdr*-west)
LL	Homozygous susceptible/ *kdr* wildtype
LLITNs	Long-Lasting Insecticide Treated Nets
LS	Heterozygous resistant (*kdr*-east)
M	Molar
MAb	Monoclonal antibody
ME	*Anopheles merus* primer
$MgCl_2$	Magnesium chloride
ML	Male
ml	Millilitre
mM	Millimolar
MRIS	Mwea Rice Irrigation Scheme
N	Sample size
NA	Not amplified
na	Not applicable
Na^+	Sodium ions
NaOAc	Sodium acetate
nm	Nanometer
NP-40	NONIDET P-40
NTC	Non template control
^oC	Degrees Celcius
OD	Optical density
P.	Plasmodium

PA	Pennsylvania
PAR	*Anopheles parensis* primer
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium falciparum*
pH	Potential of hydrogen
PMI	Presidential Malaria Initiative
PR	Possible resistance
PSI	Population Services International
qPCR	Real time Polymerase Chain Reaction
R	Resistance
RBM	Roll Back Malaria
rDNA	Ribosomal Deoxyribonucleic acid
RIV	*Anopheles rivulorum* primer
rpm	Revolution per minute
rr	Homozygous enzyme resistant
RR	Homozygous *kdr* mutant alleles
rs	Heterozygous enzyme resistant
RS	Heterozygous *kdr* mutant alleles
S	Susceptible
s.l.	sensu lato
s.s.	sensu stricto
SDS	Sodium Dodecyl Sulfate
SNPs	Single nucleotide polymorphisms
SS	Homozygous resistant (*kdr*-east)
ss	Wild type enzyme
SS	Homozygous *kdr* east
T	Thymine
TBE	Tris boric acid EDTA

TMBZ	Tetramethyl-Benzidine Dihydrochloride
TTP	Thymine triphosphates
U.S.A.	United States of America
UF	Unfed females
UN	Universal primer
UNICEF	United Nations International Children's Emergency Fund
UV	Ultra violet
VAN	*Anopheles vanadeni* primer
w/v	Weight per volume
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

ABSTRACT

Anopheles mosquitoes are vectors of malaria. Several vector control strategies have been applied to reduce human-vector contact to minimize the spread of malaria with much emphasis on control of the adult mosquitoes mainly through the use of bed nets and insecticide residual spraying (IRS). However, increasing insecticide resistance hampers the success of malaria control. The objective of this study was to determine the status and mechanism of insecticide resistance in malaria vector species from Mwea and Kwale and to determine their malaria parasite infection rates. Field collected *Anopheles* mosquitoes were reared in the insectary and subjected to bioassays, enzyme analysis and sporozoite tests. The bioassayed specimens were also screened for knockdown resistance (*kdr*) alleles. All field samples (415 from Mwea; 714 from Kwale) were identified by polymerase chain reaction. *Anopheles arabiensis* and *An. funestus* s.l. were the predominant mosquitoes in Mwea and Kwale respectively. The prevalence of malaria parasite infection was observed to be low in Mwea (0%) and slightly moderate in Kwale (10.81%). *An. arabiensis* from Mwea showed resistance against deltamethrin (79.24%) and permethrin (79.34%). *An. funestus* s.s. from Mwea were susceptible to both deltamethrin (100%) and DDT (100%). In Kwale, resistance to permethrin (71.43%) and deltamethrin (60%) was observed in *An. gambiae* s.s. Molecular analysis revealed an increasing frequency of *kdr*-east gene in Kwale (2.17%). *An. gambiae* s.l. from Kwale had increased ATCH, oxidase, β-esterase and GST activity against deltamethrin. Against permethrin, Kwale observed higher ATCH, oxidase, GST and β-esterase activity in the *An. funestus* s.l. population. The frequencies of CYP4J5 (>50%) was high in Mwea. CoE (> 25%) was high in both study sites while GSTe2 was only high in Kwale (50%). This study determined that malaria parasite infection rate was low in Mwea but high in Kwale, possibly accounting for malaria incidence in those areas. Insecticide resistance in Mwea and Kwale was observed posing a challenge on control measures. This calls for alternative control strategies to be put in place that encompasses integrated vector control methods to curtail spread of malaria in Mwea and Kwale.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria, a debilitating parasitic infection, causes about 3 million deaths every year with most of mortality cases recorded in tropical areas of Central America, Asia and Africa (Caminade *et al.*, 2014). The Sub-Saharan African countries are particularly the hardest hit by malaria infections (Rowe *et al*., 2006). The disease retards economical growth through premature deaths, affect population growth and result in high cost of drugs to treat the disease (Sachs and Malaney, 2002). High mortality rates are seen in children under the age of 5 years and pregnant women due to reduced immunity (UNICEF, 2004; WHO, 2011; WHO, 2015).

In humans, malaria is transmitted through an infective bite from a female *Anopheles* mosquito. It is caused by different species of the *Plasmodium* genus consisting of *Plasmodium falciparum* (Welch) which account for almost all fatal cases (Nicholas, 2004), *P. vivax* (Grassi and Feletti), *P. ovale* (Stephens), *P. knowlensis* and *P. malariae* (Grassi and Feletti). These species have different geographical distribution with *P. falciparum* and *P. malariae* predominantly occurs in the tropical and subtropical areas of Central and South America, Africa, and South East Asia. *P. ovale* primarily occurs in Sub-saharan Africa and *P. vivax* occurs in Central and South America, India and South East Asia. *P. knowlesi* is found in South East Asia.

The *Anopheline* mosquitoes comprise of a complex of several mosquito species including *Anopheles gambiae* and *An. funestus*. These complexes are the major malaria vector species throughout the sub-Saharan Africa and exist as sub-species with different vectoral capacity in different regions (Tsy *et al*., 2003). In Kenya, the *Anopheline* species are widely distributed. *Anopheles gambiae* s.s. is mostly found in Western Kenya (Wamae *et al*., 2010; Mutuku *et al*., 2009) and Coast region (Mwangangi *et al*.,

2007). In Mwea, Central Kenya, *An. arabiensis* were observed to be of higher density in the irrigated rice regions than the non-irrigated regions (Muturi *et al*., 2008). *Anopheles merus,* though not a dominant species, occurs along the Kenyan coast (Mbogo *et al*., 2003) while *An. funestus* complex is distributed at the Coast (Mwangangi *et al*., 2007), in Western Kenya (Munga *et al*., 2009; Mutuku *et al*., 2009) and in Central Kenya (specifically in Mwea's non-irrigated regions as compared to the irrigated areas) (Muturi *et al*., 2010). Studies in Kenya have shown that mosquito control interventions have led to shifts in the vectors originally present in a population (Bayoh *et al.*, 2010; Mutuku *et al*., 2011; Mwangangi *et al*., 2013).

Malaria vector control has been a major component in public health sector as a tool for reducing malaria transmission in the tropics (WHO, 2006). Several control measures that target different developmental stages of the mosquito lifecycle have been adopted to curtail the growth, development, maturation and eventual dispersion of malaria vectors (Walton and Eldridge, 2009). The current trend in malaria vector control is the adoption of Integrated Vector Management (IVM) strategy which involves a systemic approach to planning and implementation of vector control measures. IVM is a rational decision-making process for the optimal use of resources in the management of vector populations, in order to reduce or interrupt transmission of vector-borne diseases (WHO, 2008). These vector control measures include those targeting adult vectors such as use of insecticide treated nets (ITNs), indoor residual spraying (IRS), long-lasting insecticides treated nets (LLITNs) and those targeting premature stages especially larvicides (RBM, 2005). Several studies have observed success in vector control after implementation of IVM strategies in the field set-up (Keiser *et al*., 2005; Chanda *et al*., 2008; Killeen *et al*., 2000; Bang *et al*., 1975; Beier *et al*., 2008).

Earlier studies on *An. arabiensis* collected from Mwea found this species to be 100% susceptible to insecticides in the four classes that have been approved by WHO for indoor residual spraying, namely organochloride, organophosphates, carbamates and pyrethroids (Kamau and Vulule, 2006; Chen *et al*., 2008). These insecticides target the

central nervous system of the insect, interfering with nerve impulses on the targeted axon and /or the synaptic cleft. However, in Kwale, there is no documented evidence of the status of insecticide resistance. With the scaling up of the use of ITNs in Mwea and Kwale leading to the reduction of malaria incidences (PSI, 2004; Mutuku *et al*., 2011) and the evidence of insecticide resistance development in some parts of West and East Africa, there is need to determine the current status of insecticide resistance in these regions.

Insecticide resistance has been reported in malaria vector species, including *Anopheles gambiae* sensu strict (s.s.), *An. funestus* and *An.* a*rabiensis* as a result of wide scale use of mosquito insecticides. This has been reported in at least 64 malaria endemic countries, with pyrethroids resistance reported in 27 countries in sub-Saharan Africa (WHO, 2011; WHO 2013). It has also been suggested that the development of resistance could also be associated with cross resistance due to larval intervention by treatment of larval habitats using different chemicals. Studies have reported an emerging new adaptation in mosquitoes when faced with domestic interventions, the development of behavioral avoidance whereby the insect avoids environments where insecticides have been sprayed (Ferguson *et al*., 2010; Govella *et al*., 2010). The change in mosquito vector behavior has thus resulted in reduced contact between vectors and insecticides and hence reduced effectiveness of the malaria intervention programmes (Pates and Curtis, 2005). Resistance can thus arise due to either change in the gene sequence in the target site resulting in either East or West Africa knockdown (*kdr*) mutation in mosquitoes, increased insecticide metabolic rates by the mosquitoes or behavioral changes of the vector (IRAC, 2011).

As a result, the need to continuously monitor the status of resistance by malaria vectors against a variety of insecticides and understand the different mechanisms responsible for the widespread levels of resistance is essential for resistance management. This study primarily focused on the mechanisms used by malaria vectors in Mwea and Kwale to confer insecticide resistance against insecticides currently used in mosquito control.

1.2 Statement of the problem

In Kenya, the more frequent use of agricultural insecticides (Chen *et al*., 2008) as compared to its use in other non agricultural areas (Kamau and Vulule, 2006) might have resulted in the initial development of insecticide resistance. The wide scale use of ITNs/ LLITNs, IRS and agricultural activities in Mwea and Kwale have impacted on *Anopheles* mosquitoes differently and reduced mosquito densities (Mbogo *et al*., 1996). However, studies have shown that these vectors can reappear in regions where they were once eliminated, sometimes even with resistance to the previously used insecticides (Hargreaves *et al*., 2000; Brooke *et al*., 2001; Casimiro *et al*., 2006). Resistance to recommended insecticides can jeopardize the efforts put forth towards the control of mosquito transmitted diseases such as malaria.There is need to effectively put in place strategies that will manage and contain the spread of insecticide resistance. Effective management of insecticide resistance will therefore require activities in both public health and agriculture to be closely monitored and the sharing of data and information on the development and mechanisms of resistance.

1.3 Justification of the study

Recent research in malaria endemic regions has confirmed strong suspicions that wide scale use of a single class and related classes of mosquito insecticides has given rise to resistance (Ochomo *et al*., 2013; 2014) in several predominant malaria vector species, including *An. gambiae* s.s., *An. funestus* and *An. arabiensis*. Resistance has been reported in several malaria endemic countries, with pyrethroids resistance being the most common in sub-Saharan Africa (WHO, 2011). This might be due to increase in selection pressure caused by the use of pyrethroids in all the approved LLITNs and in most IRS programmes worldwide (WHOPES, 2011). The pyrethroids used include deltamethrin and permethrin compounds. In some regions, malaria has been reported to be on the rise even after a significant decline of malaria cases in the previous years. This rise was initially associated with insecticide resistance as a result of the continued use of ITNs (Lee *et al*., 2010; Zhou *et al*., 2011). However, Viana *et al*., (2016), recently

obsereved that continous exposure to insecticides against insecticide resistant mosquito population reduce malaria transmission. With the appearance and rapid spread of the West African *kdr* mutation and the recent increase in the frequency of the East African *kdr* mutation in mosquitoes, regular monitoring is required within different vector population in a locality. This will help understand the mechanism of resistance and thus lead to downstream implementation of improved and effective vector control strategies and ultimate decline in malaria incidences and inform insecticide resistance management strategies. The compromised mode of action should be well understood to allow for the introduction of a new class of insecticide with a different kind of mode of action against the malaria vectors. DDT, although this insecticide is currently not in use in Kenya, which has the same mode of action as the pyrethroids, was also tested in the current study in an effort to explore the existence of cross-resistance. It is important however also to understand that further extension of vector control intervention poses a big threat to the increase in vector resistance. Reducing reliance on a single intervention or a single insecticide is a major objective of any resistance management policy. The proposed study provided information on the current status of susceptibility or resistance to insecticides on mosquito vectors in Kwale and Mwea after the scaling up of vector control interventions in these areas in the recent past.

Malaria parasite infections in the vectors is an important aspect of malaria epidemiology that relate to malaria transmission. Malaria endemic areas are usually associated with poor socio-economic status with lower rateds of economic growth. This is due to effects on fertility, population growth, worker productivity, premature mortality and medical costs (Sachs and Malaney 2002). This study also provided information on the parasite infection rates and the malaria vecor species that acted as hosts for the parasites in Kwale and Mwea. This information can be used to implement future vector control strategies.

1.4 Hypothesis

1.4.1 Null Hypothesis

1. *Anopheles gambiae* s.l. and *An. funestus* s.l. mosquitoes from Mwea and Kwale are not resistant to insecticides
2. *Anopheles gambiae* s.l. and *An. funestus* s.l. mosquitoes from Mwea and Kwale have a low malaria parasite infection rates.

1.4.2 Alternative Hypothesis

1. *Anopheles gambiae* s.l. and *An. funestus* s.l. mosquitoes from Mwea and Kwale are resistant to insecticides.
2. *Anopheles gambiae* s.l. and *An. funestus* s.l. mosquitoes from Mwea and Kwale have a high malaria parasite infection rates.

1.5 Objectives

1.5.1 General objective

To determine status and mechanisms of insecticide resistance associated in malaria vectors in Mwea and Kwale and their malaria parasite infection rates.

1.5.2 Specific objective

1. To determine the main malaria vector species present in Mwea and Kwale using real-time polymerase chain reaction (PCR).
2. To assess malaria parasite infection rates in the mosquitoes from Mwea and Kwale using sporozoite Enzyme-linked immunosorbent assay (ELISA).
3. To establish the status of resistance to pyrethroids and DDT in *An. gambiae* s.l. and *An. funestus* s.l. from Mwea and Kwale using WHO bioassay tests.
4. To evaluate the mechanisms of insecticide resistance in *An. gambiae* s.l. and *An. funestus* s.l. mosquitoes from Mwea and Kwale using real-time PCR and

microplate enzyme assays to establish mutations and changes in ATCH, oxidase, β-esterase and GST enzyme levels.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Anopheles* mosquitoes

Mosquitoes are insects belonging to the order Diptera and family Culicidae. There are two sub-families of mosquitoes including the *Anophelinae* and *Culicinae*. The *Anophelinae* mosquitoes are the major malaria vectors in sub-Saharan Africa (Gillies and De Meillon, 1968) with other genus of mosquitoes transmiting other diseases like filariasis, yellow fever, Chikungunya, Dengue virus and O'nyong' nyong (Gubler and Clark, 1995; Sanders *et al*., 1996; Thompson *et al*., 1996). The *Anopheline* mosquitoes comprise of a complex of several mosquito species including *Anopheles gambiae*, which exists as sub-species with different vectoral capacity in different regions (Tsy *et al*., 2003).

Mosquitoes undergo complete metamorphosis (egg, larvae, pupa and adult) (AMCA, 2014; Bates, 1949). The eggs hatch into first instar larvae that develop to the fourth instar larvae from where they pupate then emerge into adults. Adult female *Anopheline* mosquitoes require blood meal which aids in the development of their eggs, thus serving as vectors for malaria. (AMCA, 2014; Kogan, 1990). There are approximately 400 known *Anopheline* species with about 40-50 species capable of transmitting malaria (Renshaw *et al*., 2001). However, malaria transmission in a locality is usually dominated and driven by two or three important vector species that are ecologically adapted to reproduce and survive in the area (Coetzee *et al*., 2000). For instance, *An. gambiae* s.s. Giles and *An. arabiensis* Theobald usually occur in sympatry over large geographical ranges and associate strongly with the traditional rural life of many African communities (Coetzee *et al*., 2000). In sub-Saharan Africa, the most important and predominant malaria vectors include *Anopheles gambiae* Giles complex (especially *An. gambiae* s.s. and *An. arabiensis), An. funestus* Giles complex and *An. pharoensis* Theobald (Service, 1993). In Kenya, the malaria vector distribution is mainly composed of *An. gambiae* s.s.,

An. arabiensis, An. funestus and *An. merus* majorly found in Western, Central and Coastal Kenya (Figure 2-1).

Anopheles funestus Giles complex consists of at least eleven species that are morphologically very similar but differ in vectoral behavior (Harbach, 2004). The common species found in Kenya that belong to this complex includes *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. rivulorum* Leeson, *An. parensis* and *An. leesoni* Evans.

Until recently, only seven species in the *An. gambiae* Giles complex were known (White *et al*., 2011). However, recent research has brought into light two more species discovered in South Africa (Coetzee *et al.*, 2013). Even within sub-species like *An. gambiae* s.s., further sub-divisions have been made by the different karyotypes and are known as chromosomal forms like Mopti, Savanna Bissau and Forest (Della *et al*., 2002). Of the several sub-species, *An. gambiae* s.s. and *An. arabiensis*, have been incriminated as the major malaria vectors in sub-Saharan Africa. These mosquito vector species differ in their biology to enhance their survival within the population. For instance, *An. arabiensis* often rest outdoors making it a lesser target for indoor-residual spraying (IRS) with insecticides (Githeko *et al*., 1994; Bayoh *et al*., 2010).

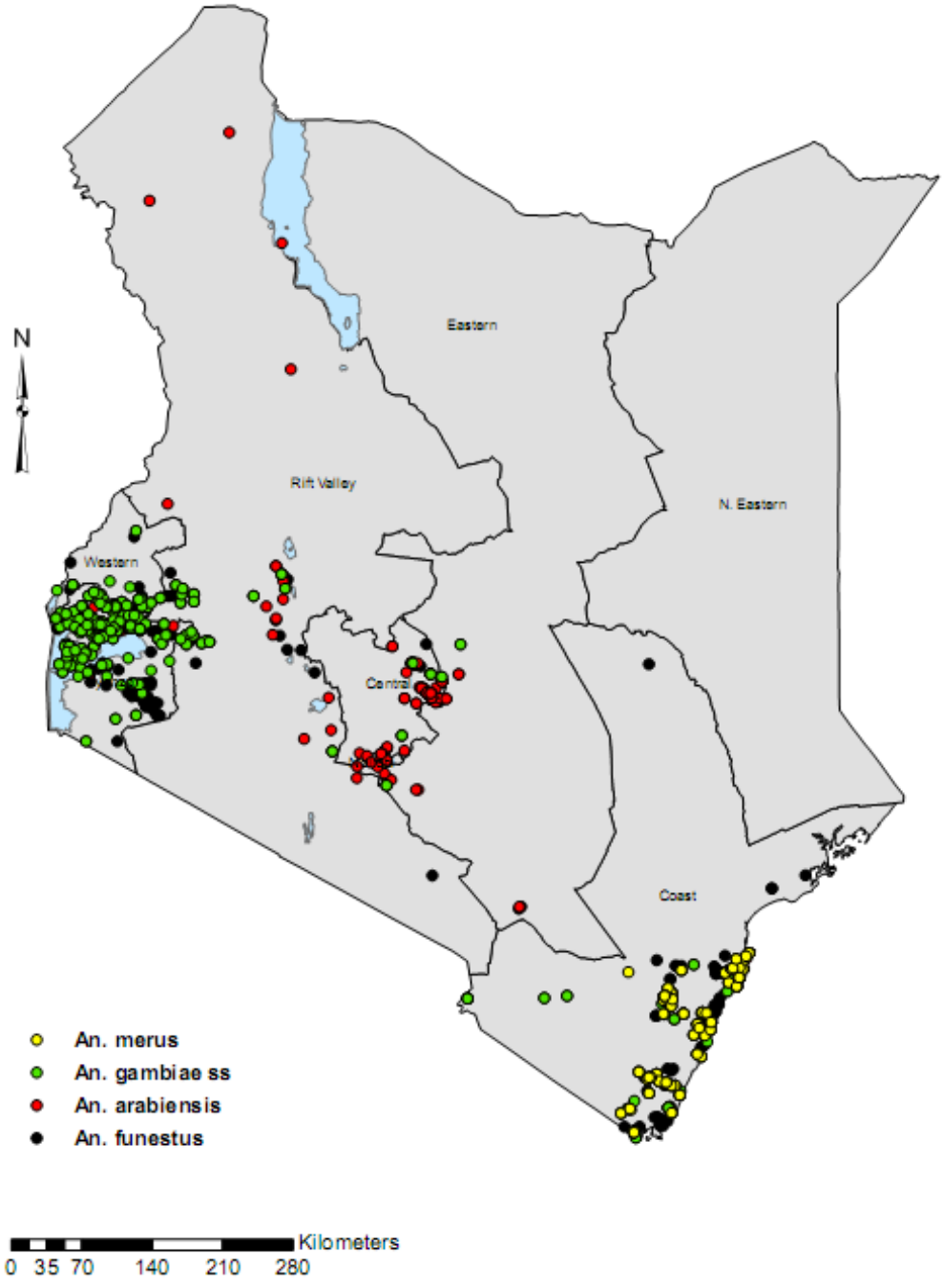


Figure 0-1: Distribution of the main malaria vectors in Kenya (Adapted from Ondeto BM, 2012).

2.2 Importance of mosquito species identification

Prior to implementation of any vector control strategy in a region, target species identification is paramount so as to shape necessary deployment policies. Correct identification of specific vector species is thus crucial so as to differentiate non-vector species from vector species. This helps to save time and resources that would otherwise be used to control non- vector species. Moreover, related mosquito species may show different levels of susceptibility to different mosquito insecticides (Ramphul *et al*., 2009), thus underlining the importance of correct taxonomic classification. Currently, the widely used technique for species identification is first morphological identification followed by polymerase chain reaction (PCR) for morphologically similar species. This technique provides markers that can be used in diagnostic assays (Collins and Paskewitz, 1996) so as to distinguish members of the *An. gambiae* complex (Scott *et al.*, 1993; Bass *et al*., 2008).

2.3 Species distribution and increase in insecticide resistance

In Kenya, the *Anopheline* species are widely distributed. *Anopheles gambiae* s.s. is mostly found in Western Kenya (Wamae *et al*., 2010; Mutuku *et al*., 2009) and Coast region (Mwangangi *et al*., 2007). In Mwea, Central Kenya, *An. arabiensis* were observed to be of higher density in the irrigated rice regions than the non-irrigated regions (Muturi *et al*., 2008). *An. merus,* though not a dominant species, occurs along the Kenyan coast (Mbogo *et al*., 2003) while *An. funestus* complex is distributed at the Coast (Mwangangi *et al*., 2007), in Western Kenya (Munga *et al*., 2009; Mutuku *et al*., 2009) and in Central Kenya (specifically in Mwea's non-irrigated regions as compared to the irrigated areas) (Muturi *et al*., 2010).

Insecticide resistance has been reported in malaria vector species, including *Anopheles gambiae* sensu stricto (s.s.), *An. funestus* and *An.* a*rabiensis* as a result of wide scale use of mosquito insecticides. This has been reported in at least 64 malaria endemic countries, with pyrethroids resistance reported in 27 countries in sub-Saharan Africa

(WHO, 2011). In Kenya, susceptibility tests against pyrethroids have been done in the Central and Western Kenya that have shown that *An. gambiae* s.l. have developed phenotypic resistance against pyrethoids (Ngala *et al*., 2015, Ochomo *et al.,* 2013, Stump *et al.,* 2004). However, the mechanisms involved in insecticide resistance in the Central Kenya population have not been tested. There are two main mechanisms of insecticide resistance that have been reported. Target site resistance (knockdown resistance, *kdr*) caused by a mutation in the voltage gated sodium channel from leucine to phenylalanine (L1014F) commonly known as the West African *kdr* and leucine to serine (L1014S) commonly referred to as East African *kdr*. L1014S was first reported in Western Kenya in 2000 (Stump *et al*., 2000) while L1014F has not been reported in Kenya. These reports have originated from Western Kenya with no reports of *kdr* from Central and Coastal Kenya. Metabolic resistance has been reported to be caused by over-expression of metabolic enzymes glutathione-s-transferases, carboxylases and monooxygenases (Hemingway, 1983; McAllister *et al*., 2012; Nikou *et al*., 2003). In Kenya, there were reports of increased metabolic activity following the implementation of bed nets in the early 1990s (Vulule *et al.,* 1994) and thereafter another report of elevated esterases and oxidases in resistant mosquitoes from Bungoma in Western Kenya (Ochomo *et al*., 2013).

2.4 Malaria Prevalence in Kenya

Malaria, a mosquito-borne infection caused by *Plasmodium* parasites, causes a significant burden of disease, both globally and regionally (Murray *et al*., 2012). Malaria is transmitted from an infective bite of female *Anopheles* mosquito. In regions with high malaria transmission, children under 5years are greatly affected. In Kenya, malaria is endemic at the Coast and the Lake region with the Coast region having malaria transmission all year round with a prevalence of less than 5% (PMI, 2014; KMIS, 2010). The Central Kenya region is termed a low risk area with little or no malaria transmission. After the initiation of Roll Back Malaria Program by the World Health Organization (WHO), several countries outside the tropics have successfully eliminated

malaria. In the tropics however, malaria has persisted and thus WHO has intensified the fight against malaria in these regions focusing on vector control measures.Malaria vector control remains the main and most effective intervention strategy in malaria control programs. Kenya has conducted a massive distribution of ITNs country wide with priority given to the endemic areas of the Lake region and the Coast. On average, by 2009, 48% of all Kenyan households own at least one ITN with those in low risk areas having the lowest percentage ownership (KMIS, 2010). At the Coast, ITN ownership is estimated at 62% while in Central Kenya it is estimated at 35% (KMIS, 2010). In the low risk areas like Mwea, environmental management is mainly used as the main vector control strategy (KMIS, 2010).

2.5 Malaria Vector Control Strategies

Malaria causes significant economic and social burden, both globally and regionally (Murray *et al*., 2012). Due to the high rates of morbidity and mortality caused by this vector- borne disease in Sub-Saharan Africa several IVM measures have been adapted to try and curb the menace (WHO, 2006). The current trend in malaria vector control is the adoption of IVM strategy which involves a systemic approach to planning and implementation of vector control measures. IVM is the targeted use of different complementary vector control methods either singly or in combination advocated globally as a strategy to prevent or reduce human-vector contact and thereby reduce or interrupt malaria transmission cost-effectively (WHO, 2006). Much has been done to prevent mosquitoes from feeding on humans through reduction of human-vector contact thus lowering malaria incidences (Mabaso *et al*., 2004; Hawley *et al.,* 2003) in Sub Saharan Africa where malaria had previously been endemic (Gimnig *et al*., 2003). The emphasis has been concentrated on the control of adult mosquitoes including the use of insecticide treated nets (ITNs), indoor residual spraying (IRS) and long-lasting insecticides treated nets (LLITNs) (Sharp *et al*., 2007; Hanson *et al*., 2003). However, other control methods targeting the various stages have also been implemented including environmental management and larval control (Service, 1996, Killeen *et al*., 2002).

Malaria vector control remains the main and most effective intervention strategy in malaria control programs (Feachem *et al*., 2009).

2.5.1 Environmental Management

Environmental management is an effective and long-term solution for malaria vector control. It involves the elimination of favorable habitats for larval survival by draining or filling mosquito breeding sites with rubble or sand (Service, 1996). However, this may not be applicable to extensive lands (WHO, 1985) and in large irrigated areas. It is also impossible to fill in all the scattered, small and temporary collections of water (Service, 1996). The approach is also labor intensive and costly. This control method however, has not been widely adapted in Kenya.

2.5.2 Larval control

Studies have shown that mosquito larval control is very effective. However, not much consideration is given to it as a strategy for reducing malaria transmission (Killeen *et al*., 2000a, 2000b, Killeen *et al*., 2002). Larval control targets the pre-mature stages of mosquitoes and is effective because the larvae are killed before they get into human habitats (Killeen *et al*., 2002). This is easier because the larvae are still less mobile and are confined in a smaller area (Killeen *et al*., 2002). Reductions in malaria infections due to larval control activities have been shown to be effective because larval control can be used to target mosquitoes feeding both indoors and outdoors before they emerge into adults unlike the use of ITNs and IRS which only target indoor feeding and resting mosquitoes (Utzinger *et al*., 2001). This control method employs the use of biological agents incorporated in microbial larvicides (Imbahale *et al.,* 2012) and larvivorous fish (Walshe *et al*., 2013). However, this method is costly and labor intensive as the identification of breeding grounds have to be continuously monitored.

2.5.3 Adult vector control

The current malaria control strategies in Africa focuses mainly on reduction of vector population targeting the adult stages which are more active and with a high dispersal range thereby posing a greater risk in malaria transmission (Fillinger and Lindsay, 2006; Kahindi *et al*., 2008). Adult vector control aims at reducing vector human contact.

Since the introduction of Dichlorodiphenyl Trichloroethane (DDT) in 1940s, much success has been experienced in the control of mosquitoes (Mellanby, 1992). DDT was widely used initially but it was banned in some countries due to its bioaccumulation capabilities (Chen and Rogan, 2003; Sadasivaiah *et al*., 2007; Bouwman *et al*., 2011). Other control methods such as use of mosquito repellants containing *N*,*N*-diethyl-3-methylbenzamide (DEET) were later introduced (McCabe *et al*., 1954). Other insecticides were thereafter introduced to be used for indoor spraying. These include organophosphates (Malathion, fenitrothion), carbamates (propoxur), pyrethroids (permethrin, deltamethrin, lambdacyhalothrin), and organochlorine (DDT, dieldrin) (WHO, 1993). The insecticides only protect the environment where they are sprayed, that is, they only protect from endophagic and endophilic mosquitoes (Padonou *et al*., 2012; Sharp *et al*., 2007). Pyrethroids use has been advised in most IRS programmes because they are highly effective with high knockdown rate and low mammalian toxicity (WHO, 1993; Hemingway *et al*., 2004; Liu *et al*., 2006). Insect repellents reduce human-vector contact (Das *et al.*, 2003) by preventing the mosquito from landing on humans especially against outdoor biting mosquitoes. Some vector control methods like the IRS and ITNs act in combination by killing the vector and as a repellants as well (Lengeler, 2004; Pluess *et al*., 2010).

Insecticide treated nets (ITNs) and long-lasting insecticides treated nets (LLITNs) have widely been used and have proven effective in reducing malaria transmission through reducing the longevity of the vectors and decreasing human-vector contact (Lengeler and Sharp, 2003). The use of ITNs has been demonstrated to be cost effective in the control of mosquitoes as compared to other vector control interventions (Hanson *et al*.,

2003) with pyrethroids being the recommended chemicals used in ITNs/LLITNs (Protopopoff *et al*., 2013).

2.6 Insecticide Resistance

Resistance has been defined as 'the developed ability in a strain of insects to tolerate doses of toxicants that would prove lethal to the majority of individuals in a normal population of the same species' (WHO, 1957). This can arise due to either change in the gene sequence in the target site, increased insecticide metabolic rates by the mosquitoes or behavioral changes of the vector (Hemingway *et al*., 2004). Insecticide resistance spreads from its focal point as a result of selection pressure caused by continued use of a single class of insecticide.

Most of these vector control strategies involve the use of chemicals which are xenobiotics (Hemingway *et al*., 2002). Insecticide resistance, especially against pyrethroids which are the major class of chemical insecticide used on all approved LLITNs and in most IRS programme worldwide (WHOPES, 2011) has been confirmed in some parts of sub Saharan Africa in the mosquitoes. Should this be allowed to spread to other parts where resistance has not been reported, it would threaten the sustainability and operational impact of IVM programmes.

In Africa, insecticide resistance has been reported to be widespread in West Africa (Yawson *et al*., 2004; Diabate *et al*., 2002; Okoye *et al*., 2008; Corbel *et al*., 2007). There has also been evidence of resistance to some insecticides in various regions that seem to be localized to specific regions. In Mozambique, for instance, *An. funestus* s.s. remained fully susceptible to DDT and malathion despite a high level of pyrethroid (lambda-cyhalothrin) resistance in *An. funestus* s.s. populations in Southern Mozambique (Casimiro *et al*., 2006). In South Africa, *An. funestus* was found to be resistant to pyrethroids (Hargreaves *et al.*, 2000, Nikou *et al*., 2003) while in Tanzania (Kulkarni *et al*., 2006) and Sudan (Abdalla *et al*., 2014) *An. arabiensis* was shown to have developed resistance to pyrethroids. In Kenya, earlier studies found presence of

low level of the knock down resistance (*kdr*) gene in some parts of the country (Vulule *et al*., 1994; Kamau *et al*., 2007; Stump *et al*., 2004) indicating the presence of insecticide resistance in major malaria vectors. Recent studies indicate a reduced susceptibility to pyrethroid insecticides in *An. gambiae* sensu latu (s.l.) in Western Kenya (Kawada *et al*., 2011; 2011b; Ochomo *et al*., 2013; 2014).

As a result, there is need to continuously monitor resistance against all the classes of insecticides and understand the different mechanisms responsible for the widespread levels of resistance. If resistance is observed, another class of insecticides with a different mechanism of action against the mosquito vectors should be deployed. This will keep malaria incidences in check and not jeopardize the efforts so far put forth towards the reduction of malaria incidences (WHO, 1992; Krogstad, 1996).

2.6.1 Resistance Mechanisms

Two main mechanisms of insecticide resistance have been reported (Hemingway and Ranson, 2000; Corbel *et al*., 2007). The first is propelled by changes at the insecticide target site resulting in mutations (knock down rate, *kdr* mutations) (Figure 2-2) (Corbel *et al*., 2007). Pyrethroids and DDT insecticides act against the sodium ions (Na^+) channels, disrupting their operation (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000). Acetylcholinesterases on the other hand are the target for organophosphates and carbamantes action (Eldefrawi, 1985). Another mechanism is through increased rate of insecticide metabolism (Hemingway and Ranson, 2000). The rate of insecticide metabolism can be increased due to changes in enzyme composition due to overproduction of the enzyme or alteration in the catalytic activity of the enzyme (Matowo *et al.*, 2010). The main enzyme groups involved in insecticide resistance are the esterases, monooxygenases and glutathione-S-transferases (Hemingway and Ranson, 2000).

With the impact of insecticide resistance on ITNs not being clear yet, the different mechanisms of resistance have been studied in different regions where resistance have

been seen (Vulule *et al*., 1999). In Kenya, metabolic resistance (Vulule *et al*., 1999; Ochomo *et al*., 2013; 2014) and voltage-gated Na^+ channel knock down resistance (*kdr*) (Ochomo *et al*., 2013) to permethrin has been found in *An. gambiae*.

Permethrin resistance associated with target site insensitivity, *kdr*, has arisen independently at least twice in this species. Widespread permethrin resistance in West Africa is due to a leucine-phenylalanine substitution at position 1014 of the sodium channel gene (L1014F *kdr* allele), in the S6 hydrophobic segment of domain II (Martinez-Torres *et al.*, 1998). A different mutation at the same amino acid position, causing a leucine-serine substitution (L1014S *kdr* allele), is associated with permethrin resistance in *An. gambiae* from Kenya (Ranson *et al*., 2000).

Development of resistance has been shown to occur in some species but not in others (Ramphul *et al*., 2009). With resistance being a constantly evolving process that needs to be constantly monitored for better management and control, the current study determined the distribution and mechanisms on insecticide resistance in malaria vectors in Mwea and Kwale due to continuous use of agricultural pesticides and sustained use of ITNs/ LLITNs in the study sites respectively.

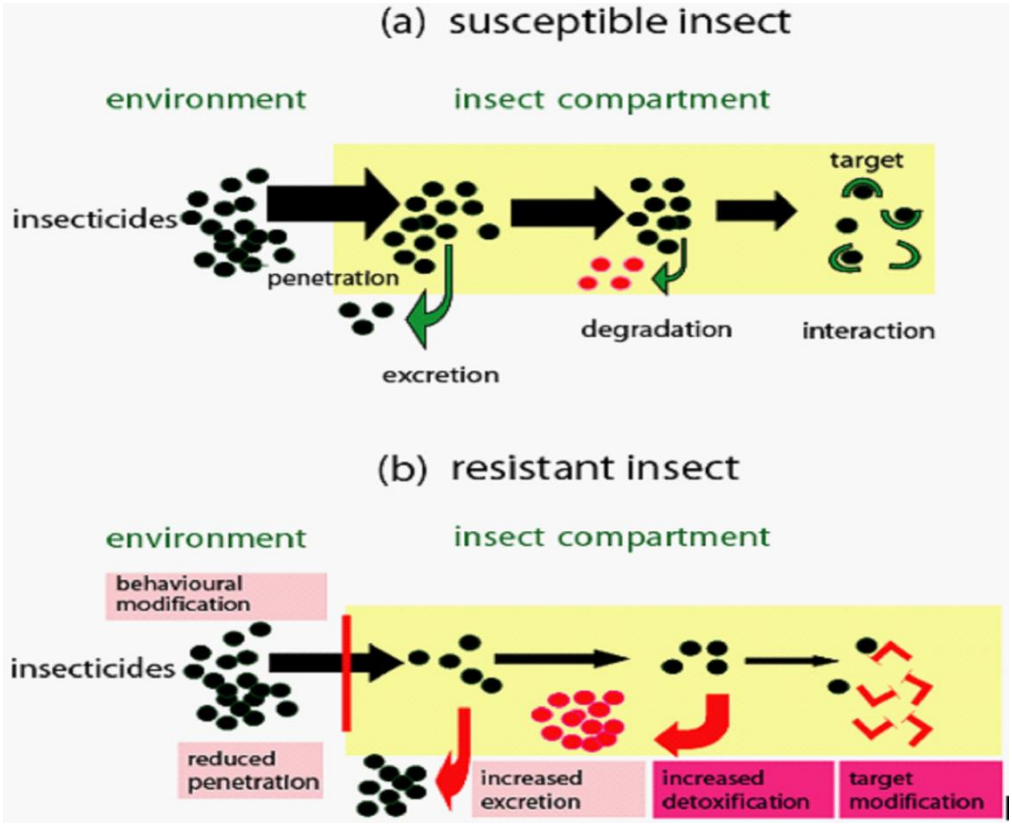


Figure 0-2: Mechanisms of resistance (Adapted from Lapied *et al*., 2009).

(a) When a susceptible insect is exposed to an environment with an insecticide, the insectide molecules penetrate its body compartment. Some of the insectide is excreted, others degraded then the rest is bound at the target site thus the insect dies

(b) When a resistant insect is exposed to an environment with an insecticide, some of the insectide molecules penetrate its body compartment. The insect intensifies its excretion and detoxification processes to reduce the amount of insectide molecules that get to the target site where however it does not bind.

2.6.2 Monitoring Insecticide Resistance

Over the years, WHO has produced and published several guidelines and instructions for investigating the presence of insecticide resistance, including the use of a standardized bioassay technique in adult mosquitoes (WHO 1981a; 1981b; 1998, 2013). Currently, WHO bioassay kit is the recommended kit for testing development of resistance (WHO, 1970) in any species of mosquito vector, however these kits are very expensive and their use cannot be implemented in the detection of low frequency resistance within a vector population (Brogdon, 1989; WHO 2013). This test is based on the time taken for the insecticide to penetrate the vector's body compartment and get to the target site and cause death. This is usually the initial sign that the insecticide is losing its effectiveness against the mosquito vector. Resistance is assumed to have developed if some of the mosquitoes that were initially exposed to the insecticide survive the test after the test time. Generally, WHO, (2013) recommends that insecticide resistance is characterized as follows: Susceptibility is thus seen when the mortality is recorded to be between 98% - 100%. Mortalities less than 98% are a representation of a possibility of resistance development and further tests should be done to confirm. Mortality less than 90% is evidence of resistance in the test species (WHO, 2013).

Susceptibility to all the four insecticide classes (organophosphates, organochlorine, carbamates, and pyrethroids) ought to be monitored frequently. Insecticide resistance management strategies must be implemented before the mechanism in action becomes common and stable in the population; otherwise, the gene will not recede. These resistance management strategies include: rotations of insecticides, use of interventions in combination and mosaic spraying. Potential future strategies include use of mixtures (WHO, 2012). In some settings, resistance management strategies may be implemented in the broad context of integrated vector management. If nothing is done and insecticide resistance eventually leads to widespread failure of the insecticides, the progress achieved so far in reducing the burden of malaria would be lost. This would result in

vector management failure and reduced effectiveness of malaria control resulting in increased malaria incidences of malaria morbidity and mortalities.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

This study was part of the bigger study on vector behavior that was aimed at evaluating the variations in malaria vector behavior after sustained vector control interventions. The study was conducted in selected villages in Mwea and Kwale where mosquito samples were collected. The bioassay experiments were conducted at the Centre for Biotechnology, Research and Development (CBRD), Molecular Biology and Ecology laboratory in Kenya Medical Research Institute (KEMRI), Nairobi and the Division of Vector-Borne Diseases (DVBD), Msambweni for Mwea and Kwale samples respectively. Additional laboratory tests were carried out in KEMRI, Nairobi and PCR laboratory at the Centre for Disease Control (CDC), Kisumu for all the study areas.

3.1.1 Mwea sub-county

Mwea sub-county (0°45'0" S and 37°28'60" E) is located in Kirinyaga County in Central Kenya at the foot of Mount Kenya. This area is well known for its vast practice in rice irrigation producing up to 50% of Kenyan rice. The Mwea Rice Irrigation Scheme (MRIS) is located approximately 120km, North of Nairobi with an altitude of 1000-1200 meters above sea level. This area is an agricultural area with over 50% of the area being used for the cultivation of irrigated rice and a large proportion (over 70%) of residents being small scale farmers. The region experiences an annual temperature range of between 12°C and 26°C, humidity of 80% and annual precipitation of about 1250mm. Mwea experiences two rainy seasons; the long rains in March to May and the short rains in October to December. The communities in this area live in houses made of iron sheet roofs with mud or stone walls (Figure 3-1B).

The main mosquito species in Mwea have been documented to be *An. arabiensis* (Mutero *et al.*, 2004; Mwangangi *et al*., 2010; 2006; Muriu *et al*., 2008; Mutero *et al.*,

2000; Chen *et al*., 2006;) with *An. funestus* being rare in this region (Muturi *et al*., 2009).

The study was done in 3 villages in the former Mwea sub-county; Karima, Kiamaciri and Murinduko. Karima is a village within Mwea irrigation scheme and is largely covered with paddy regions of cultivated rice for commercial purposes. The area is characterized by presence of clay soil which greatly supports rice growing. The population also keeps some cattle. Kiamaciri is approximately 5km from the rice irrigation scheme. However, a considerable amount of land is used for rice cultivation for domestic use. Murinduko, approximately 20km from Mwea Irrigation Scheme, is a village with sloppy topography and does not allow for the cultivation of rice. This village is at the periphery of the Mwea and boarders Embu County to the east. The population in Murinduko practices subsistence farming of maize, beans, bananas and vegetables. However some rice is grown along river valleys like River Kii and small streams.

3.1.2 Kwale County

Kwale County (4° 11' 0" S, 39° 27' 0" E) is situated along the coast of Kenya, approximately 40km south of Mombasa with an altitude of 100-462 metres above sea level. The county borders Tanzania to the South West, Taita Taveta to the West, Kilifi to the North, Mombasa to the North East and the Indian Ocean to the East. The county covers a total surface area of 8,270.2 km^2 with a population of 649,931 people as indicated by the 2009 Kenya population and housing census. The main sources of livelihood include mixed farming, livestock keeping, fishing and formal employment mainly in the tourism sector. The area is characterized by sandy loam soils required by the crops grown here. The locals in this region practice subsistence farming of cashew nuts, coconuts, fruits and sisal.

Kwale experiences hot and dry weather between January and April and cool weather between June and August with two rainy seasons. The short rains occur between

October and December while the long rains are usually experienced between March and July. The average amount of temperature and rainfall received is 24.2^{o}C and 400-1200mm per year respectively. The climate is generally tropical humid due to the high amounts of humidity that originate from the Indian Ocean.

The major malaria vector species in Kwale are *Anopheles gambiae* and *Anopheles funestus* which occur all the year round but their numbers increase during the rainy seasons (Mutuku *et al*., 2011; Mbogo *et al*., 2003).

The study was done in Marigiza (Msambweni Sub-county), Gwadu (Kinango Sub-county) and Kidomaya (Lunga Lunga Sub-county) villages in Kwale County. The human population in these villages mainly lives in stick- and mud-built houses with coconut –leaves thatched roofs (Figure 3-1A). Homesteads are scattered and separated from one another by agricultural land. Marigiza village is located on the interior parts of the Indian Ocean. The village is characterized by the presence of sandy soil with large coconut plantations. Gwadu village is located in the interior rural parts of the coastal mainland and is a representation of a hilly semi-arid inland part of the county. The population cultivates maize for livelihood. This region is arid with few mango trees and no coconut plantations. The inhabitants keep small herds of cattle. Kidomaya is located along the shores of the Indian Ocean. The human population is mainly agriculturalists, planting maize, green grams, beans and other grains. They also keep large herd of cattle which are used to plough farm lands. There are some coconut plantations and the region is characterized by black loam soils.

3.2 Sampling

This study was an experimental study and specifically, a randomized control study. Sampling was done in Mwea in September 2014, just before the commencement of the short rains and in Kwale, in December 2014 immediately after the short rains. Purposive sampling was used so as to collect as many adult and larval mosquitoes as possible because the main purpose of the study required a large number of the *Anopheles*

mosquitoes; at least 75 mosquitoes, per village per insecticide to be tested. The sampling sites were selected from previously identified productive sites as had been seen in the vector behavior project which this was part of.

3.2.1 Adult collection

Adult mosquitoes were collected from selected houses in the study areas using window exit traps, prokopack and indoor aspiration. Sampling was done from 5 houses in each village while selecting one house per homestead. Mosquitoes were trapped overnight using the window exit traps and in the same houses, indoor aspiration was done followed by prokopack aspiration. The *Culicine* species were discarded.

A **B**

Figure 0-1: House types in (A) Kwale and (B) Mwea

3.2.1.1 Window exit traps

Window exit traps are wooden or wired frames covered with a netting material. These traps are made to fit on windows of houses and they measure approximately 75cm x 75cm (Figure 3-2 A). These are aimed at capturing mosquitoes that are exiting the house either after a blood meal for outdoor resting or for oviposition. These traps were tied every evening (6:00pm) onto windows where humans would sleep that night and the occupants requested not to close the window or cover the entry to the trap. Mosquitoes were collected every morning (between 6:00am and 7:00am) from the traps before indoor aspiration was done in the same houses and in additional houses. The collection was done between 7:00am and 10:00am. From the window exists of each selected house, the *Anopheline* mosquitoes were mouth aspirated (Figure 3-2 B) into paper cups and fed on 6% glucose soacked in cotton wool during transportation to the laboratories.

3.2.1.2 Day resting indoor collection

This is a combination of several methods used to collect mosquitoes that rest inside the houses (indoors), either after a blood meal or in-wait for a blood meal. These include mouth aspiration and prokopack collection.

A **B**

Figure 0-2: Window exit trap (A) and mouth aspiration (B) from a window exit trap

3.2.1.2.1 Mouth aspiration

This is an oral aspiration collection method that uses an aspirator made using a special glass tube and rubber. The aspirator is used to gently suck mosquitoes from their resting positions on the walls into the tube then into paper cups. For the mouth aspiration, mosquito vectors were visually searched from all possible resting places in the houses using torches. *Anopheline* mosquito vectors were morphologically identified and orally aspirated into paper cups covered with a net at the top, fed with 6% glucose soaked in cotton wool and taken to the laboratory for further processing.

3.2.1.2.2 Prokopack aspiration

A prokopack aspiarator is a hand-held mechanical aspirator that uses pressure to suck mosquitoes into the paper cup fitted on it. This collection method collects all insects and sorting has to be done immediately to discriminate against the different mosquito species and to remove mosquito predators that might have been trapped alongside the mosquitoes. Prokopack aspiration was done after mouth aspiration as it collected mosquitoes that were not visible or were far from the reach of the collector. Each house used a different paper cup and sorting was done immediately.

3.2.2 Larval Collection

Larval collection was done by the use of standard dipping technique using a standard dipper of 350ml (WHO, 1975). Larvae were collected from rice paddies and canals in Mwea (Figure 3-3 A) and potential habitats (water pools and drainages) in Kwale (Figure 3-3 B). These sampling sites had been previously identified from earlier collections of the vector behavior project. Purposive sampling was done so as to collect as much larvae as possible. *Anopheline* larvae were sorted to separate them from other larvae species and predators using dropper pippetes. These were transported to the laboratory insealable and labeled whirl paks, indicating the place and date of collection. In Karima, 2 larval sites were picked; 1 right in the village and the other at the periphery of the village. This was because the the rice paddies in the village had been applied with

fertilizers and drained so there were few larvae. In Kiamaciri, most of the paddies had been drained and so most of the sampling was done in the main irrigation canal. The coordinates of these sites were taken and recorded in the field data forms. These samples were then taken to Kimbimbi Sub County Hospital laboratory for rearing to be used in the bioassay tests. In Kwale, *Anopheline* larvae were collected from all potential habitats within the villages and taken to Msambweni District Hospital for rearing and bioassay tests.

A **B**

Figure 0-3: Larval collection from rice paddy in Mwea (A) and water pool in Kwale (B).

3.3 Initial Processing of the Samples from the Field

Mwea samples were taken to Kimbimbi Sub County Hospital laboratory while Kwale samples were taken to Msambweni District Hospital for further processing, sorting and identification. In the laboratories, every adult mosquito was individually morphologically identified as either *An. gambiae* or *An. funestus* and the female mosquitoes were sorted according to their abdominal physiological status as either blood fed, unfed, gravid and half gravid. All the males and unfed female *Anopheles* mosquitoes from each village were put in a single cage (7cm by 8cm) and allowed to mate in preparation for blood feeding. The fed, gravid and half gravid female mosquitoes were placed singly in 50ml centrifuge tubes stuffed with cotton wool then layered with Whatmann filter paper soaked in tap water to provide a conducive environment for them to oviposit. The top to these tubes were sealed with netting material secured with rubber bands. These were then stood on 50mls eppendorf tube racks and the specimens were then fed daily with 6% glucose. Each specimen that was gravid, half gravid or fed was assigned a unique code to capture the collected site, house number, date of collection and collection method.

The larvae were sorted, removing all the *Culicine* larvae and predators like tadpoles. The *Anopheline* larvae were put in larval trays and and the water from the source habitat sieved using a cloth to sieve out predator eggs that might be present in the water. The larvae were then fed with Sera® (Sera North America, Inc, PA, U.S.A.) larval food once a day.

3.4 Transportation of the Samples

Transportation of samples was only done from Kimbimbi Sub County Hospital laboratory, Mwea to KEMRI insectary in Nairobi. Bioassay tests on the Kwale samples were done in Msambweni District Hospital, Division of Vector Borne Diseases (DVBD) insectary. The carcases after the bioassays were put individually in correctly labeled 1.5ml microfuge tubes containing 3 or 4 pellets of anhydrous calcium sulfate, Drierite®

(anhydrous calcium sulfate, W.A. Hammond Drierite Company, Xenia, Ohio, U.S.A), lined with cotton wool. These were then transported to KEMRI, Nairobi Molecular Biology and Ecology lab for further molecular analysis.

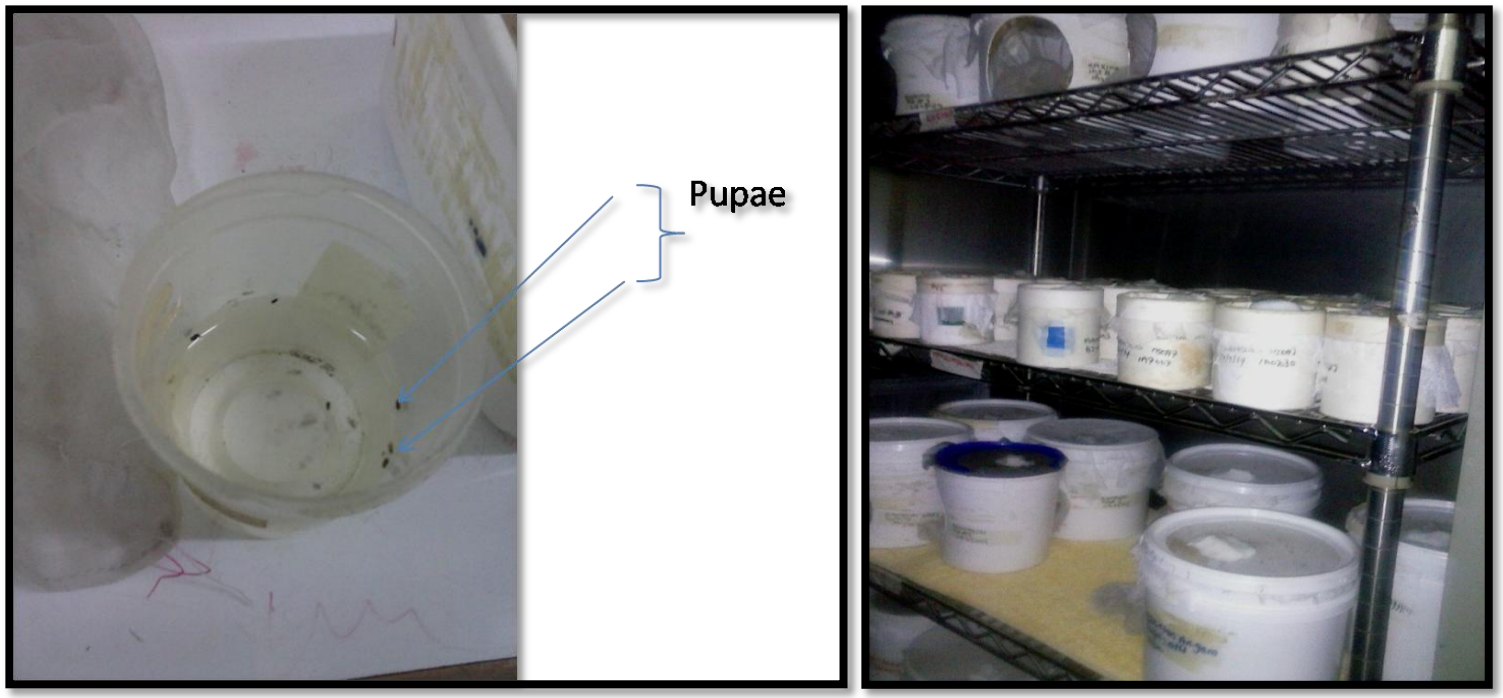
For the samples from Mwea, the adult mosquitoes in cages and ovipositioning tubes were packed in cool boxes and fed with 6% glucose soaked in cotton wool during transportation to the KEMRI, Nairobi insectary. The larvae were placed in water bottles that were not fully corked, packed in cool boxes and brought to KEMRI, Nairobi insectary for rearing until they emerged into adults.

3.5 Experimental Techniques

3.5.1 Rearing and Maintenance of mosquitoes in the Insectary

3.5.1.1 Adult maintenance

The adults were placed in the adult chambers on arrival in the insectary (Figure 3-4), maintained at temperatures and humidity of between 25^{o}C - 27^{o}C and 84% - 87%. The adults were fed on 6% glucose soaked in cotton wool. The ovipositioning tubes were checked daily for eggs on the filter paper and if present, the eggs were flooded in larval trays and placed in the larval chambers, maintaining the same numbering codes until emergence into adults.



A **B**

Figure 0-4: Adult mosquito rearing in the insectary

Collected pupae (A) before being kept in the emergent cages (B)

3.5.1.2 Larval rearing

The field collected larvae were placed in larval trays in the larval chambers (Figure 3-5) on arrival in the insectary. Daily, the larvae were fed using fish food, Sera® and the temperature in this chamber maintained between 28°C -31°C while the humidity was maintained betweeen 80%-85%. The larvae hatched from the flooded eggs were also monitored daily alongside the field collected larvae and pupae were collected and transferred into plastic cups in emergence cages and paper cups, where they emerged into adults (Figure 3-4). These mosquitoes that emerged in the insectary (F_1 and F_0) were used in bioassays and enzyme assays. Dead mosquitoes were removed daily from the cages and the 50mls centrifuge (oviposition) tubes using forceps and preserved in 1.5ml eppendorf tubes with 3 or 4 pellets of anhydrous calcium sulfate, Drierite®, lined with cotton wool for species identification and sporozoite analysis. After each adult individual field collected mosquito was preserved, the forceps was cleaned using 70% ethanol to avoid cross contamination. These specimens maintained the same numbering codes as when they were still alive and the field collected larvae were assigned an identification code on emergence.

BALB/c laboratory mice were use to blood feed the field collected unfed mosquitoes contained in the cages where they were mixed with the male mosquitoes. The blood fed mosquitoes were mouth aspirated from the cages and placed individually in ovipositioning tubes. After 3 blood feeding occasions, the males and the unfed females were killed by freezing at -20°C and preserved for species identification.

Pupae from the field collected larvae were collected every morning and evening, counted and recorded before being placed in emergence cages. These were used for bioassays and enzyme analysis.

A **B**

Figure 0-5: Rearing larvae in the insectary

3.5.2 Bioassays

Two to five day old adults from field collected larvae (F_0) and F_1 progeny of field collected females were used in bioassay tests in accordance with WHO resistance testing guidelines (2013). Susceptibility to deltamethrin and permethrin were tested for all mosquitoes from Mwea and Kwale. Additionally, susceptibility to DDT was only tested for samples from Mwea. At lest 50 mosquitoes from each study village were tested to include either *An. gambiae* or *An. funestus* per insecticide per village. Each test was accompanied by a positive and negative control whereby the positive control comprised of insectary reared susceptible *An. gambiae* s.s. Kisumu strain and the negative control were the field collected mosquitoes. The positive control exposure tubes were lined with diagnostic concentrations of insecticide impregnated papers (0.05% deltamethrin, 0.75% permethrin and 4% DDT) while the negative controls were exposed to the bioassay control papers which were not impregnated with any insecticide. These papers were held firm on the walls of the tubes with rings. Resistance was determined using the recently revised WHO standard of classification (WHO 2013). There were between 15-25 mosquitoes, both male and female, in each WHO tube for a single test with at least 2 replicates depending on the available mosquitoes. Each exposure test was observed and knockdown recorded every 10 minutes for 60 minutes. A knocked down mosquito was recorded as one that after exposure to insecticide was morbid regardless of the number of legs still remaining (WHO, 2013). At the end of the exposure, the mosquitoes were transferred into a holding chamber and fed with 6% glucose (Figure 3-6). The final mortality was recorded 24 hours post exposure to insecticide. These susceptibility tests were done at recorded temperatures of between 24^{o}C – 27^{o}C and relative humidity of between 83% - 89%.

After recording the mortality at 24hrs post exposure, the live mosquitoes were aspirated into a separate paper cup and killed by freezing at -20^{o}C for 20minutes and then together with the dead mosquitoes, individually stored in correctly labeled 1.5ml microfuge tubes. The labels captured the collection site, date of bioassay, insecticide tested and

whether the mosquito was dead or alive after 24hrs. The percentage mortalities for the villages were calculated and the Abborts formula was used to correct for the mortalities in the negative control. The species of each specimen was then determined by PCR.

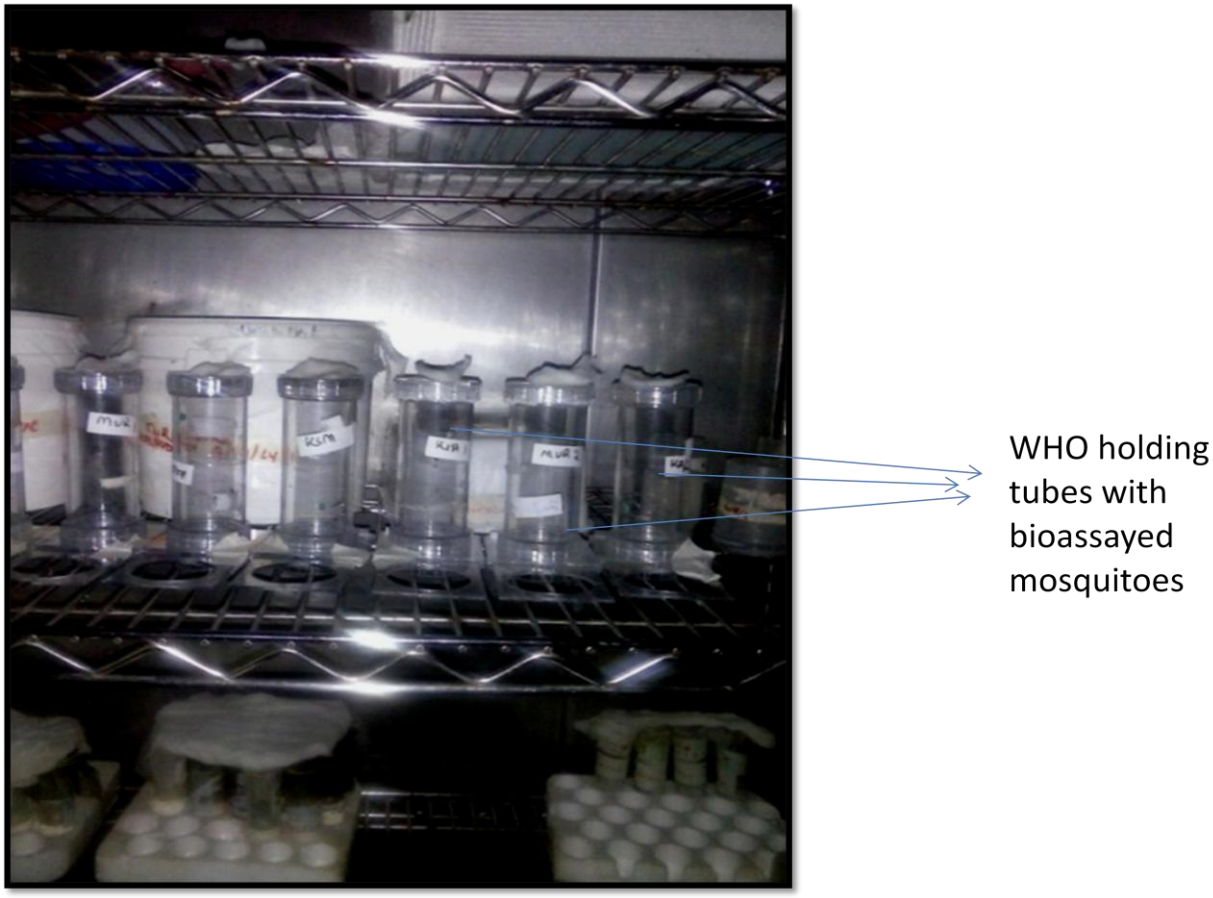


Figure 0-6: WHO Bioassay setup awaiting 24hr mortality reading.

3.5.3 DNA Extraction

All field collected specimen, *An. gambiae* s.l. and *An. funestus* s.l. were subjected to DNA extraction following Collins *et al.,* (1987) protocol. In Mwea, 415 were analysed while 714 samples were analysed from Kwale. Field collected female specimens that had dried were cut to separate the head and the thorax for sporozoite analysis from the rest of the body for species identification. The abdomen, legs and wings were subjected to DNA extraction using the alcohol precipitation technique. For the field collected males and the F_0 specimens, DNA was extracted from the whole mosquito. Each mosquito was put in a 1.5ml microfuge tube and ground using 100µl grinding buffer (Appendix1). The samples were then placed in 65^{o}C water bath for 30 minutes. 14µl potassium acetate (58.89g of potassium acetate in 75ml of distilled water) was added and immediately placed in ice for 30 minutes. The samples were then centrifuged at high speed; 14000 revolutions per minute (rpm) for 10 minutes at 4^{o}C. The supernatant was carefully transferred in a new and labeled microfuge tube and the precipitate discarded. 200µl of 95% ethanol was added and the samples were stored overnight at -20^{o}C. The samples were then microfuged at 14000 rpm for 20 minutes at 4^{o}C. Ethanol was poured off and 200µl of 70% ethanol added to wash the pellets then poured off. Another washing was done using 200µl of 95% ethanol which was then poured off and the tubes inverted and allowed to air dry overnight. The pellets were suspended in 100µl PCR water and vortexed then stored at -20^{o}C awaiting species identification by PCR.

3.5.4 Species Identification

The morphologically similar members of the *An. gambiae* s.l. and *An. funestus* s.l. species complex were further distinguished using ribosomal-DNA (rDNA) PCR (Scott *et al*., 1993). This was done on all DNA extracted field collected mosquitoes, both larvae and adults. This discrimination of the sub species within the *Anopheles* species complex was done to distinguish between *An. funestus* s.l. (*An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. rivulorum* Leeson, *An. parensis* and *An. leesoni* Evans) and *An. gambiae* s.l. *(An. gambiae* s.s., *An. arabiensis* and *An. merus)*. This PCR

is based on species-specific single nucleotide polymorphisms (SNPs) in the internal transcribed spacer region 2 (ITS2).

DNA amplification was done using 15µl reaction; 13µl master mix and 2µl DNA template. In every reaction there was provided 5.86µl PCR water, 3µl of 5x green reaction buffer which contains the blue and the yellow dye acting as the loading dye, 1mM $MgCl_2$, 100µl (25ρmole/µl) of each dinucleotides triphosphates (dNTPs) which were a composition of adenine (dATP), guanine (dGTP), cytosine (dCTP) and thymine (dTTP) and 0.25 units of Taq polymerase. 625.2 pmole/µl of GA primer, 579.8 pmole/µl of UN primer and 607.0 pmole of AR primer was used. Bovine Serum albumin (BSA) was used to enhance the yield PCR amplification and was prepared by dissolving of 0.01g albumin serum in 1ml of distilled water.

For samples from Mwea, the *An. gambiae* complex ribosomal DNA (rDNA) primers were GA: 5' CTG GTT TGG TCG GCA CGT TT 3', AR: 5' AAG TGT CCT TCT CCA TCC TA 3', UN: 5' GTG TGC CCC TTC CTC GAT GT 3' (Scott *et al*., 1993). The basepair (bp) length of *An. arabiensis* is 315 and 390 for *An. gambiae.* These regions amplified between the UN primer and the species specific primers. *An. gambiae* s.l. amplification was done in a GeneAmp PCR system 9700 Thermo cycler version 3.05 which was set as follows: Initiation 5minutes at 94°C, 30 seconds of denaturing at 94°C, 30 seconds of annealing at 50°C, 30 seconds of extension at 72°C, 10 minutes of auto-extension at 72°C and the cycle was repeated 30 times. This process took about 1hour 30minutes after which the machine goes to 4°C until the samples are removed from the machine. For the *An. gambiae* s.l. from Kwale, *An. merus* primer was included in the master mix (ME: 5' TGA CCA ACC CAC TCC CTT GA 3') whose bp length is 466.

For *Anopheles funestus* complex, a 15µl reaction was performed under the following PCR conditions: 13µl master mix and 2µl DNA template inclusive of 5.35µl PCR water. 3µl of 5x green reaction buffer which contains the blue and the yellow dye acting as the loading dye. 100µl (25ρmole/µl) of each dNTP, 1mM $MgCl_2$, 0.25 units of GoTaq polymerase. *Anopheles funestus* complex ribosomal DNA (rDNA) internal transcribed

spacer 2 diagnostic primers were: VAN: 5' TGT CGA CTT GGT AGC CGA AC 3', PAR: 5' TGC GGT CCC AAG CTA GGT TC 3', RIV: 5' CAA GCC GTT CGA CCC TGA TT 3', LEES: 5' TAC ACGGGC GCC ATG TAG TT 3', FUN: 5' GCA TCG ATG GGT TAA TCA TG 3' and UN: 5' TGT GAA CTG CAG GAC ACA T 3' (Koekemoer *et al*., 2002). The nucleotide basepair lengths of these sequences were *An. vaneedeni* (587bp), *An. parensis* (252bp), *An. rivulorum* (411bp), *An. leesoni* (146bp) and *An. funestus* (505bp). These regions amplified between the UN primer and the species specific primers. *An. funestus* s.l. amplification was done in a BIO-RAD T100^{TM} thermal cycler which was set as follows: initiation took 4 minutes at 94°C, 30 seconds of denaturing at 94°C, 30 seconds of annealing at 58°C, 45seconds of extension at 72°C, 7 minutes of auto-extension at 72°C and the cycle was repeated 30 times. This process took about 1hour 30minutes after which the machine went to 4°C until the samples were removed from the machine.

The amplification was then scored using 3% agarose gel electrophoresis and visualized under ultra violet (UV) radiation. The agarose gel was prepared with ethidium bromide (EtBr) to enhance visualization. Identification of *An. funestus* s.l. species from both study sites and *An. gambiae* s.l. from Kwale was done based on DNA size marker while that of *An. gambiae* s.l. from Mwea was done by comparison to previously identified *An. gambiae* s.s. and *An. arabiensis* specimens that were included in the PCR amplification and electrophoresis.

3.5.5 Agarose Gel Preparation and Electrophoresis

On completion of the PCR reaction, 3% agarose gel was prepared by heating 1.5g agarose in 50ml Tris boric acid EDTA (TBE) buffer (Appendix2) in a microwave for 45seconds. After heating the agarose in TBE buffer, the solution was allowed to cool but not allowed to solidify, 1.3µl of ethidium bromide was added and mixed by swirling before it was poured into the electrophoresis tank which had been prepared and the combs put in place. The solution was left to solidify after which the combs were removed. The tank was flooded with electrophoresis buffer (TBE) and the sample

amplicons loaded into the wells. Where the DNA size marker was used, the 100bp DNA ladder was prepared using 4µl PCR water, 1µl 6x blue loading dye and 1µl DNA ladder giving a total volume of 6µl. This was mixture was vortexed and approximately 5µl was added to designated wells in the gel. The tank was connected to the mains and allowed to run at between 90-105volts for 15-20 minutes. The fragments were visualized under ultraviolet illuminator and scoring done.

For a set where previously identified amplicons were included, the samples were scored as they aligned with their respective species. Every band that was in line with previously identified *An. arabiensis* was scored as *An. arabiensis* while that which was in line with *An. gambiae* s.s. was scored as *An. gambiae* s.s. The amplicon fragments that were run with the DNA ladder were also scored against the DNA ladder fragments.

3.5.6 Sporozoite ELISA test

The protocol used was sandwich ELISA method by Wirtz *et al.,* (1987). The head and thoraces of individual field collected female adult mosquitoes were placed into labelled 1.5ml microfuge tubes. 50µl of blocking buffer (BB) and NONIDET P-40 (NP-40) was added into each microfuge tube and the samples ground using pestles. Blocking buffer was prepared as in Appendix3 while BB and NP-40 was prepared by adding 5µl of NP-40 to 1ml of BB. After grinding, the pestles were rinsed with 200µl of BB. These samples were then stored at -20°C overnight. 50µl of monoclonal antibody (MAb) solution diluted in plain PBS was used to coat each well of the 96-well polyvinyl microtitre plate resulting in 0.2µg *Plasmodium falciparum* /50µl PBS in each well. This was covered with microplate sealers and incubated at room temperature for 30 minutes away from light. The mosquito triturates were removed from the freezer and allowed to thaw in readiness for testing. After 30 minutes the MAb solution was dumped by banging on paper towels and the wells were filled with 200µl of blocking buffer, covered and incubated for 1 hour at room temperatures away from the light. The blocking buffer was then dumped and 50µl of negative (BB) and positive controls were added in the first two column of the microplate respectively. To prepare the positive

control, the lyophilized positive control (5µg) was diluted with 1000µl blocking buffer resulting in the 10,000ρg/µl BB stock solution. 20µl (200,000ρg) of this stock solution was then transferred to vial I containing 1000µl BB (100ρg/µl BB). From vial 1000µl (1000ρg) is transferred to vial II containing 500µl of BB to give a final concentration of 2ρg/µl. The positive controls were made from vial II from where the positive controls are made by serial dilutions of 20µl *Plasmodium falciparum* (*Pf*) control in 1000µl of BB. These results into serial dilutions of 100ρg, 50ρg, 25ρg, 12.5ρg, 6.25ρg, 3.125ρg, 1.5625ρg and 0ρg of the positive control per 1000µl BB.

The mosquito triturates were then added to rest of the wells, each in its well. This was then incubated for 2hours and then the microplates were banged and the wells washed twice with 200µl PBS- Tween 20 (500µl of Tween 20 in 1 litre PBS) solution and banged to dryness. 50µl of MAb-peroxidase conjugate (for 1 microplate, add 10µl of conjugate to 5ml BB) were added to each well resulting in 0.05µg peroxidase conjugate /50µl BB and incubated for one hour at room temperature. The lyophilized MAb and the conjugate were diluted in 2ml of glycerol: distilled water (1:1) to make the stock solution which was stored at -20^{o}C. After one hour, the solutions were dumped and the plates washed thrice with PBS-Tween 20 solution, banging after every wash. 100µl peroxidase substrate (solution A, ABTS and solution B, hydrogen peroxide in the ratio of 1:1) was added to each well and incubated for 30 minutes. This procedure was done using a multichannel pipette. The samples were then assessed visually to check for positivity and then the optical densities read using an ELISA reader at 405nm. Positive samples were calculated as follows:

2*Mean of the OD of the negative samples

3.5.7 Determination of the frequency of *kdr* gene

DNA was extracted from all mosquitoes that were subjected to bioassay tests. The mosquitoes were subjected to conventional PCR to determine their species. Real time polymerase chain reaction was done on *An. gambiae* s.l. samples to detect the *kdr* gene.

This followed the Bass *et al*., 2007 protocols to test the West African and East African forms *kdr* mutations. DNA was extracted from the mosquito samples. The master mix was prepared by mixing PCR water, 2x TaqMan mix (TaqMan® Gene expression Master mix), forward and reverse primers, wild type probe (LL) and the *kdr* allele in question; either *kdr* east (SS) or *kdr* west (FF). For a 10µl reaction, 2.15 µl of PCR water was mixed with 5µl TaqMan mix, 0.2µl of forward primers, reverse primers and wild type probe each and 15µl of *kdr* allele. 1.5µl of the DNA sample was then added into the real time PCR tubes. 410µM of forward and reverse primers were used. The *kdr*-east probe sequence used was 5'-ACGACTGAATTT-3' while *kdr*-west probe sequence used was 5'-ACGACAAAATTT-3'. The wild type probe sequence used was 5'-CTTACGACTAAATTT-3'. Samples and controls were loaded on a 96-well PCR plate. The controls were loaded in the last four wells of the plate which consisted of FAM positive control, buffer, HEX positive control and non template control (NTC) respectively. FAM (blue) dye was used to detect the mutant allele while the HEX (green) dye detected the wildtype allele. The reporter dye used was ROX (red). The temperature profile was set as 95°C for 10minutes for initiation followed by 40 cycles of denaturation at 92°C (for *kdr*-east) or 95°C (for *kdr*-west) for 15 seconds and annealing at 60°C for 1minute on an Stratagene® MX3005 real-time PCR machine. HEX and FAM fluorescence dyes were captured at the end of each cycle and genotypes called from endpoint fluorescence using the MXPro software. *Kdr*-east reactions took approximately 1hour 20minutes while *kdr*-west reactions took approximately 1hour 15minutes.

3.5.8 Assessment of enzyme activities

The biochemical tests were used to test the altered enzyme activities in the *Anopheles* mosquitoes. This was used to determine the levels, activity and alteration of oxidases, non-specific β-esterases, glutathione- S- transferase (GST) and acetylcholinesterase following Brogdon *et al*., (1988) protocol. This was done using microplate assays for *An. gambiae* s.l. and *An. funestus* and TaqMan assays for *An. gambiae* s.l. The

mosquitoes were dissected to remove the legs for the body to be used in microplate analysis and the legs for TaqMan assays. For the microplate assays, each mosquito was homogenized using 100µl potassium phosphate (KPO_4) buffer. KPO_4 was prepared by mixing 6.6g dibasic KPO_4 and 1.7g monobasic KPO_4 in 1000ml of distilled water. The pH was adjusted to 7.2 and stored at room temperature. The mosquito homogenate was diluted with 900µl KPO_4 buffer. 500µl of this was further aliquoted in a different tube and further diluted with 500µl. The microplate optical density (OD) readings were done using a microplate reader with SoftMax Pro software.

3.5.8.1 Protein assay

The protein microplate assay was used to measure the amount of total proteins in each mosquito sample. It was used to correct for size differences between mosquitoes considering larger mosquitoes possibly have higher protein levels. 20µl of mosquito homogenate was put in each well of the ELISA plate in triplicate (i.e. A1, A2 and A3). Negative controls were added to wells G10, G11, G12, H10, H11 and H12. 80µl of KPO_4 buffer was added to each well. 200µl Protein dye reagent was then added in each well. The plates were read immediately with microplate reader at 620nm and the optical densities recorded. The Protein Dye Reagent was made by mixing 20ml Protein dye concentrate in 80ml distilled water and stored at 4^oC in a light proof bottle (aluminium foil covered bottle). To correct for the size differences in the mosquitoes, the following was done for each mosquito:

Corrected enzyme OD reading = Enzyme OD reading/ Protein OD reading

3.5.8.2 Elevated non-specific β-esterase assay

This assay was used to measure the levels of non-specific β-esterases present in the mosquito homogenate sample.100µl of the mosquito homogenate was pipetted in each well of an ELISA plate in triplicate (i.e. A1, A2 and A3). Positive and negative controls were added to each of 3 designated wells (G10, G11, G12 and H10, H11, H12

respectively) on each plate. 100µl of β-naphthyl acetate was added to each well and incubated at room temperature for 10minutes then 100µl of Dianisidine was added to each well and incubated for 2 minutes and ODs read at 540nm. β-naphthyl acetate was made by dissolving 56mg β-naphthyl acetate in 20ml acetone and 80ml KPO_4. This was stored at 4^oC. Dianisidine was prepared by dissolving 100mg of 0-dianisidine tetrazotized in 100ml of distilled water in a light proof bottle immediately before use. The color of this reagent was checked to ensure it was pale yellow, with amber colored reagent discarded. Positive controls were prepared by making Esterase stock solution (50mg β-naphthyl in 10ml acetone and 90ml KPO_4). Aliquots of 1ml were put in 1.5ml microfuge tubes and frozen and covered with aluminium foil to keep off light. A standard solution was made from this by diluting the esterase stock in the ratio of 1:35 (35µl β-naphthyl stock, 1.2ml KPO_4 buffer). The dilution was also made by diluting the esterase stock in the ratio of 1:70 (i.e.17.5µl β-naphthyl stock, 1.2ml KPO_4 buffer). KPO_4 buffer was used as the negative control.

3.5.8.3 Oxidase reaction

This assay was used to measure heme peroxidase levels in mosquito sample. 100µl of mosquito homogenate, positive and negative controls were added in appropriate wells. 200µl of Tetramethyl-Benzidine Dihydrochloride (TMBZ) was added then 25µl of 3% hydrogen peroxide [H_2O_2] and the plates incubated for 5minutes before the ODs were read at 620nm. TMBZ was made by dissolving 50mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride (TMBZ) in 25ml methanol. After this solution has dissolved, 75ml of 0.25M sodium acetate (NaOAc) buffer was added. This solution was stored at 4^oC and the color change noted (colorless). A light blue solution was discarded. NaOAc buffer was made by mixing 83 ml of 3M NaOAc (408.1g of NaOAc in 800ml of water) with 900 ml distilled water. The pH was adjusted to 5 with glacial acetic acid and the volume adjusted to a final volume of 1 liter. This buffer was stored at room temperature. Positive controls were prepared by making oxidase stock solution (10mg Cytochrome-C (from bovine heart) dissolved in 100ml NaOAc). Aliquots of 1ml were put in 1.5ml microfuge

tubes, frozen and covered with aluminium foil to keep off light. A standard solution was made from this by diluting the oxidase stock in the ratio of 1:55 (i.e. 22µl cytochrome-C stock, 1.2ml KPO_4 buffer). The dilution was also made by diluting the oxidase stock in the ratio of 1:110 (i.e. 11µl cytochrome-C stock, 1.2 ml KPO_4 buffer). KPO_4 buffer was used as the negative control.

3.5.8.4 Glutathione-S-Transferase assay

This assay gives an indication of the level of Glutathione S-Transferase present in the samples.100µl mosquito homogenate was put in appropriate wells in triplicates. Wells G10, G11, G12, H10, H11 and H12 were left blank. 100µl of reduced glutathione was added then 100µl of 1-chloro-2,4'-dinitrobenzene (cDNB) was added. The ODs were read immediately (T_0) at 340nm. The plates were then incubated at room temperature then the ODs read after 5minutes (T_5) at 340nm. The T_0 readings were subtracted from the T_5 readings and these values were used for analysis. Reduced glutathione was made by mixing 61mg reduced glutathione with 100ml KPO_4 buffer and stored at 4^oC for 3-4 days. cDNB was made by dissolving 20mg 1-chloro-2,4'-dinitrobenzene (cDNB) in 10 ml acetone and 90ml KPO_4 buffer stored at 4^oC for 3-4 days.

3.5.8.5 Acetylcholine esterase assay

This assay was done to determine the amount of acetylcholine esterase present. 100µl mosquito homogenate was put in appropriate wells in triplicates. Negative controls were added to wells G10, G11, G12, H10, H11 and H12. 100µl of Acetylthiocholine iodide was added to each well. 100µl of Dithio-bis-2-nitrobenzoic acid (DTNB) was added to each well and the ODs were read immediately (T_0) and then after 10 minutes (T_{10}) at 414 nm. The T_0 readings were subtracted from the T_{10} readings and these values were used for analysis. Acetylthiocholine iodide was made by dissolving 75mg Acetylthiocholine iodide in 10ml of Acetone and 90ml of KPO_4 bufferand stored at 4^oC for 3-4 days. DTNB was made by mixing 13mg Dithio-bis-2-nitrobenzoic acid in 100ml KPO_4 buffer and stored at 4^oC for 3-4 days.

3.5.9 TaqMan Enzyme assays

This was done to determine the presence of elevated glutathione-s-transferase epsilon 2 (GSTe2), cytochrome P450 (CYP4J5) and carboxylesterase (CoE). DNA was extracted from the legs of An. *gambiae* s.l. that were taken through enzyme microplate analysis. DNA extraction was done using ethanol precipitation method before real-time PCR (qPCR) was done. The mastermix was prepared using PCR water, 2x sensimix II probe (Bioline) and primers (probes) for the specific enzyme being tested and this was done away from the light because the probes are light sensitive.

For a 10µl reaction, 3.875µl of PCR water was mixed with 5µl sensimix probe and 0.125µl of GSTe2, CYP4J5 or CoE probes. 1µl of the DNA sample was then added into the real time PCR tubes. Samples and controls were loaded on a 96-well PCR plate. The controls were loaded in the last four wells of the plate which consisted of FAM positive control, buffer, HEX positive control and non template control (NTC) respectively. FAM (blue) dye was used to detect the mutant allele while the HEX (green) dye detected the wildtype allele. The reporter dye used was ROX (red). The temperature profile was set as 95°C for 10minutes for initiation followed by 40 cycles of denaturation at 92°C for 15seconds and annealing at 60°C for 1minute on an Stratagene® MX3005 real-time PCR machine. HEX and FAM fluorescence dyes were captured at the end of each cycle and genotypes called from endpoint fluorescence using the MXPro software. GSTe2 reactions took approximately 1hour 30minutes while CYP4J5 and CoE reactions each took approximately 1hour 40minutes.

3.6 Data management

During field work, all information was recorded in previously prepared field forms. This data was later entered in Microsoft Excel sheets. In the insectary and laboratory, data was recorded in respective laboratory processing forms and this data was also later entered in Ms Excel sheets. The field forms and laboratory processing forms are shown in Appendix 4 to Appendix 12.

Statistical analysis was done on Ms Excel to determine the distribution of the *Anopheles* mosquitoes and sporozoite rates analysis.

3.7 Data Analysis

3.7.1 Susceptibility Test

The 10minute knock down and final mortality at 24hours was recorded for all experimental mosquitoes together with their controls; both negative and positive controls. Abbots formula was used to correct percentage mortality in cases where the negative control mortality was between 5 and 20%. Using WHO criteria, mortality of 98% - 100% in the sample population was viewed as an indication that the population was still susceptible to the tested insecticide. A mortality of between 90% - 98% suggested possible resistance and further tests are recommended should the species population indicate possible resistance. Mortality less than 90% indicated resistance in the test species from that particular village.

3.7.2 Species Identification

The number and proportions of the different species collected from the study sites were calculated per village per study site.

3.7.3 Sporozoite analysis

The proportions of the positive samples were calculated against the sample size in every village in the study sites.

3.7.4 Microplate enzyme assays

Microplate enzyme assay results were entered in SPSS software and the means of each enzyme per village per species was calculated. The means were plotted on bargraphs and compared against the susceptible Kisumu strain. The upper absorbance limit for the susceptible Kisumu strain was used as the cut-off for for determining elevated enzyme activity. The proportion of individuals within the specific test populations that were

above this threshold was then determined. The Z-test was used to determine whether the proportion of mosquitoes in which enzyme activity was elevated was significantly different from the proportion of mosquitoes that were phenotypically resistant to the insecticides tested according to the bioassays conducted.

3.7.5 TaqMan enzyme and *kdr* analysis

The allele frequencies within a population were calculated as follows:

$$\frac{2(RR) + 1(RS)}{N*2}$$

Where; RR is the frequency of homozygous mutant alleles

RS is the frequency of heterozygous mutant alleles

N is the sum of the population

3.8 Ethical considerations

Prior to sample collection, verbal consent was obtained from village chiefs, area leaders and household heads or their representatives. This study aimed at indoor collection of adult mosquitoes and mosquito larvae from suitable larval sites in the study areas. Human involvement was not invasive but limited to their acceptance that mosquitoes would be collected from their houses. Field workers who assisted in the collection of mosquitoes were trained before they were allowed to go to the field to ensure that they acquired good data collection techniques along with good communication skills.

CHAPTER FOUR

RESULTS

4.1 Distribution of the main malaria vector species in Mwea and Kwale

A total of 423 adults (Table 4-1) and 754 larvae (Table 4-2) *Anopheline* mosquito species were collected from the two study sites (Mwea and Kwale).

Of all the adult mosquitoes collected from Mwea, there were 361 *An. gambiae* s.l. and 12 *An. funestus* s.l. collected. In Mwea *An. funestus* s.l. was only collected from Murinduko but not from Karima and Kiamaciri. In Kwale, 62 adult mosquitoes were collected. From Marigiza village, 6 *An. gambiae* s.l. and 53 *An. funestus* s.l. were collected while from Kidomaya, 3 *An. funestus* s.l. were collected. No adult *Anopheles* mosquito was collected from Gwadu village. The adult female mosquitoes that were either gravid, half gravid or blood fed were put singly in ovipositioning tubes and monitored daily for presence of eggs.

The larvae collected from Mwea were 102 in total, 49 from Karima, 8 from Kiamaciri and 45 from Murinduko. From Kwale, however, larval collection was most targeted due to the low adult mosquito densities resulting in 652 larvae being collected, 237 from Kidomaya, 114 from Marigiza and 301 from Gwadu.

Table 0-1: Number of adult *Anopheles* collected from each village per species

Site	**Village**	***An. gambiae* s.l.**	***An. funestus* s.l.**	**Total (Adults)**
Mwea	Karima	172	0	172
	Kiamaciri	54	0	54
	Murinduko	82	53	135
Kwale	Kidomaya	0	3	3
	Marigiza	6	53	59
	Gwadu	0	0	0

4.1.1 Mosquito distribution in Mwea

In Mwea, a total of 313 adult female *Anopheles* mosquitoes were collected with 241 being either blood fed, half gravid or gravid females and 72 were unfed. Of the females that were blood fed, half gravid or gravid, 135 (56.02%) were collected by indoor mouth aspiration, 91 (37.76%) by window exit traps and 15 (6.22%) by prokopack (Figure 4-1). 99 (41.08%) of these laid eggs which were flooded and only 78 (78.79%) of the flooded eggs hatched.

From Mwea, larval collection resulted in 49 (48.04%) from Karima, 8 (7.84%) from Kiamaciri and 45 (44.12%) from Murinduko. The entire field collected adults and larvae that emerged in the insectary; a total of 463 samples were morphologically identified as *An.funestus* s.l. and *An. gambiae* s.l. These were then identified to their sibling species by conventional PCR with only 408 (88.12%) samples being amplified. Gel electrophoresis revealed that 401 (98.28%) were *An. gambiae* s.l. and 7 (1.72%) were *An. funestus* s.s. Analysis by PCR on *An.gambiae* s.l. specimens revealed 399 (99.5%) were *An. arabiensis* and 2 (0.5%) were *An. gambiae* s.s. (Figure 4-3). *An. funestus* s.l. was collected only in Murinduko village and *An. gambiae* s.s. was collected only from Karima.

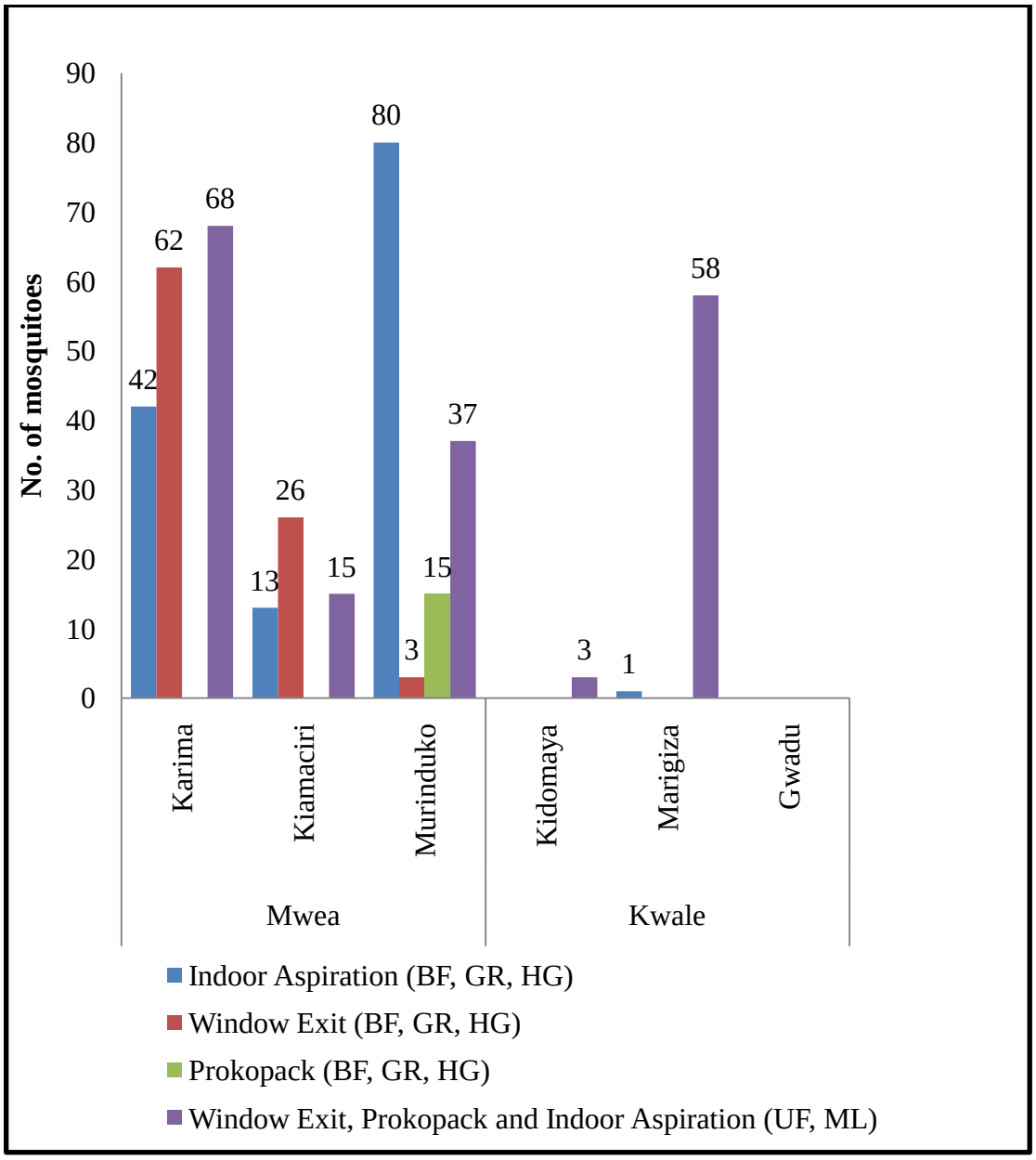


Figure 0-1: Number of mosquitoes collected per their abdomen physiological status

KEY

BF - blood fed **GR** – Gravid **HG** – Half gravid

UF – Unfed **M** – Males

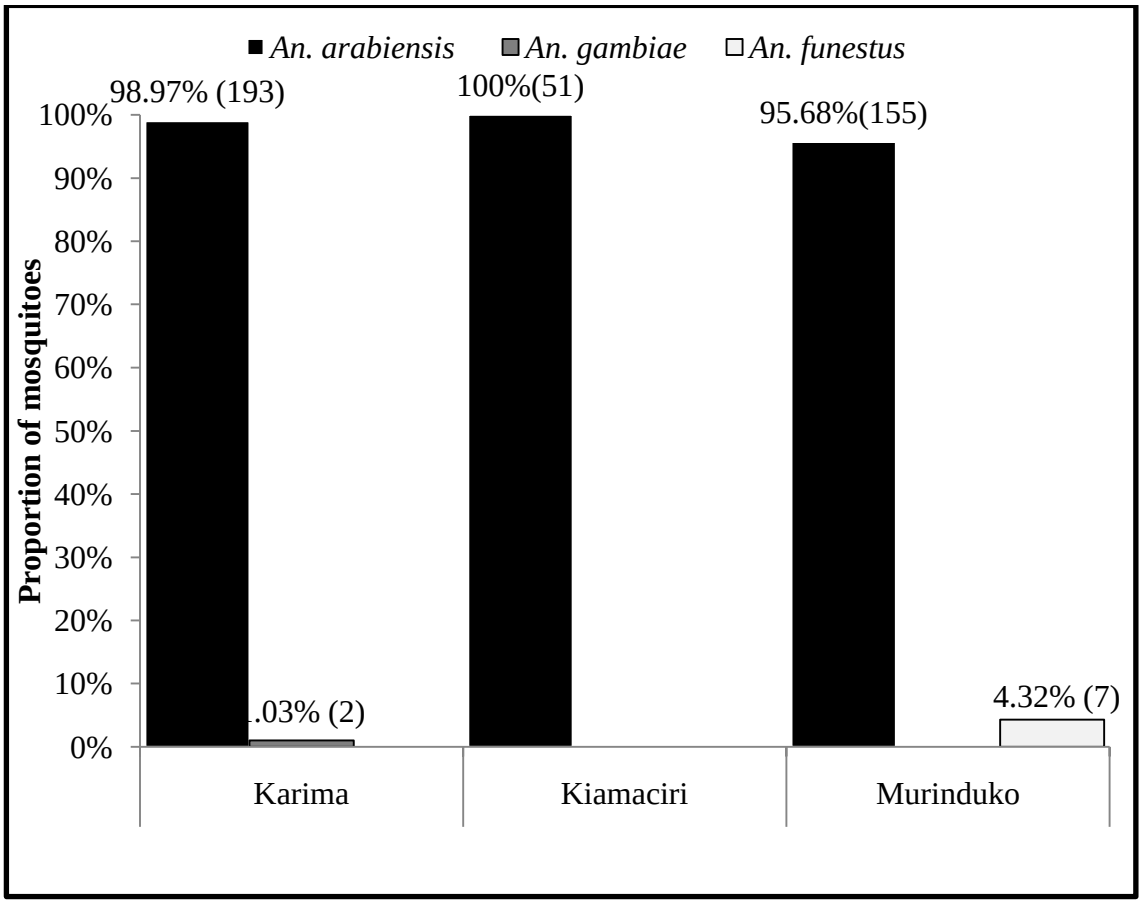


Figure 0-2: Proportion of the different species in Mwea with total number of mosquitoes (in brackets)

4.1.2 Mosquito distribution in Kwale

In Kwale, the densities of adult *Anopheles* mosquitoes were extremely low and a total of 62 adults were captured with the main mosquitoes species collected being *Culicines*. A total of 40 adult female *Anopheles* mosquitoes were collected from Kidomaya and Marigiza villages. Only one mosquito that was blood fed was captured from Marigiza village using indoor mouth aspiration, however, it never laid eggs.

A total of 652 *Anopheline* larvae were collected, 237 (36.35%) from Kidomaya, 114 (17.48%) from Marigiza and 301 (46.17%) from Gwadu (Table 4-2). Those that survived to emerge into adults were 453 larvae. The entire field collected adults and larvae that emerged in the insectary; a total of 514 samples were morphologically identified as *An. funestus* s.l. and *An. gambiae* s.l. These were identified by to their sibling species by conventional PCR with 421 (81.91%) samples being amplified. From the adult collection, 61 mosquitoes were subjected to species identification with 33 (54.1%) amplifying. Of these, there were 26 (78.79%) *An. funestus* s.l. and 6 (18.18%) were *An. gambiae* s.l. Analysis by PCR on *An.gambiae* s.l. specimens revealed all the 6 (100%) were *An. arabiensis*. In the *An. funestus* complex, there were 21 (80.77%) *An. funestus* s.s., 4 (15.38%) *An. rivulorum* and 1 (3.85%) *An. leesoni* (Figure 4-4).

From the Kwale larval collection 453 mosquitoes were subjected to species identification with 388 (85.65%) amplifying. Of these, there were 294 (75.77%) *An. funestus* s.l. and 94 (24.23%) were *An. gambiae* s.l. Amplification by PCR on *An.gambiae* s.l. specimens revealed 93 (98.94%) were *An. gambiae* s.s. and 1 (1.06%) *An. merus* while PCR on *An. funestus* reveled that there were 288 (97.96%) *An. leesoni* and 6 (2.04%) *An. rivulorum* (Figure 4-4). Gel electrophoresis reveled higher numbers of *An. rivuloruma* and *An. leesoni*. (Figure

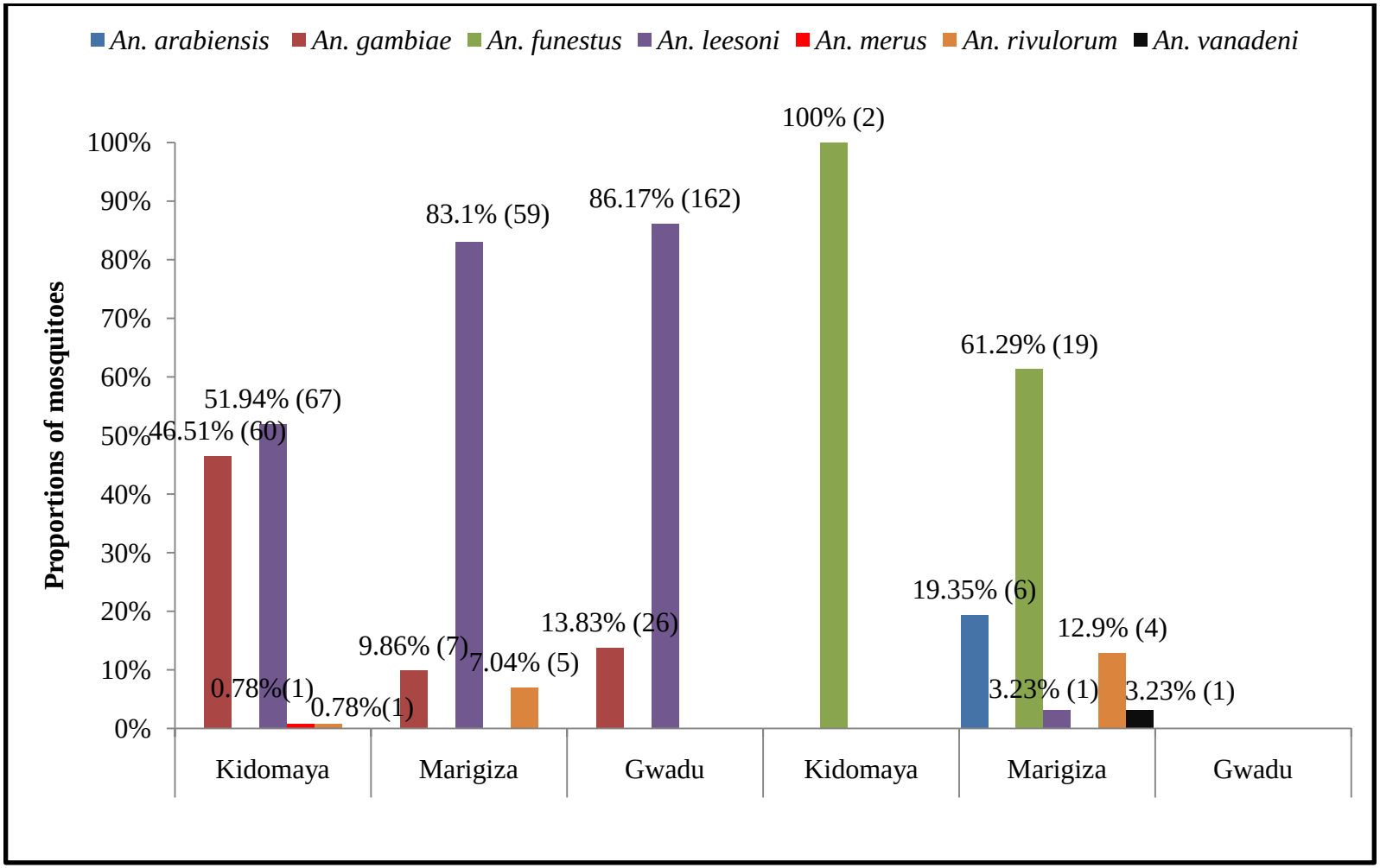


Figure 0-3: Proportion of the different species in Kwale with number of mosquitoes (in brackets)

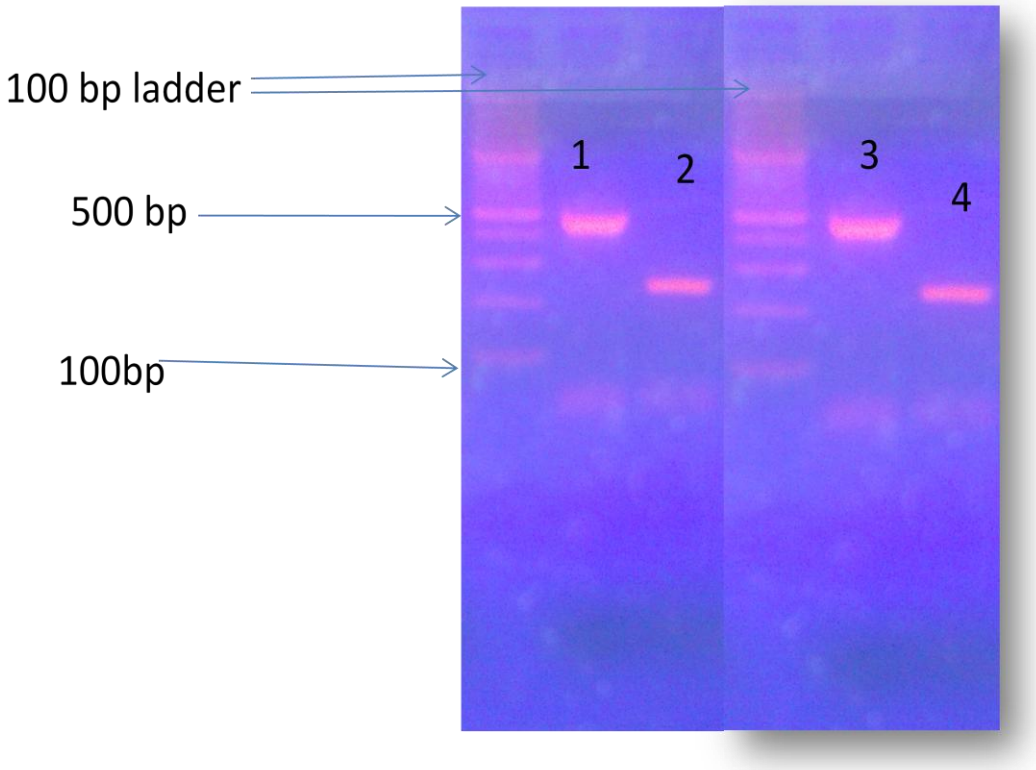


Figure 0-4: Gel image after fragment amplification and electrophoresis with 100bp ladder.

Bands 1, 3 and 6 - *An. rivulorum*

Bands 2, 4 and 5- *An. leesoni*

4.2 Mosquito parasite infection rates

All female field-collected *Anopheles* were subjected to sporozoite analysis by ELISA. In Mwea, 288 samples; 133 from Karima, 47 from Kiamaciri and 108 from Murinduko were tested for the presence of circumsporozoite proteins (CSP). There was however no sporozoite positive sample from Mwea. From Kwale, 40 samples; 3 from Kidomaya and 37 from Marigiza were tested for presence of CSPs. Four (10.81%) *An. funestus* s.s. samples from Marigiza tested positive for *Plasmodium falciparum* sporozoite. All the positive samples were collected from Marigiza village.

4.3 Status of insecticide resistance in the main malaria vectors

Resistance in the mosquitoes was tested against permethrin, deltamentrin and DDT for Mwea's Karima and Murinduko villages; against deltamethrin for Kiamaciri and against permethrin and deltamentrin for all villages in Kwale. This was primarily determined by the number of mosquitoes available for use. In Mwea, 460 *An. arabiensis* and 114 *An. funestus* were tested while in Kwale, 166 *An. gambiae* s.s. and 446 *An. funestus* s.l. mosquitoes were subjected to the bioassay tests. The percentage mortalities for the villages were calculated (Table 4-2). In *An. arabiensis* from Mwea, the percentage mortalities against deltamethrin was highest in Murinduko and lowest in Kiamaciri while against permethrin was highest in Karima but low in Murinduko with permethrin not being tested in Kiamaciri. *An. arabiensis* was seen to be susceptible in Murinduko but low resistance was seen in Karima. *An. funestus* s.l. was only collected from Murinduko in Mwea and these were susceptible to DDT and deltamethrin with possible resistance to permethrin. In Kwale, only deltamethrin and permethrin were tested and deltamethrin was not tested in *An. gambiae* sampled from Marigiza. Mortalities against deltamethrin in *An. gambiae* from Kidomaya and Gwadu ranged between 60% and 90%. Against permethrin, *An. gambiae* from Kidomaya and Gwadu were susceptible (100% mortality) while those from Marigiza were resistant (71.43% mortality). *An. funestus* s.l. were susceptible against deltamethrin in Marigiza with resistance being seen in

Kidomaya and Gwadu. Mortalities in *An. funestus* s.l. against permethrin ranged between 78% to 100% with the highest mortalities in Kidomaya and lowest in Gwadu.

Table 0-2: Phenotypic resistance of *An. gambiae* and *An. funestus* from Mwea and Kwale, September and December 2014

Village	Insecticide	*Anopheles gambiae* s.l.				*Anopheles funestus* s.l.			
		Species	Sample Size	% Mortality	Status	Species	Sample Size	% Mortality	Status
Mwea									
Murinduko	Deltamethrin	*An. arabiensis*	76	84.39	R	*An. funestus* s.s.	47	100.00	S
	Permethrin	*An. arabiensis*	61	31.79	R	*An. funestus* s.s.	29	93.64	PR
	DDT	*An. arabiensis*	102	100.00	S	*An. funestus* s.s.	38	100.00	S
Karima	Deltamethrin	*An. arabiensis*	63	79.24	R		0	NA	NA
	Permethrin	*An. arabiensis*	69	79.34	R		0	NA	NA
	DDT	*An. arabiensis*	47	96.76	PR		0	NA	NA
Kiamaciri	Deltamethrin	*An. arabiensis*	42	59.17	R		0	NA	NA
Kwale									
Marigiza	Deltamethrin		0	NA	NA	*An. leesoni* *An. rivulorum*	69 7	100.00 100.00	S S
	Permethrin	*An. gambiae* s.s.	53	71.43	R	*An. leesoni* *An. rivulorum*	71 9	98.03 95.43	S PR
Kidomaya	Deltamethrin	*An. gambiae* s.s.	29	90.00	PR	*An. leesoni* *An. funestus* s.s.	66 15	69.70 92.00	R PR
	Permethrin	*An. gambiae* s.s.	31	100.00	S	*An. leesoni*	42	100	S
Gwadu	Deltamethrin	*An. gambiae* s.s.	30	60.00	R	*An. leesoni*	90	88.89	R
	Permethrin	*An. gambiae* s.s.	23	100.00	S	*An. leesoni*	77	78.00	R

KEY

R- Resistance

S - Susceptible

PR- Possible resistance

NA – Not Applicable

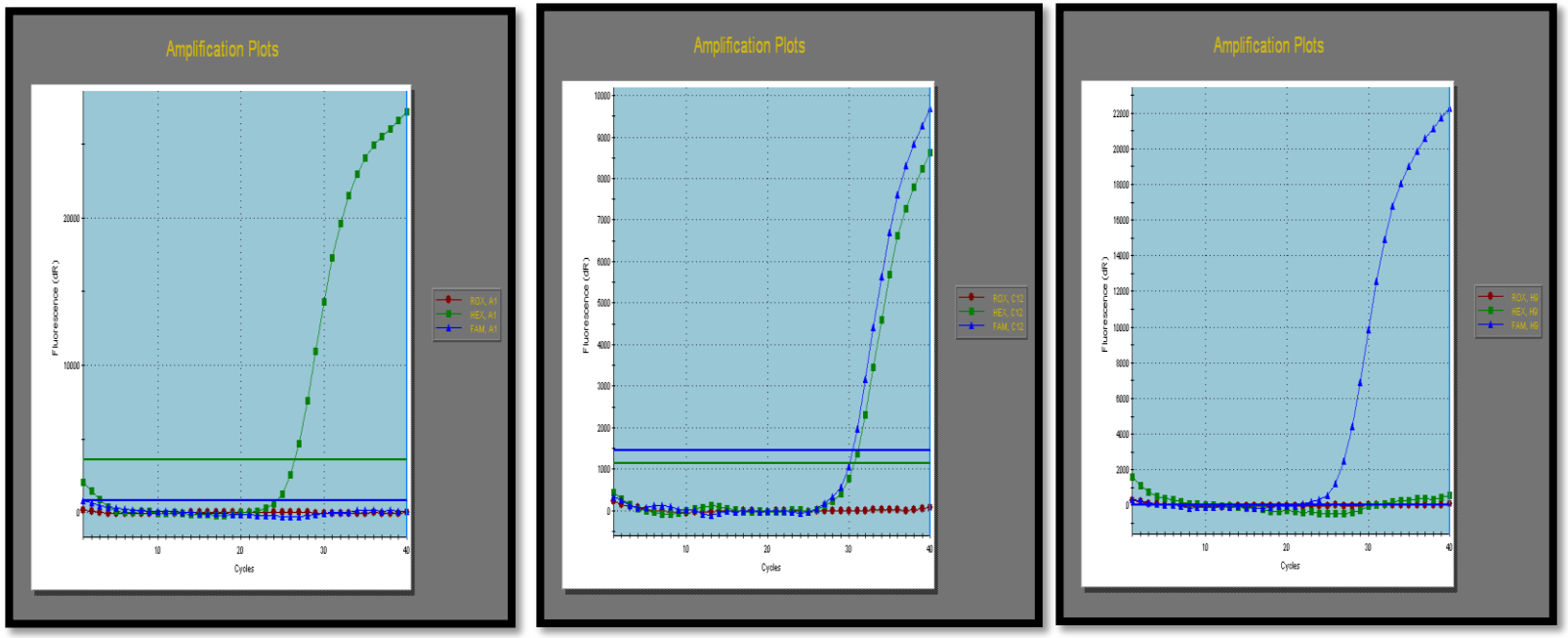
4.4 Mechanisms of insecticide resistance

4.4.1 Knockdown resistance (*kdr*) analysis

Tests for the *kdr* mutation were done on 218 samples; 95 from Mwea (33 from Karima, 30 from Kiamaciri and 32 from Murinduko) and 103 from Kwale (73 from Kidomaya, 7 from Marigiza and 23 from Gwadu). In addition to these field collected samples, there were 20 Kisumu strain that were used as controls. Amplification plots were retrieved to assess for the presence of the *kdr* mutation (Figure 4-5).

For *kdr*-east, PCR amplification failed in one Kisumu strain, 2 Kidomaya strains and 2 Gwadu strains. No *kdr* east gene was detected in Mwea (Karima, Kiamaciri and Murinduko) and Kwale (Kidomaya and Marigiza). However, in Gwadu, one sample amplified for the heterozygous (LS) *kdr*-east gene (Table 4-3) giving a frequency of 2.17%. All other samples amplified for the wildtype/ unmutated (LL) gene.

In all samples tested from both study sites, no *kdr*-west gene (Table 4-3) was setected. However, 5 samples from Kisumu, 6 from Kidomaya, 1 from Gwadu and 2 samples Marigiza villages did not amplify.



LL **LS** **SS**

Figure 0-5: Real-time PCR amplification plots

KEY

LL- Homozygous susceptible/ wildtype

LS – Heterozygous resistant (*kdr*-east)

SS – Homozygous resistant (*kdr*-east)

Table 0-3: *KDR* results in *An. gambiae* s.l. from the study sites

Study site		**No. of samples**	***Kdr* East**			***Kdr* West**		
Site	**Village**		**Non Mutated (LL)**	**Mutated (SS/LS)**	**Frequency of *kdr*-east (%)**	**Non Mutated (LL)**	**Mutated (FF/LF)**	**Frequency of *kdr*-west (%)**
MWEA	Karima	33	33	-	-	33	-	-
	Kiamaciri	30	20	-	-	20	-	-
	Murinduko	32	32	-	-	32	-	-
KWALE	Kidomaya	73	71	-	-	67	-	-
	Marigiza	7	7	-	-	5	-	-
	Gwadu	23	20	1	2.17	22	-	-

KEY

LL- Homozygous susceptible/ wildtype

SS – Homozygous resistant (*kdr*-east)

LS – Heterozygous resistant (*kdr*-east)

FF - Homozygous resistant (*kdr*-west)

LF - Heterozygous resistant (*kdr*-west)

4.4.2 Microplate Enzyme assays

Enzyme levels variation was tested in 521 samples. There were 392 samples from Mwea (Murinduko-255, Kiamaciri-47, Karima-90), 107 from Kwale (Gwadu-75, Kidomaya-26, Marigiza- 6) and 22 Kisumu strain which was used as the control group. Protein assays was done to correct for the sizes of the mosquito. Different enzyme tests gave different colour changes (Figure 4-6). The mean ODs for each sample were done as each test was done in triplicate. These means were rounded off to 2-decimal places and plotted on bargraphs comparing the enzyme activities of mosquito populations from each village with that of the susceptible Kisumu strain. The Kisumu strain was used to determine the enzymes activity cut offs and the individuals with enzyme activity above that of the susceptible Kisumu strain were assumed to be resistant (Table 4-4; Table 4-5). The proportions of the resistant population were calculated and this was compared to the phenotypically resistant proportions within the same population.

Generally in the *An. arabiensis* from Mwea, it was seen that oxidase and β-esterase activity were elevated in all the 3 villages. ATCH activity was elevated in Karima and Murinduko while GST activity was elevated in Karima and Kiamaciri. However, there was not seen any net association between the observed phenotypic resistance with these elevated enzyme activities in Kiamaciri and Murinduko. However, all the tested enzymes were elevated in Karima giving a net association with phenotypic DDT resistance in Karima.

In Kwale, *An. funestus* s.l. from Kidomaya and Gwadu had ATCH, oxidase and β-esterase elevated activity while GST enzyme activity was only elevated in Kidomaya. There was seen no enzyme activity elevation from *An. funestus* s.l. collected from Marigiza. The phenotypic resistance in Gwadu against deltamethrin was seen to have an association to β-esterase activity elevation while that against permethrin was seen to be associated with ATCH enzyme elevation. Oxidase and β-esterase enzyme activity elevation was seen to be as a result of *An. funestus* phenotypic resistance against deltamethrin.

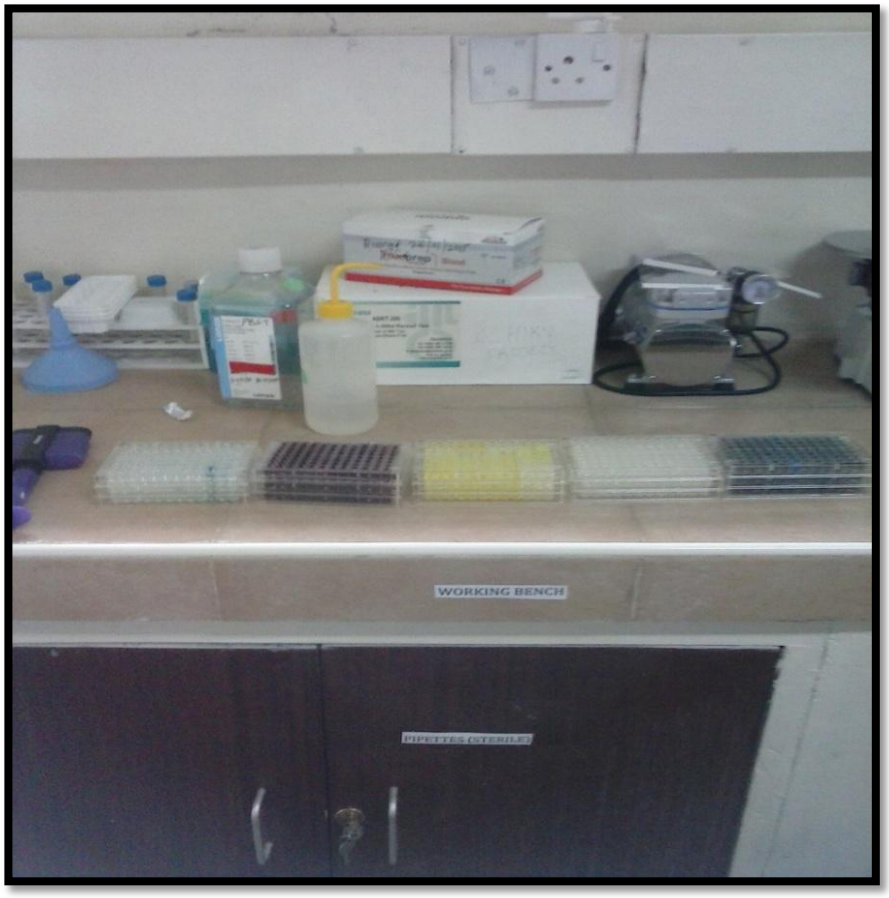


Figure 0-6: Microplate enzyme assay test results

From left to right: Oxidase (colourless), β-esterase (maron), ATCH (yellow), GST (colourless) and Protein (blue) tests.

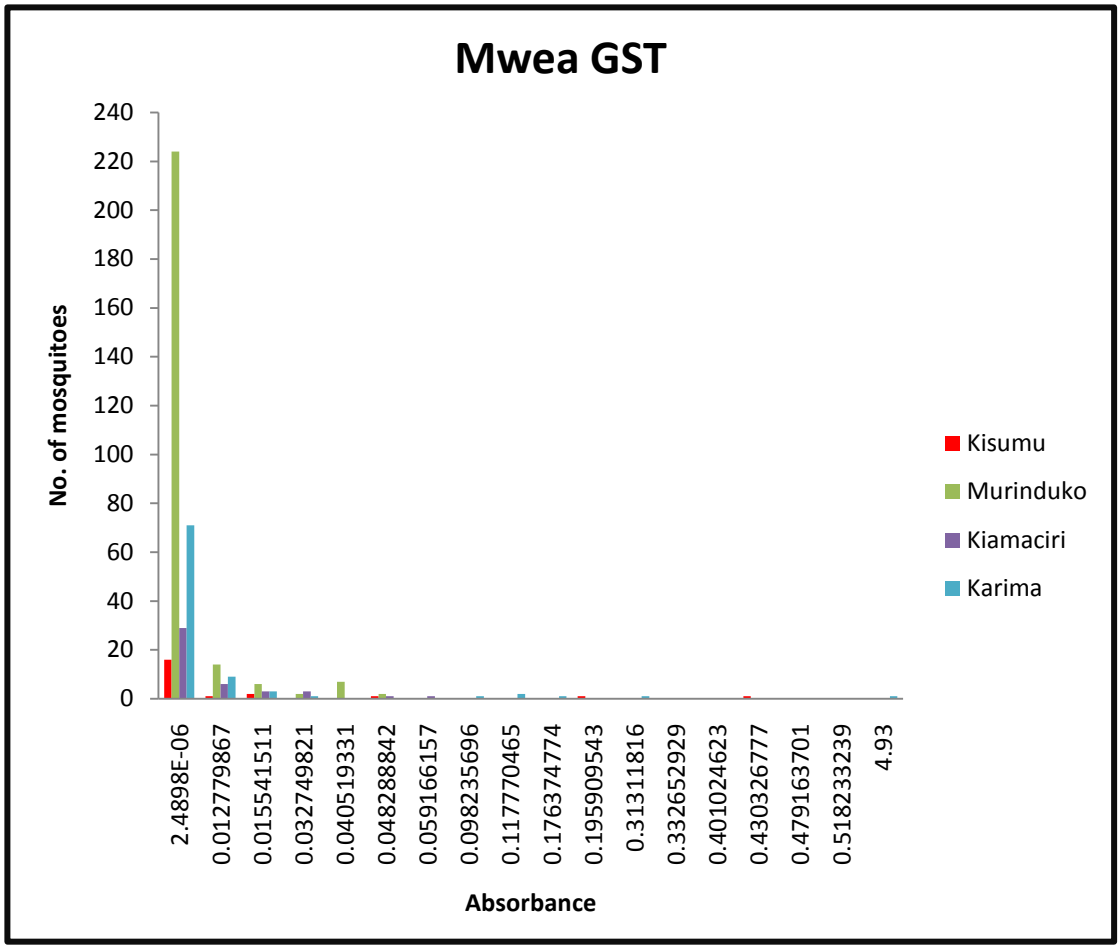


Figure 0-7: Mean ODs comparing GSTs of Mwea mosquito populations with the susceptible Kisumu strain

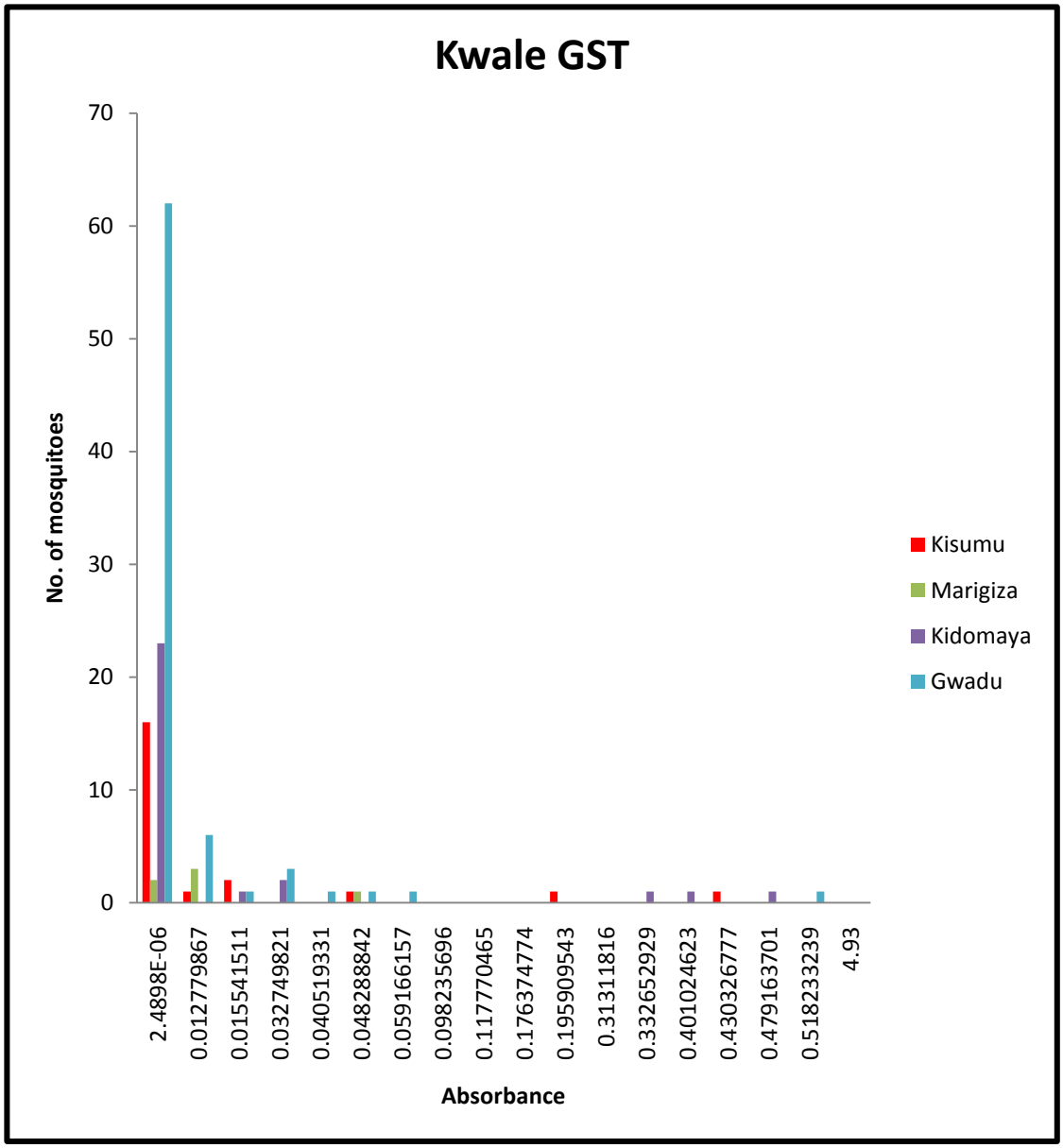


Figure 0-8: Mean ODs comparing GSTs of Kwale mosquito populations with the susceptible Kisumu strain

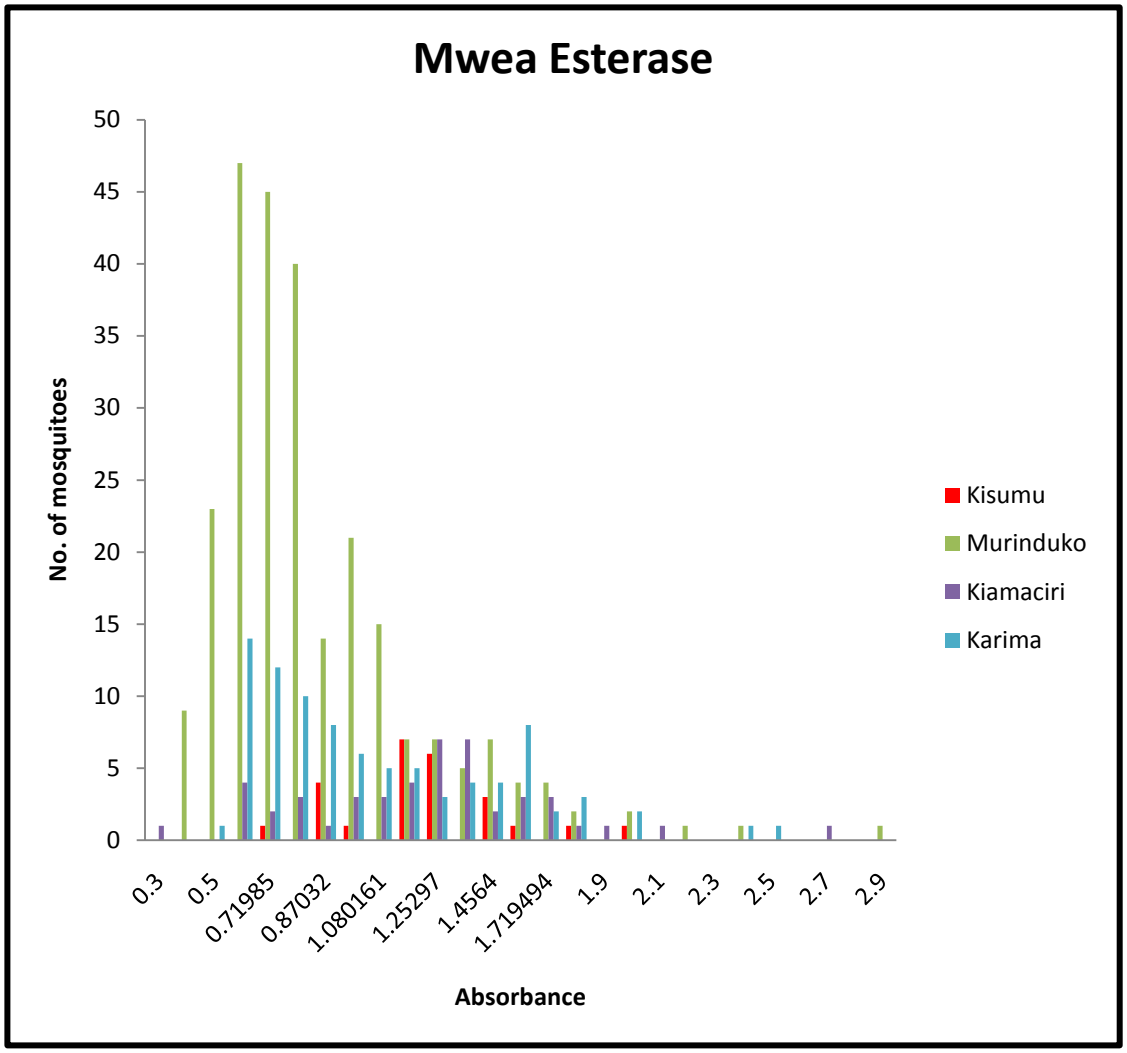


Figure 0-9: Mean ODs comparing esterase of Mwea mosquito populations with the susceptible Kisumu strain

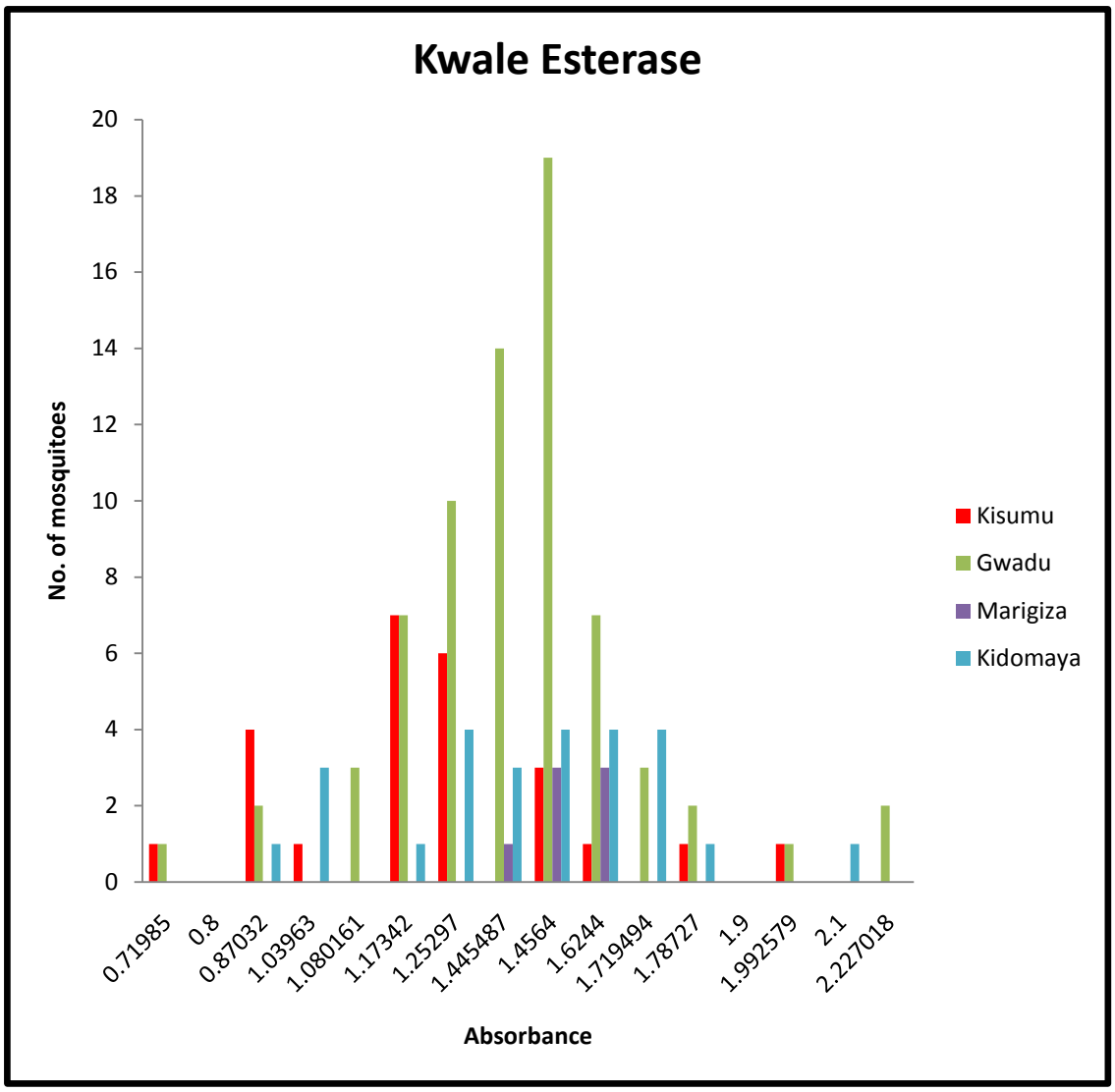


Figure 0-10: Mean ODs comparing estarese of Kwale mosquito populations with the susceptible Kisumu strain

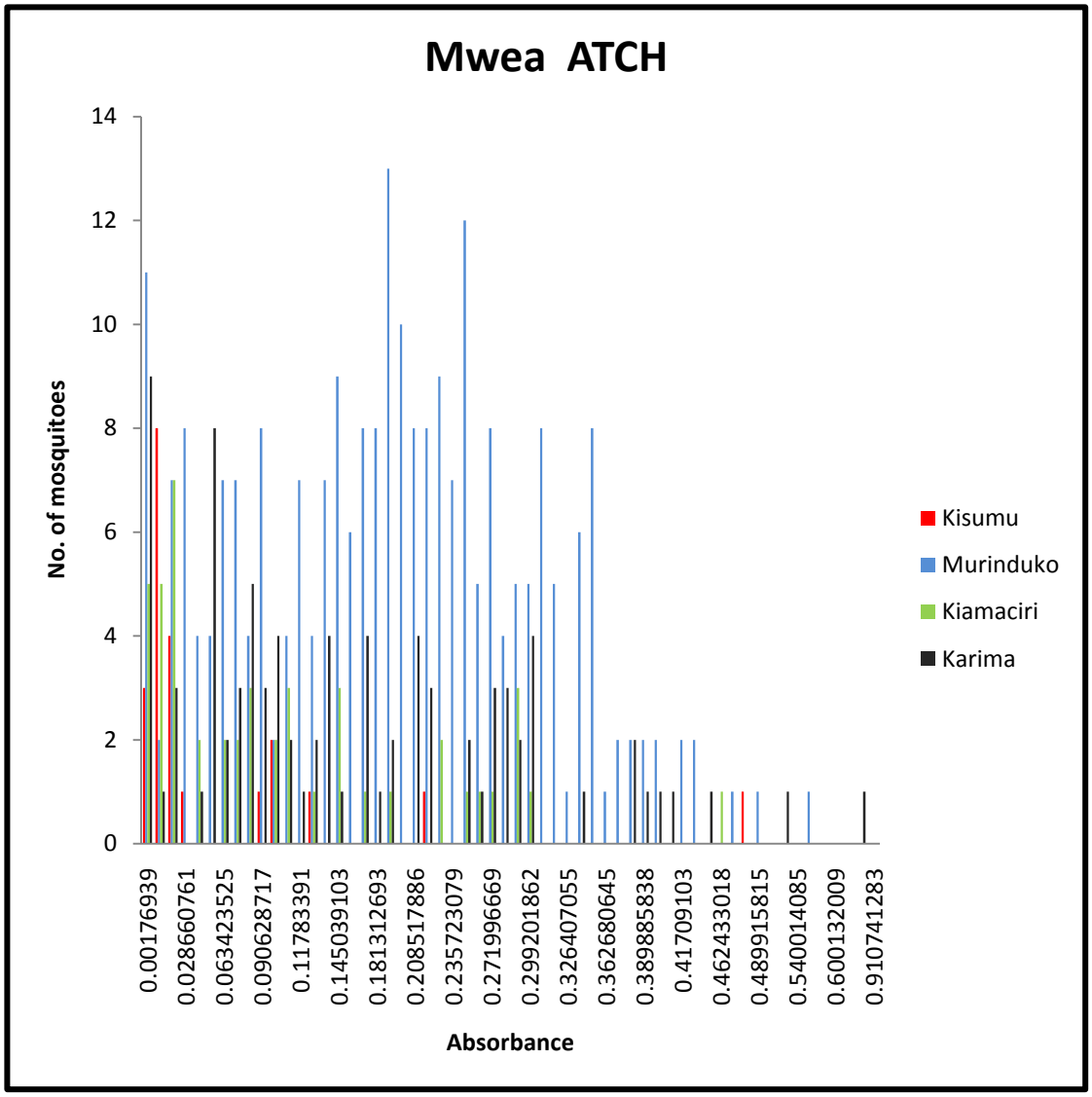


Figure 0-11: Mean ODs comparing ATCH of Mwea mosquito populations with the susceptible Kisumu strain

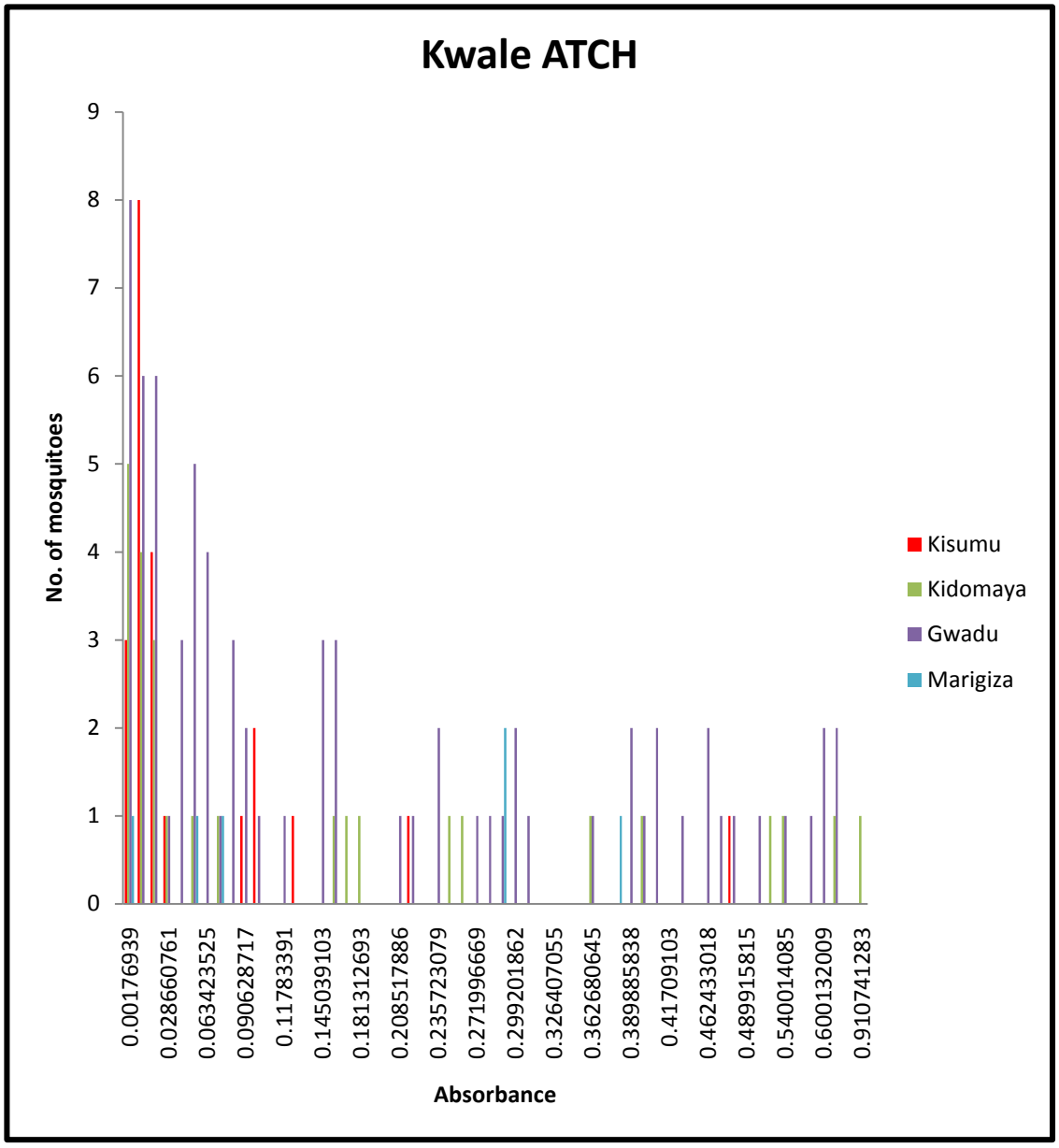


Figure 0-12: Mean ODs comparing ATCH of Kwale mosquito populations with the susceptible Kisumu strain

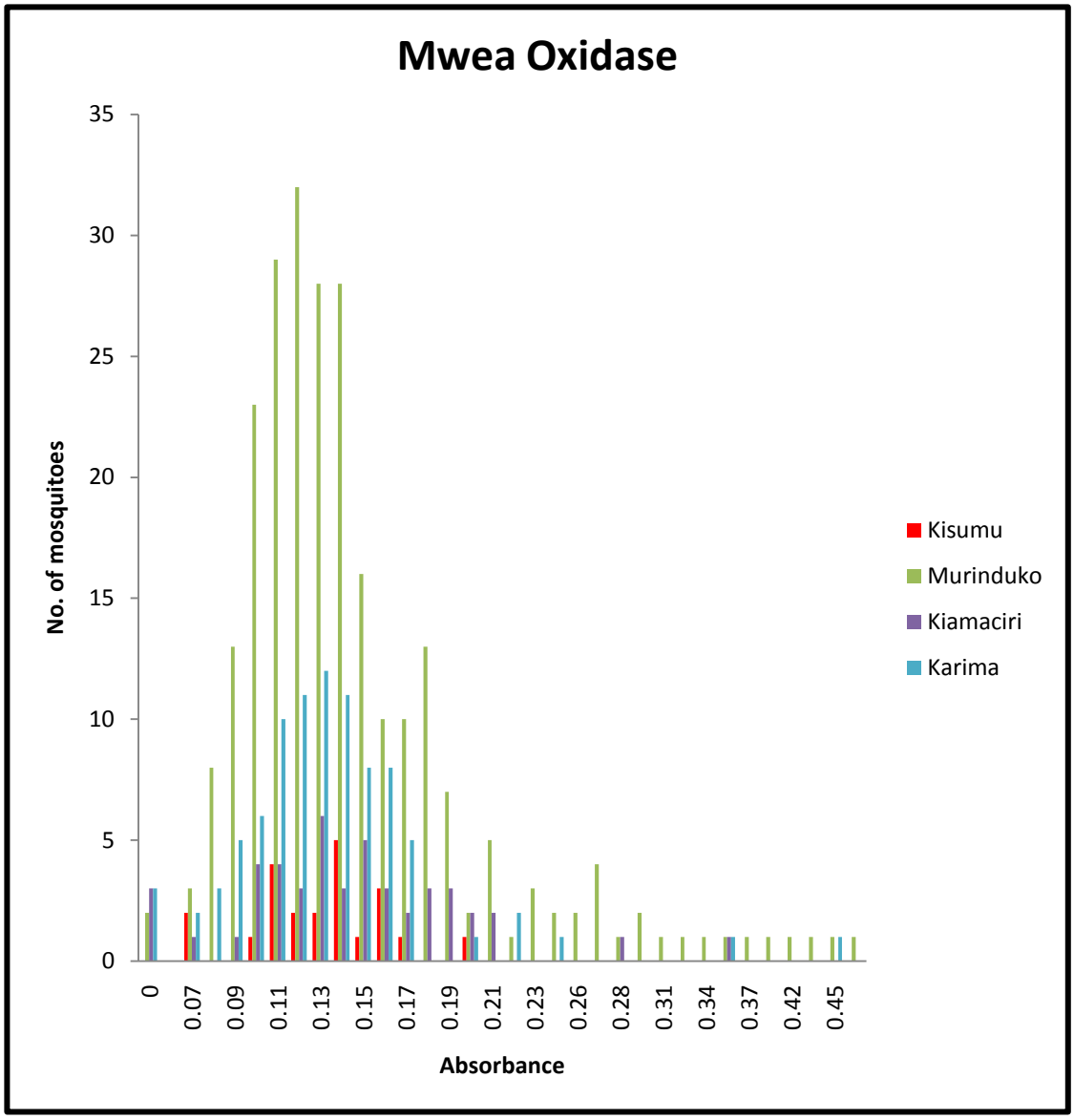


Figure 0-13: Mean ODs comparing oxidase of Mwea mosquito populations with the susceptible Kisumu strain

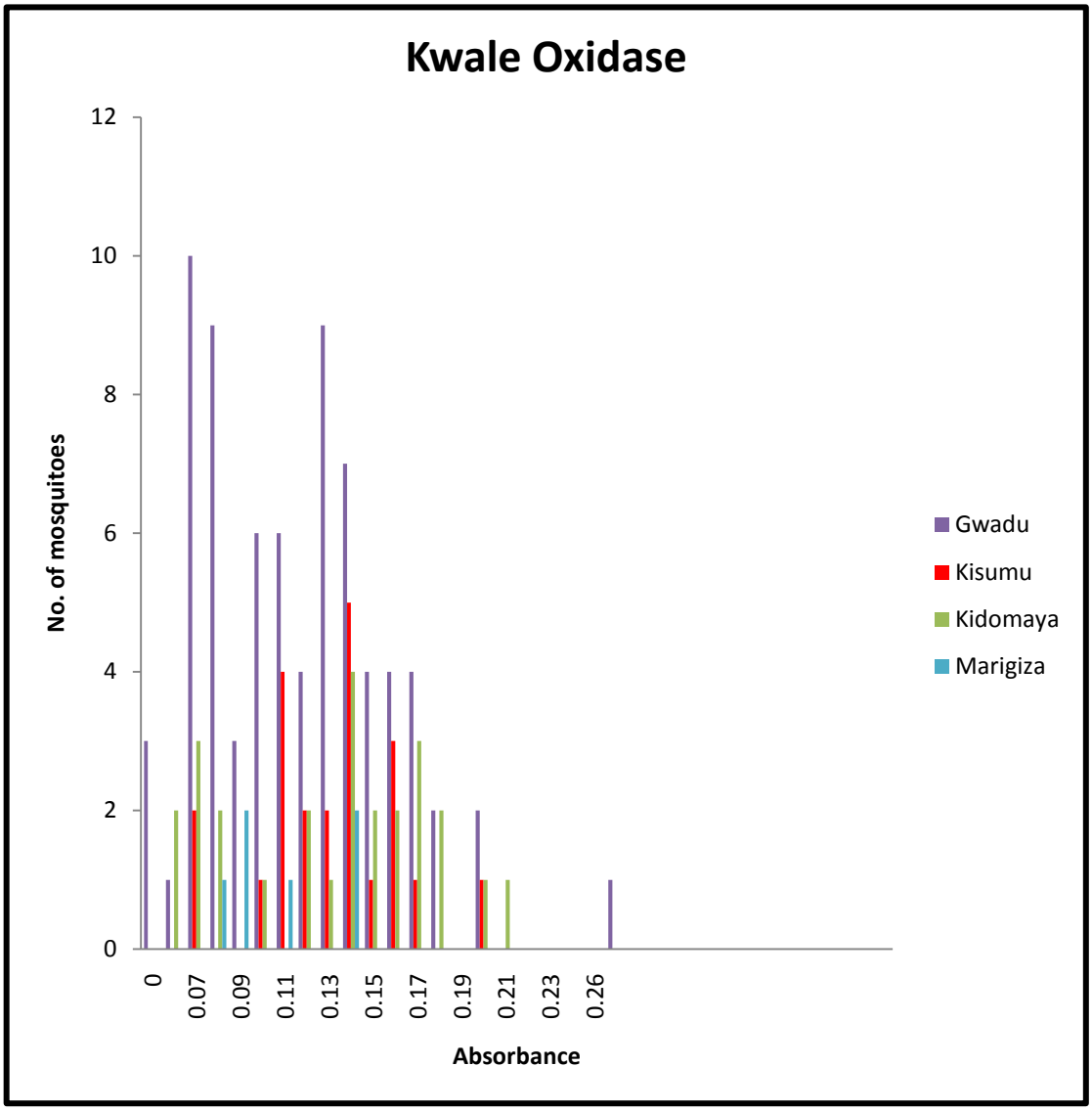


Figure 0-14: Mean ODs comparing oxidase of Kwale mosquito populations with the susceptible Kisumu strain

Table 0-4: Relationship between the proportions of phenotypically resistant *An. gambiae* s.l. individuals and high enzyme activity

Insecticide	Village	%age with phenotypic resistance (n)	%age with ATCH activity above Ksm Strain	Z-Test		%age with oxidase activity above Ksm Strain	Z- Test		%age with GST activity above Ksm Strain	Z-Test		%age with β-esterase activity above Ksm Strain	Z-Test	
				Z-Value	P-Value		Z-Value	P-Value		Z-Value	P-Value		Z-Value	P-Value
Deltamethrin	Karima	20.76 (63)	2.22	5.30	0.00	5.56	4.26	0.00	1.11	5.59	0.00	2.22	5.30	0.00
	Kiamaciri	40.83 (42)	0.00	-	-	8.51	7.45	0.00	2.13	8.80	0.00	4.26	8.39	0.00
	Murinduko	15.61 (76)	0.78	7.16	0.00	11.76	4.49	0.00	0.00	-	-	1.96	6.84	0.00
Permethrin	Karima	20.66 (69)	2.22	5.01	0.00	5.56	3.96	0.00	1.11	5.31	0.00	2.22	5.01	0.00
	Murinduko	68.21 (61)	0.78	18.86	0.00	11.76	17.23	0.00	0.00	-	-	1.96	18.7	0.00
DDT	Karima	3.24 (47)	2.22	1.23	0.22*	5.56	-0.06	0.95*	1.11	1.74	0.08*	2.22	1.23	0.22*

KEY: *No significant difference between the phenotypic resistant proportion and the enzyme activity

Total number of samples tested for enzyme assays (Murinduko-255, Kiamaciri-47, Karima-90, Gwadu-2 and Kidomaya-2)

n- Sample size

Table 0-5: Relationship between the proportions of phenotypically resistant *An. funestus* s.l. individuals and high enzyme activity

Insecticide	Village	%age with phenotypic resistance (n)	%age with ACTH activity above Ksm Strain	Z-Test		%age with oxidase activity above Ksm Strain	Z- Test		%age with GST activity above Ksm Strain	Z-Test		%age with β-esterase activity above Ksm Strain	Z-Test	
				Z-Value	P-Value		Z-Value	P-Value		Z-Value	P-Value		Z-Value	P-Value
Deltamethrin	Gwadu	11.11 (90)	20.55	-2.64	0.01	1.37	2.64	0.01	1.37	2.64	0.01	2.74	1.84	0.07*
	Kidomaya	30.30 (81)	16.67	-2.92	0.00	4.17	1.87	0.06*	0.00	-	-	4.17	1.87	0.06*
Permethrin	Gwadu	22.00 (77)	20.55	-0.03	0.98*	1.37	4.62	0.00	1.37	4.62	0.00	2.74	4.02	0.00
	Marigiza	4.57 (80)	0.00	-	-	0.00	-	-	0.00	-	-	0.00	-	-

KEY: *-No significant difference between the phenotypic resistant proportion and the enzyme activity

Total number of samples tested for enzyme assays (Gwadu-73, Kidomaya-24 and Marigiza- 6)

n – Sample size

4.4.3 TaqMan Enzyme Assays

TaqMan enzyme assays for the epsilon class glutathione-S-transferase (GSTe2), carboxylesterase (CoE) and cytochrome P450 (monooxygenases/ CYP4J5) enzymes were done on 119 samples; 93 from Mwea (30 from Karima, 33 from Kiamaciri and 30 from Murinduko) and 4 from Kwale (2 from Kidomaya and 23 from Gwadu). In addition to these field collected samples, there were 22 Kisumu strain that were used as controls.

The frequency of the GSTe2 gene mutation ranged between 0% - 50% and was was highest Kidomaya (50%) with a low frequency of this gene in Murinduko. There was no mutation of GSTe2 gene in Kiamaciri, Karima and Gwadu. The frequency of the CYP4J5 gene ranged between 53.03% -60% in Mwea. However, in Kwale none of the samples tested amplified for this gene while the frequency of the CoE gene was between 25% and 34.85% (Table 4-6).

Table 0-6: Frequency of mutated TaqMan Enzyme genes in *An. gambiae* s.l.

Study Sites	Village	Number of samples	% GSTe2	% CYP4J5	% CoE
MWEA	Kiamaciri	33	0	53.03	34.85
	Karima	30	0	58.33	25
	Murinduko	30	6.67	60	30
KWALE	Kidomaya	2	50	NA	25
	Gwadu	2	0	NA	25

KEY

NA- Not amplified

CHAPTER FIVE

DISCUSSION

The current study provides information on the prevelance of malaria vector species in Mwea (Karima, Kiamaciri and Murinduko villages) and Kwale (Kidomaya, Marigiza and Gwadu villages) and their insecticide resistance status. The collection methods used for sampling adult mosquitoes were window exit traps to target exophilic vectors and the indoor aspiration and prokopack for the endophilic mosquito vectors. This study indicated that most of the *An. arabiensis* mosquitoes from Karima and Kiamaciri are exophilic in nature, resting outdoor after a blood meal, as was captured by the window exit traps. This is consistent with earlier studies by Bayoh *et al*., (2010) which indicated that *An. arabiensis* are exophilic vectors. In contract, indoor aspiration collected more mosquitoes in Murinduko. In Kwale however, this could not be conclusively determined due to low adult mosquito populations.

The presence of small pockets of rain water that had resulted from the rains that occurred resulted in the higher collection higher numbers of larvae as compared to the adult mosquitoes in Kwale. *Anopheles gambiae* s.l. breeds more prolifically in temporary and turbid water bodies such as ones formed by rain while in contrast, *An. funestus* s.l. prefers more permanent water bodies (Gillies and De Meillon, 1968). This could explain why most of the *An. funestus* s.l. was collected from the more permanent water bodies in Kwale. The larval habitats in Mwea, especially in Karima, were largely covered by *Azolla* vegetation.

The main vector species in Mwea and regions around MRIS are the *An.gambiae* s.l. with a high distribution of *An. arabiensis* which has been the predominant species in this region (Muturi *et al*., 2006) as shown in Figure 4-3. A small proportion of *An. gambiae* s.s. (0.5%) was sampled from Karima whose presence has not been documented in this area (Muturi *et al*., 2007; Mutero *et al*., 2000). There was also collected a small

proportion of *An. funestus* s.s. from Murinduko, consistent with the studies by Muturi *et al*., (2009).

In Kwale, the collections were done in December 2014, immediately after the short rains. The main mosquito species collected was *An. funestus* s.l. (Figure 4-4). Studies by Keating *et al*., (2005) also revealed that their November/December collection from Kwale had the highest number of *An. funestus* s.l. collected in comparison to other months. Non-amplified samples might have resulted due to possible presence of other *Anopheline* species whose primers were not included in the amplification as it has been recorded the presence of other species in these study areas (Mwangangi *et al*., 2007; Muturi *et al*., 2006; 2007; 2008) or due to either due to experimental errors or DNA degradation as a result of preservation problems.

Earlier studies that determined the distribution of malaria vectors in Kwale collected indoor adult mosquitoes and thus only targeted the endophilic vectors (Mbogo *et al*., 2003; Mwangangi *et al*., 2004). The combination of the both the adult and larval collection in the present study captured both indoor and outdoor feeding and resting mosquitoes and was thus a richer sampling approach.

Studies have shown that malaria is transmitted by a number of species in Africa (Temu *et al*., 1998). This transmission is primaryly done by *An. gambiae* s.s., *An. arabiensis* and *An. funestus* s.s. and secondarily by *An. pharoensis*, *An. coustani* and *An. rivurolum* (Gillies and Smith 1960; Bekele e*t al*., 2012; Hargreaves *et al*., 2000; Kawada *et al*., 2012). In this study, only *An. funestus* s.s. and *An. rivulorum* were the possible malaria transmitters collected. Tests on Kwale, a malaria hyper endemic region in Kenya (Zurovac *et al*., 2006), revealed presence of *P. falciparum* infection in *An. funestus* s.s. collected from Marigiza. In Mwea however, none of the specimens tested positive for *P. falciparum*. This is likely due to the malaria vector control interventions in place.

Insecticide resistance has been spreading in Africa leading to the adaptation of knockdown as an indication of resistance in mosquitoes (Kang *et al*., 1995) and is thus

being used to test for insecticide resistance. In Kenya, insecticide resistance has been extensively studied in Western Kenya showing reduced susceptibility in the *An. gambiae* population (Mathias *et al*., 2011; Ochomo *et al*., 2014; 2013; Stump *et al*., 2004).

From table 4-2, phenotypic resistance to deltamethrin and permethrin was observed in *An. arabiensis* mosquitoes collected from Mwea. Possible resistance against DDT was seen in *An. arabiensis* from Karima (96.76% mortality 24hours post exposure) while *An. arabiensis* from Murinduko were fully susceptible. For *An. funestus* s.s. collected from Murinduko (the only village where this species was collected), there was indication of developing resistance against permethrin (93.64% mortality 24h post exposure) but moquitoes were still susceptible to deltamethrin and DDT. Resistance in Mwea can largely be attributed to control interventions (use of LLITNs) and the use of pesticides used in the rice farms (ALPHA®; a pyrethroid based pesticide). In Kwale, resistance to permethrin was only seen in *An. gambiae* s.s. from Marigiza while Kidomaya and Gwadu population remain susceptible. Resistance to deltamethrin in *An. gambiae* s.s. was only tested in Kidomaya and Gwadu and resistance was seen in Gwadu while evidence of developing resistance was seen in Kidomaya. *An. funestus* s.s. that were sampled and tested for phenotypic resistance were collected from Kidomaya. These were only tested against deltamethrin resistance and they revealed possible resistance. *An. rivulorum* was also tested from Marigiza for deltamethrin revealing susceptibility and permethrin showing possible resistance. However, the numbers of *An. funestus* s.l. mosquitoes analysed were very small. This trend in resistance in malaria vectors may be as a result of up-scaling of bed nets in the region (KMIS, 2010).

The above results suggest that patterns of resistance vary greately but that resistance to chemicals used for the treatment of bed nets and possibly in agriculture is a real problem in these areas and may pose a challenge to the continued efficacy of their use. Furthermore, there was evidence of developing resistance to DDT due to cross-resistance despite it not being used in the country.

Insecticide resistance is seen as a result of selection pressure in the natural populations (Soderlund and Bloomquist, 1989; Scott and Kasai, 2004). This results in either target site mutations or alterations of detoxifying enzyme. Pyrethroid resistance and DDT resistance are both associated with mutation in the knockdown resistance (*kdr*) gene (Martinez-Torres *et al*., 1998; Ranson *et al*., 2000) and this can lead to cross resistance between the two insecticides (Prapanthadara *et al*., 1995). The *kdr*-west mutation was not found in all the samples tested in the present study; however this mutation has just recently been reported in western Kenya [Ochomo *et al*., 2015]. The *kdr*-east gene was only found in Gwadu in Kwale which had a frequency of 2.17% with the heterozygous mutated gene within the *An. gambiae* s.s. population. This shows that this gene is slowly gaining entrance in this region as opposed to the western region where frequencies as a high as 100% have been found (Ochomo *et al*., 2013).

Although this study found elevated ATCH, oxidase, esterase and GST activity in *An. gambiae* s.l., there was no evidence that this elevated activity was associated with phenotypic resistance for all insecticides that were tested except for DDT. For this insecticide, levels of phenotypic resistance were similar to the levels of elevated enzyme activity for all the four enzymes, suggesting an association, although it is notworthy that both the levels of resistance and elevation of enzyme activity were generally low (less than 6%). Esterases have in other studies been associated with organophosphate, carbamate and pyrethroid resistance (Hemingway, 1983; McAllister *et al*., 2012). ATCH, a more specific esterase, measures the amount of acetylcholine esterase and is usually associated with carbamates and organophosphate resistance (WHO, 2010). GSTs have also been found to be involved in DDT resistance (Hemingway, 1983; Ffrench-Constant, 2014; Riveron *et al*., 2014) while oxidases measure the level of heme peroxidase and are used to determine resistance in several classes of insecticides (WHO, 2010). Studies have shown that increased levels of some cytochrome P450 genes are linked to increase in detoxifying oxidase enzyme (Amenya *et al*., 2008). For *An. funestus* s.l., results from this study suggest that elevated ACTH activity may be associated with resistance to permethrin while elevated oxidase and esterase activity

may be associated with resistance to deltamethrin, in agreement with findings from other studies.

Increased ATCH, oxidase, and β-esterase activity seen *An. gambiae* s.l. microplate assay tests were accompanied by higher frequencies of the mutations of CoE and CYP4J5 enzyme genes and these results are therefore in concordance. However, for GSTe2, the frequency of mutation was low to none. This was in agreement with the no or very low GST activity seen in the microplate enzyme assays.

While insecticide resistance poses a great threat to the fight against malaria, worse still is the development of cross resistance which has been reported in *An. gambiae* s.s. (Kwiatkowska *et al*., 2013; Edi *et al*., 2012; Corbel *et al*., 2007), *An. arabiensis* (Yewhalaw *et al*., 2011) and *An. funestus* (Djouaka *et al*., 2011) in Africa. More permanent vector control tools would maintain the effectiveness of vector control program thus understanding the mechanism of resistance present in the resistant vector species through molecular or biochemical techniques is vital. Due to the over-reliance on pyrethroids in mosquito control, mainly IRS and ITNs, diversity and IVM strategies should be included in vector control. This should include physical barriers (Kirby *et al*., 2009), larviciding (Fillinger *et al*., 2009), environmental management (Imbahale *et al*., 2011), selection of insecticides with alternative mode of action (Blanford *et al*., 2011). There are also several other tools under development that can be used including order baited traps (Hiscox *et al*., 2012), biopesticides (Scholte *et al*., 2005), use of parasites like Wolbachia (Iturbe-Ormaetxe *et al*., 2011) and using genetically modified male mosquitoes (Lacroix *et al*., 2012).

Singly, most of these vector control methods face possible unsustainability but alternative methods would complement one another thus IVM can provide a more sustainable and effective vector management. Vector control programs must strive to sustain impact so far attained.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has revealed that *An. gambiae* s.l. and *An. funestus* s.l. species from Mwea (Karima, Kiamaciri and Murinduko) and Kwale (Kidomaya, Marigiza and Murinduko) are the common vectors of malaria in these regions as collected from indoors and as larvae. The prevalence of malaria is low in Mwea but there were cases seen that existed in Kwale. Evidence of phenotypic resistance against pyrethroids in these vector species was seen. Occurrence of *kdr*-west gene was not seen in both study sites and no existance of *kdr*-east was witnessed in Mwea. In Gwadu however, the *kdr*-east gene was witnessed. Enzyme levels in these malaria vectors have generally been elevated, a possible indicator of presence of cross-resistance. The frequency of mutation was also seen to be high in genes associated to resistance.

6.2 Recommendations

Based on the data obtained from this study it is recommended that a combination of both indoor and outdoor collection methods to be used in studies that aim at describing accurately the vector distribution and composition within a locality. This would provide a clearer indication of vector distribution. Due to the high numbers of malaria vector species in Mwea and presence of circumsporozoite proteins in mosquitoes from Kwale, mosquito vector interventions should be enhanced in the study areas to prevent residents from malaria and nuisance bitting.

It is also recommended that continuous and monitoring of the state of insecticide resistance should be carried out more often so as to understand the state of insecticide resistance within a vector population. In addition to this, IVM strategies should be taken up to provide a more sustainable vector management programme.

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APENDICES

Appendix1: Grinding buffer

1) Homogenization buffer (100ml at ph 8.0)
   0.59g 0.1M sodium chloride
   6.84g 0.20M sucrose
   0.37g 0.01M Ethylenediaminetetraacetic acid (EDTA)
   0.36g 0.03M trizma base
   100ml distilled water
2) Lysis buffer (100ml at ph 9.2)
   9.28g 0.25M EDTA
   1.88g 2.5% w/v Sodium Dodecyl Sulfate (SDS),
   6.03g 0.5M trizma base
   100ml distilled water

Mix the homogenization buffer and the lysis buffer at a ratio of 4:1 to make the grinding buffer

Appendix 2: TBE buffer

To make 1 litre of TBE buffer:

5.5g boric acid,

10.8g trizma base,

0.93g of EDTA

1 litre distilled water.

Stir this solution until all the solutes dissolve.

Appendix 3: Blocking buffer (BB)

To prepare 1 litre blocking buffer,

Plain PBS, pH 7.4

I packet of powdered phosphate buffered saline (PBS)

1 litre distilled water

10g BSA

5g casein

0.1g thimersol

0.02g phenol red

Stir this solution for atleast 2hours

Appendix 4: Adult mosquito collection field form

ADULT COLLECTION FIELD FORM

SITE…….. VILLAGE……. DATE…….…….

HOUSE ID	COLLECTION METHOD	HOUSE TYPE			COORDINATES	ALTITUDE
		WALL	ROOF	SIZE OF EAVES		

Appendix 5: Larval field collection form

LARVAL COLLECTION FIELD FORM

SITE…..……..……. VILLAGE……..…DATE……..……

HABITAT TYPE	COORDINATE	WATER TURBIDITY	TOTAL VEGETATION COVER	PREDATORS PRESENT (Y/N)	DISTANCE TO NEAREST HOMESTEAD (M)	EXPOSED TO SUNLIGHT (Y/N)	EARLY INSTARS (L1/L2)	LATE INSTARS (L3/L4)

Appendix 6: Laboratory processing form

MOLECULAR ENTOMOLOGY LABORATORY

Laboratory Processing Form

Mosquito ID	Collection Date	Species	DNA Extraction Date	Species ID	Sporozoite ELISA Date	Sporozoite ELISA Result

Appendix 7: Bioassay data form

CBRD-Molecular Entomology Laboratory

Resistance Bioassay Data Form
Experiment
Start Time: **Stop Time:**

Collection Site: _____ Insecticide: _____
Bioassay Date: _____ Sample: _____

Time in mins	-ve control	+ve control	T1	T2	T3	T4	T5	T6	TOTAL
	Number of Mosquitoes Knocked Down								
10									
20									
30									
40									
50									
60									
80	N/A	N/A	N/A						
Sample Size									
Mortality 24h									

Collection Site: _____ Insecticide: _____
Bioassay Date: _____ Sample: _____

Time in mins	-ve control	+ve control	T1	T2	T3	T4	T5	T6	TOTAL
	Number of Mosquitoes Knocked Down								
10									
20									
30									
40									
50									
60									
80	N/A	N/A	N/A	N/A					
Sample Size									
Mortality 24h									

Appendix 8: Conventional PCR form

MOLECULAR ENTOMOLOGY LABORATORY
PCR ASSAY LAB DATA SHEET

USER NAME_____ DATE_____

PURPOSE: _____
PROGRAM Name: _____
PCR Cycle: Denature- _____; Anneal-____; Extend -____ Auto extend-____ No. of Cycles: _____

	1X	**X_____**
dH_2O		
5X /10X PCR BUFFER		
DNTPS		
$MGCL_2$		
PRIMERS		
BSA		
TAQ POLYMERASE		
DNA TEMPLATE		

Lane 1		**Lane 2**		**Lane 3**		**Lane 4**	
Specimen ID	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**	**Specimen ID**	**Score**
1.		1.		1.		1.	
2.		2.		2.		2.	
3.		3.		3.		3.	
4.		4.		4.		4.	
5.		5.		5.		5.	
6.		6.		6.		6.	
7.		7.		7.		7.	
8.		8.		8.		8.	
9.		9.		9.		9.	
10.		10.		10.		10.	
11.		11.		11.		11.	
12.		12.		12.		12.	
13.		13.		13.		13.	
14.		14.		14.		14.	
15.		15.		15.		15.	
16.		16.		16.		16.	
17.		17.		17.		17.	
18.		18.		18.		18.	
19.		19.		19.		19.	
20.		20.		20.		20.	
21.		21.		21.		21.	
22.		22.		22.		22.	
23.		23.		23.		23.	

Appendix 9: TaqMan enzyme data form

MOLECULAR ENTOMOLOGY LABORATORY

TaqMan Enzyme Lab Data Form

Site…… Village ……..

Mosquito ID	PCR Date	GSTE2	CYP4J5	CoE

Appendix 10: *KDR* data form

MOLECULAR ENTOMOLOGY LABORATORY

***KDR* Lab Data Sheet**

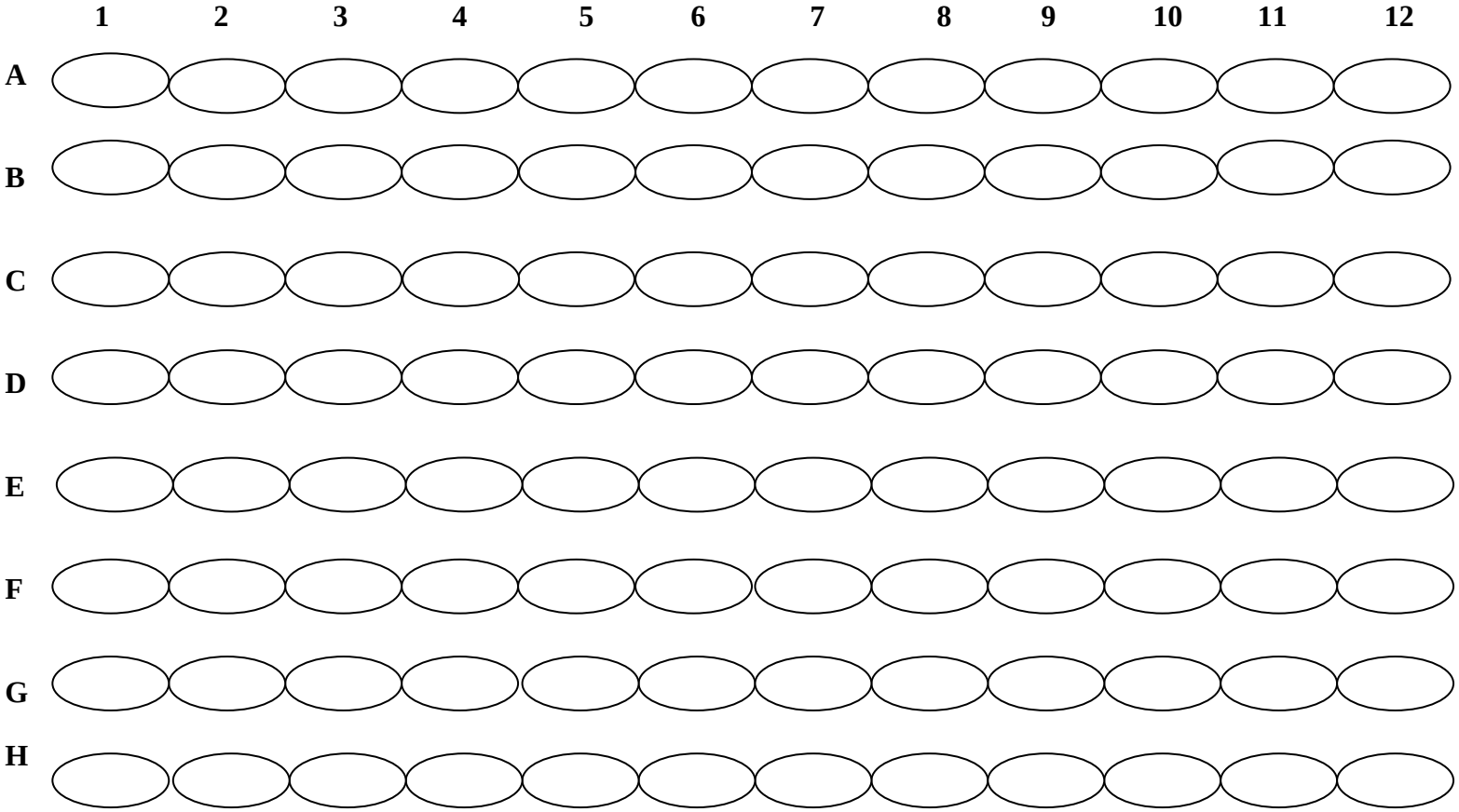
Site……..…….. Village ……..

Mosquito ID	**Test**	**Bioassay Date**	**Status (Dead/Alive)**	**DNA Extraction Date**	***KDR* East**		***KDR* West**	
					RT- PCR Date	***KDR* result**	**RT- PCR Date**	***KDR* result**

Appendix 11: Sporozoite ELISA plate form

SPOROZOITE ELISA PLATE FORM

DATE:…….. PLATE NUMBER:…… TEST: (Screen or *Pf*)…….



Appendix 12: TaqMan enzyme /*kdr* plate form

ENTOMOLOGY *KDR*/TAQMAN ENZYME PLATE FORM

DATE:…….. PLATE NUMBER:…… TEST:……..….