

**RESIDUAL EFFECTS OF INSECTICIDE-BASED MALARIA
CONTROL INTERVENTIONS ON MALARIA VECTORS AND THE
STATUS OF INSECTICIDE RESISTANCE IN WESTERN KENYA**

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DEDICATION

This work is dedicated to my parents Mr. and Mrs. Maurice Wanjala Wakhungu.

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ABBREVIATIONS AND ACRONYMS

AChE	Acetylcholinesterase
ANOVA	Analysis of Variance
CDC	Center for Disease Control
DDT	Dichlorodiphenyltrichloroethane
IRAC	Insecticide Resistance Action Board
IRS	Indoor Residual Spraying
ITN	Insecticide Treated Nets
KDR	Knockdown Resistance
LLIN	Long Lasting Insecticidal Nets
MOH	Ministry of Health
NGOs	Non Governmental Organizations
RBM	Roll Back Malaria
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
WP	Wet Powder
IVM	Integrated Vector Management

DEFINITION OF TERMS

Cross resistance - occurs when resistance to one insecticide confers resistance to another insecticide, even where the insect has not been exposed to the latter product.

Endophagic - Insects that feed indoors.

Endophilic - Insects that rest indoors after feeding.

Exophagic - Insects that feed out doors

Exophilic - insects that rest outdoors after feeding.

Insecticide resistance – selection of a heritable trait in an insect population that results in an insecticide no longer functioning as intended.

Multiple resistance – occurs when insects are resistant to more than one class of pesticide.

Pyrethroids - a group of synthetic chemicals that are structurally modified from natural pyrethrins derived from *Chrysanthemum* flowers

ABSTRACT

Malaria is a human disease caused by a sporozoan from the genus *Plasmodium*, transmitted by a bite of Anopheles mosquitoes. Insecticides remain the major tool for control of malaria vectors in Kenya and therefore the potential of such programs to be compromised by the reported insecticide resistance is a major concern. Studies in western Kenya have reported reemergence of morbidity and malaria attributed child mortality which has been linked to reported spread of insecticide resistance in anophiline mosquitoes. The aim of this study was to evaluate the residual effect of insecticides used for indoor residual spraying and impregnated on long lasting insecticide nets, and also determine the status of insecticide resistance in malaria vectors from western Kenya. Wall bioassays were performed on mud slabs and filter papers sprayed with lambda-cyhalothrin and deltamethrin using mosquitoes collected from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula. Net bioassays were performed on long lasting insecticide nets (LLINs) collected from the field using wild caught mosquitoes from Emutete and Kabula. Kisumu strain, a susceptible reference strain was used as a control. Chemical analysis of the netting material was performed using gas chromatography. World Health Organization tube bioassays was conducted using standard diagnostic dosages of Lambda-cyhalothrin, Deltamethrin, Permethrin, DDT, Bendiocarb and Malathion tested on *Anopheles* mosquitoes collected from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula. Biochemical assays, where the enzymatic activity of three enzymes (monooxygenases, esterases and Glutathione S-transferases) were performed on susceptible and resistant mosquitoes preserved after WHO tube bioassays. Mosquitoes were identified to species level using Polymerase Chain Reaction. Genotyping was done on the susceptible and resistant mosquitoes after the WHO tube bioassays using Real-Time Polymerase Chain Reaction. Pyrethroid susceptible *An. gambiae* and *An. arabiensis* colonies from Bungoma and Ahero was raised and their genetic and biochemical changes monitored from generation to generation. The mortality of mosquitoes from all sites decreased significantly with time after spraying (75% mortality after six months) and with the age of the LLINs (60% mortality after 24 months). Insecticide concentration decreased significantly from 0.14 µg/ml in new LLINs to 0.077 µg/ml in LLINs older than 18 months. WHO susceptibility tests indicated that *An. gambiae* has developed high level of resistance to pyrethroids and DDT in Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Bungoma. Resistant to bendiocarb in Iguhu and Kabula and susceptible to Malathion (100% mortality) in Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Bungoma. There was an elevation of monooxygenases and esterases enzymatic activities in resistant *An. gambiae* mosquito populations exposed to Lambda-cyhalothrin, Permethrin, Deltamethrin and DDT but no elevation in glutathione s-transferases. A high frequency of L1014S allele was detected in

An. gambiae s.s. population but there was no *kdr* allele found in *An. arabiensis* mosquitoes. Successive selection for deltamethrin resistance showed a steady increase in the mosquito mortality with 100% mortality in fifth and third generations for Kabula and Ahero mosquitoes respectively. The frequency of the homozygous ss (L1014s) allele was high in the first generation of Kabula mosquito populations but reduced in the subsequent generations. Long lasting insecticide nets should be replaced with new nets every three years and not five years as recommended and also high levels of ITN coverage and usage should be maintained. There is also an urgent need for development and deployment of non-pyrethroid based vector control tools.

CHAPTER ONE

1 GENERAL INTRODUCTION

1.1 Background information

The World Health Organization (WHO) reported that there were approximately 655,000 malaria deaths in the year 2010 in Kenya, with 86% occurring in children under 5 years (WHO, 2011). The massive scaling up of malaria vectors control measures which include indoor residual spraying (IRS), distribution and use of long lasting insecticide nets (LLINS) and larval control, has led to a tremendous decline of morbidity and mortality associated with malaria (WHO, 2012a). Currently, Long lasting insecticide treated nets (LLINs) usage and indoor residual spraying (IRS) with pyrethroids are the major components of malaria vectors control in Kenya.

There are four major classes of insecticides which include; pyrethroids, organochlorines, organophosphates and carbamates. Pyrethroids are the only class of insecticides licensed to be impregnated on long lasting insecticide nets, and also commonly used in Kenya for indoor residual spraying. Unlike indoor residual house spraying where other classes of insecticides can be deployed, LLINs can only be impregnated with Pyrethroids; therefore their efficacy is greatly affected by pyrethroid resistance (WHO, 2013a). In a recent studies, massive scale up and usage of LLINs was linked to selection of resistance in *Anopheles* mosquitoes in Senegal, where morbidity associated with malaria also increased (Trape *et al.*, 2011).

The mechanisms of insecticide resistance include: target-site resistance, metabolic resistance, and cuticular resistance. Target-site resistance to pyrethroids in *An. gambiae* and *An. arabiensis* is caused by a non-silent point mutation (either L1014F or L1014S) in the sodium channel gene, which is referred to as the knock-down resistance (*kdr*) genotype (Martinez Torres *et al.*, 1998; Ranson *et al.*, 2009). Metabolic resistance is caused by elevation of the activity of three large multi- gene families (monooxygenases, glutathione s-transferases, and carboxylesterases) that are able to metabolize or sequester the insecticide, thereby preventing it from reaching its target (Hemingway *et al.*, 2004). Cytochrome P450s (Monooxygenases) have been found to be responsible for the majority of cases of metabolic resistance, with a secondary role for the glutathione s-transferases (Muller *et al.*, 2008; Mohammed *et al.*, 2008; Awolola *et al.*, 2009; Matowo *et al.*, 2010; Mitchell *et al.*, 2012). Cuticular resistance has also been thought to contribute to resistance observed in malaria vectors, but this aspect requires further analysis (Mohammed *et al.*, 2008; Awolola *et al.*, 2009; Wood *et al.*, 2010). Various studies have reported an increase in phenotypic resistance and *kdr* frequency following the introduction and extensive usage of LLINs in some areas, which could greatly affect the efficacy of LLINs (Stump *et al.*, 2004; Ranson *et al.*, 2009; Ndiath *et al.*, 2012).

There is need for comprehensive information on residual effect of LLINs and indoor residual sprayed walls and the status of insecticide resistance in malaria

vectors. This will help the malaria control programmes to plan for the replacement of nets and also for the implementation of effective vector control programmes including resistance management strategies. This study was carried out as part of continuing vector insecticide resistance surveillance in the contrasting ecosystems such as low lands, highlands and rice irrigation scheme. The study aimed at assessing insecticidal decay on sprayed walls and insecticide treated nets and insecticide resistance as indicated by WHO cone assays, to determine the status of insecticide resistance of malaria vectors in Western Kenya.

1.2 Statement of the problem

Effective malaria control involves the use of LLINs and IRS for the control of malaria vectors. Currently pyrethroids are the predominant insecticides for control of malaria vectors (WHO, 2013a). They comprise 40% of the insecticides used annually worldwide for indoor residual spraying against malaria vectors and 100% of the WHO-recommended insecticides for the treatment of long lasting insecticide nets (Hemingway and Ranson, 2000).

In Kenya, LLINs have been mainly distributed to the groups at risk which includes; pregnant women and children under five years old through programs run by the Ministry of Health (MOH) and non-governmental organizations (Noor *et al.*, 2007; Wacira *et al.*, 2007). Consequently, LLINs coverage for children under five years old has increased rapidly from 7% in 2004 to 67% in 2011; this increase has been associated with a 44% reduction in malaria deaths

(Fegan *et al.*, 2007). Previous studies in western Kenya indicate that there is a reemergence of morbidity and malaria-attributed child mortality (Zhou *et al.*, 2010), which has been linked to the reported spread of insecticide resistance in anopheline mosquitoes.

The resistance of *Anopheles* mosquitoes to pyrethroids is one of the main obstacles against the effective control of malaria (Hemingway *et al.*, 2004). On the other hand the effect of insecticide decay in long lasting insecticide nets and indoor residual spraying programmes for malaria control is not clear with the reported insecticide resistance in malaria vectors. Therefore there is need to investigate the residual effect of insecticides on the sprayed walls and impregnated on long lasting insecticide nets on malaria vectors, and the status of insecticide resistance of malaria vectors in western Kenya.

1.3 Justification of the study

This study was conducted in seven sites; Kabula (Bungoma county), Iguhu (Kakamega county), Emutete and Emakakha (Vihiga county), Kisian, Ahero and Chulaimbo (Kisumu County). Kabula, Iguhu, Emutete and Emakakha are located in the highland fringe of Western Kenya, where malaria occurs as epidemics. Indoor residual spraying and use of long lasting insecticide nets are the key tools for malaria control in these regions. Kisian, Ahero and Chulaimbo (Kisumu County) are in the low lands of western Kenya along Lake Basin of Lake Victoria where malaria is endemic. Insecticide resistance

of malaria vectors to insecticides has also been reported in these regions (Vulule *et al.*, 1999, Stump *et al.*, 2004, Ochomo *et al.*, 2012). The aim of the study was to determine the duration of insecticides impregnated on long-lasting insecticide nets (LLINs) and sprayed on walls, which are the major malaria control interventions in these regions. This information will help to establish the efficacy of malaria vector control measures. The insecticide susceptibility profile of malaria vectors to the following classes of insecticides was also studied; Organochlorine (DDT), Organophosphate (malathion), Carbamates (bendiocarb) and Pyrethroids (Permethrin, deltamethrin and lambda-cyhalothrin), to establish the alternative insecticide to be used for malaria control. Although DDT usage was banned in Kenya, this insecticide was tested because of cross resistance with pyrethroids in mosquitoes which is brought about by their same mode of action. Furthermore the generational and biochemical changes during the selection process of pyrethroid resistant *An. gambiae* and *An. arabiensis* was studied to establish the mechanisms and selection for insecticide resistance in malaria vectors from Western Kenya.

1.4 Research questions

- i. What is the residual effect of insecticides sprayed on walls and impregnated on long lasting insecticide nets on malaria vectors in Western Kenya?
- ii. What is the status of insecticide resistance of malaria vectors in Western Kenya?

- iii. What are the genetic and biochemical changes involved during the selection of pyrethroid susceptible populations of *An. gambiae* and *An. arabiensis*?

1.5 Hypotheses

- i. There is no difference in the mortality of mosquitoes exposed to sprayed surfaces at different time interval after spraying.
- ii. There is no difference in the mortality of mosquitoes from different sites exposed to different insecticides.
- iii. There are no generational and biochemical changes involved during the selection of pyrethroid resistant populations of *An. gambiae* and *An. arabiensis*.

1.6 Objectives

1.6.1 General Objective

To determine the residual effect of the insecticides used for malaria control on sprayed surfaces and impregnated nets on malaria vectors and the status of insecticide resistance in Western Kenya.

1.6.2 Specific objectives

- i. To determine the residual effect of insecticides sprayed on walls and impregnated on long-lasting insecticide nets on malaria vectors in Western Kenya.
- ii. To determine the status of insecticide resistance of malaria vectors in Western Kenya.

- iii. To determine the generational and biochemical changes involved during the selection of pyrethroid resistant populations of *An. gambiae* and *An. arabiensis*.

1.7 Significance of the study

Results from this study have contributed to the advancement of knowledge by determining the susceptibility of *Anopheles* mosquitoes to various classes of insecticides available in Kenya. Knowing the residual effect of the insecticide on the sprayed surface and nets is important for vector control program rotations in Kenya since it indicates the minimal time interval between spraying. Knowledge about the status of insecticide resistance in malaria vectors in western Kenya is essential for public health sector in Kenya, since it helps in the identification of effective insecticides and thus management of insecticide resistance for effective malaria control in Kenya. Knowing the generational and biochemical changes involved during the selection of pyrethroid resistant populations of *An. gambiae* and *An. arabiensis* will help to identify the insecticide resistance mechanisms present in malaria vectors.

CHAPTER TWO

2 GENERAL LITERATURE REVIEW

2.1 The burden of malaria in Africa

Malaria accounts for a third of all hospital admissions and up to a quarter of all deaths of children under the age of five years (Hay *et al.*, 2004). Despite intervention programs to suppress the disease, malaria is still highly prevalent in sub-Saharan Africa (Lengeler, 2004). Some areas that were considered to be malaria-free earlier are now experiencing frequent malaria cases due to land use change, drug resistance, population pressure, environmental degradation and climatic change (Githeko and Ndegwa, 2001; Zhou *et al.*, 2004; Afrane *et al.*, 2006; Zhou *et al.*, 2010; Zhou *et al.*, 2014).

A recent upsurge of malaria in endemic areas with explosive epidemics in many parts of Africa has been attributed to many factors which include resistance of malaria parasites to anti malarial drugs, resistance of malaria vectors to insecticides, climate change and movement of human populations from highlands to lowlands (WHO, 2004; Githeko *et al.*, 2006; Wanjala *et al.*, 2011; Zhou *et al.*, 2014). Malaria transmission in the lowlands of Western Kenya is intense with residents receiving up to 300 infectious bites per person per year in endemic areas (Beier *et al.*, 1994; Hawley, 2003).

2.2 Malaria vectors and their distribution in Africa

Approximately 140 *Anopheles* species have been recorded in Africa, eight species out of these are effective malaria vectors (Gillies and de Meillon, 1968; Gillies and Coetzee, 1987). The major vectors of human malaria are *An. gambiae sensu stricto* (*An. gambiae*) and *An. arabiensis* (White, 1974), members of the *An. gambiae* complex. Other species that have also been reported include *An. quadriannulatus*, *An. melas*, *An. merus*, and *An. bwambae*. *Anopheles melas* and *Anopheles merus* have been found to breed in salt-water and therefore they are found along western and eastern coasts in Africa respectively, while *An. bwambae* prefer mineral springs in the Semliki forest in Uganda (Coluzzi, 1984). *Anopheles quadriannulatus*, found in south-east Africa (Coluzzi, 1984) and *An. quadriannulatus*, which was described in Ethiopia (Hunt *et al.*, 1998) are generally zoophilic (Coluzzi, 1984).

In addition to the *An. gambiae* complex, other species known to be important malaria vectors in Africa are the Funestus group which includes; *An. nili*, *An. moucheti* and *An. funestus*. Two African subgroups have also been described and they include: Funestus subgroup which comprises of *An. aruni*, *An. confusus*, *An. funestus*, *An. parensis*, *An. vaneedeni* and Rivulorum subgroup which comprises of *An. brucei*, *An. fuscivenosus*, *An. rivulorum*, and *An. rivulorum*-like species) (Gillies and Coetzee, 1987; Harbach, 2004). Other species, such as *An. paludis*, *An. mascarensis* and *An. hancocki* play only a limited, secondary and localized role where they are found (Fontenille and

Simard, 2004). Most of these Anophiline species have been found to occur in Africa and their role in malaria transmission varies depending on behaviour (example, biting activity, feeding and resting preferences), seasonal prevalence and vectorial capacity (Coluzzi, 1984; Fontenille and Simard, 2004). This difference is responsible for the variation in malaria epidemiological patterns observed in Africa and subsequently, different areas may require different tools and strategies for effective malaria vector control (Appendix II).

2.3 Distribution of malaria vectors in Kenya

Anopheles arabiensis has been found to be distributed in most regions in Kenya where malaria is prevalent; this species has been reported along the coast, across Western Kenya, central Kenya and north west in Turkana counties (Mushinzimana *et al.*, 2006; Bayoh, 2010). *Anopheles gambiae s.s.*, *An. arabiensis*, *An. merus* and *An. funestus* (Diptera : Culicidae) are the most important vectors of human malaria in coastal Kenya (Mbogo *et al.*, 2003). *Anopheles pharoensis* has been reported in counties along the coast, Kirinyaga, Embu, Migori, Siaya, Homabay and Kisumu counties (Beier *et al.*, 1999). In Western Kenya, the main malaria vectors are *An. gambiae sensu stricto*, *An. Arabiensis*, and *An. funestus* (Githeko *et al.*, 1996). *Anopheles gambiae* generally increases in density after the start of the long rains, while *An. funestus* density is seen to vary in direct proportion to the proximity of permanent breeding grounds rather than rainfall (Githeko *et al.* 1996).

2.4 Control of malaria vectors

Malaria vector control is important for successful malaria prevention. It has been proven to successfully reduce or interrupt malaria transmission when coverage is sufficiently high (WHO, 2013a). The two core broadly applicable measures for malaria vector control are use of long lasting insecticide nets (LLINs) and Indoor Residual Spraying (IRS) which fall under chemical control. These core vector control interventions can be supplemented by other methods such as biological control, physical control and environmental manipulation to interrupt the larval source (WHO, 2013a).

2.4.1 Chemical control

Classes of chemicals recommended for use in indoor residual spraying programs include; organochlorines, organophosphates, carbamates and pyrethroids (Himeidan *et al.*, 2012). In Kenya, lambdacyhalothrin (ICON™), a synthetic pyrethroid has been recommended for indoor residual spraying in the highlands to control malaria epidemics (Mulambalah *et al.*, 2010). The only recommended insecticides for treating bed nets are pyrethroids; permethrin, deltamethrin, cypermethrin, alphacypermethrin, cyfluthrin, and lambdacyhalothrin (WHO, 1998). The continual use of a single class of insecticide to treat nets might not be effective for long because of the threat of selecting of insecticide resistance in malaria vectors.

2.4.1.1 Indoor residual spraying

Indoor residual spraying can be defined as the spraying of all the surfaces inside and around human habitation using a suitable insecticide with a residual effect, for example, pyrethroids (permethrin, deltamethrin and lambdacyhalothrin), carbamates (bendiocarb), organochlorines (DDT) and organophosphates (malathion) (Najera and Zaim, 2001; WHOPEP, 2015; Table 2.1).

Earlier in malaria eradication campaigns, IRS was the most commonly used method for malaria vector control and was effective in developed countries (WHO, 2004). For an IRS program to be effective, it must have a high

coverage of households (usually above 70% coverage) (Guyatt *et al.*, 2002). Though IRS has been shown to be effective in control of endophilic mosquitoes, increased insecticide resistance by malaria vectors and environmental contamination due to accumulation of slowly degradable compounds are a major concern (Kere *et al.*, 1996).

Table 2.1: Insecticides used for indoor residual spraying

Insecticide	Dosage (g/m³)	Duration of Insecticidal	Insecticidal Action
Organochlorines			
DDT	1-2	6 or more	Contact
Lindane	0. 2-0.5	3 or more	Contact
Organophosphates			
Malathion	1-2	1-3	Contact
Fenithrothion	1-2	1-3 or more	Contact, airborne
Pirimiphos Methyl	1-2	2-3 or more	Contact, airborne
Carbamates			
Bendiocarb	0.2-0.4	2-3	Contact, airborne
Propoxur	1-2	2-3	Contact, airborne
Pyrethroids			
Alphacypermethrin	0.03	2-3	Contact
Cyfluthrin	0.025	3-5	Contact
Cypermethrin	0.5	4 or more	Contact
Deltamethrin	0.05	2-3	Contact
Lambdacyhalothrin	0.025-0.05	2-3 or more	Contact
Permethrin	0.5	2-3	Contact, airborne

2.4.1.2 Factors influencing the persistence of insecticides on sprayed surfaces

The duration of an insecticide on a sprayed surface depends not only on the type of insecticide and its formulation but also on the nature of the surface it is sprayed on (Mulambalah *et al.*, 2010). In most cases, insecticides last longer on wood and thatched surfaces than it does on mud. Mud surfaces absorb some insecticide leaving little insecticide on the surface to kill mosquitoes, other types of mud may also break down the insecticide chemically. For example, when malathion is sprayed on wood, it lasts for up to three months or more, whereas on mud surfaces it lasts for only three weeks (Mulambalah *et al.*,

2010). High dosages may be used, when the insecticide is applied on mud surfaces or when the insecticide is required to persist on the walls for long. The duration of an insecticide can also be influenced by its formulation (WHOPES, 2015).

2.4.1.3 Insecticide treated nets (ITNs)

Insecticide treated nets are mosquito nets impregnated with insecticides. Currently, they are the most preferred method of controlling malaria transmission (WHO, 2014). They have been shown to reduce morbidity and mortality associated with malaria in endemic regions. The insecticides impregnated nets also have repellent properties that reduce the number of vectors that enter the house thus vectors avoid houses with insecticide treated nets (Hawley, 2003). Other studies have also shown that if the ITN coverage in the community is high, the longevity of the mosquitoes is reduced (Hawley, 2003). Consequently, all the members of the community receive protection regardless of whether they have or do not have insecticide treated nets.

After introduction of long lasting insecticidal nets (LLINs), the potential to offer a superior sustainable protection was envisioned and confirmed by various studies. Long lasting insecticidal nets are currently preferable and recommended for large scale malaria control programs (Mendis, 2009; WHO, 2014). World Health Organization recommended a change from insecticide treated bed nets to long lasting insecticide nets (LLINs) combined with malaria case management for malaria control (Mendis, 2009; WHO, 2014). Malaria

control programs in endemic areas are now focusing on distribution of long lasting insecticidal nets to vulnerable populations in rural areas at subsidized prices.

2.4.1.4 Long-lasting insecticide nets (LLINs) recommended by WHO

The effect of insecticidal decay in LLINs, in areas where malaria vectors are resistant to pyrethroids is not clear. World Health Organization has recommended three types of Long lasting insecticide nets to be used: Monofilament Polyethylene nets impregnated with 2% permethrin, multifilament polyester netting treated with deltamethrin, and lastly multifilament polyester netting treated with alphacypermethrin (WHO, 2004; WHO, 2006).

Eleven brands of LLINs have been recommended for public use by WHO, they include; Duranet (polyethylene net treated with Alpha-cypermethrin); Interceptor (polyester nets impregnated with Alpha-cypermethrin); LifeNet (polypropylene nets treated with Deltamethrin); MAGNet (polyethylene nets coated with Alpha-cypermethrin); DawaPlus (polyester nets coated with Deltamethrin); Olyset Plus - (polyethylene nets treated with Permethrin and PBO); Olyset Net (polyethylene nets coated with Permethrin); PermaNet (polyester nets treated with deltamethrin); PermaNet (Combination of deltamethrin coated on polyester with strengthened border (side panels), deltamethrin and PBO incorporated into polyethylene (roof); Yorkool LN

(Deltamethrin coated on polyester) and Royal Sentry- Alpha-cypermethrin incorporated into polyethylene) (WHO 2004; WHO, 2006).

When the nets are used appropriately as recommended by manufacturers, the insecticide should last for 3 years on the treated nets. Many studies have reported reduced efficacy of LLINs before the expected time (Lindblade *et al.*, 2005; Graham *et al.*, 2005; N'Guessan *et al.*, 2007). Some nets have been shown not to interrupt mosquito feeding as expected (Okia *et al.*, 2013). The efficacy of LLINs has also been further affected by wear and frequent washing of the nets (Atieli *et al.*, 2010).

2.4.1.5 Target sites of insecticides used for malaria vector control

There are four classes of chemicals used as insecticides for controlling mosquito vectors; organophosphates, organochlorines, Carbamates and pyrethroids (Himeidan *et al.*, 2012). Pyrethroids and organochlorines are modulators of voltage-gated sodium channels. They open the sodium ion channels in neurons of insects, causing them to fire continuously, which leads to spasms and eventual death of the insect. If mutation occurs in the insect's sodium channel genes, it confers resistance to DDT and pyrethroids (Denholm *et al.*, 2002). Carbamate and organophosphate esters inhibit the action of acetylcholinesterase which is the enzyme that catalyses the hydrolysis of neurotransmitting agent acetylcholine resulting in the accumulation of the acetylcholine at the nerve synapse which leads to the paralysis of the insect (Fukuto, 1990).

2.4.2 Physical control

Various physical methods have been developed either to reduce vector population density or to prevent man-vector contact. They are designed for use in situations where insecticides are inadequate or inappropriate. Mesh screen for houses and bed nets have been in use for a long time. Mesh proofing for outlets of septic tanks, animal shades, and domestic drinking water containers effectively serves to control vector breeding in domestic environment. Impregnation of bed nets with synthetic pyrethroids enhances personal protection in malaria endemic regions (WHO, 2013a). House modification, involves designing houses with insect screen windows, ceiling to prevent mosquitoes from entering the houses. House screening can significantly reduce human-mosquito contact, especially for endophilic species which are potential vectors for malaria in humans (Lindsay *et al.*, 2003). A study conducted in a rice irrigation scheme area of western Kenya indicated that house modifications involving insect screen ceilings reduced the exposure of human to malaria vectors, and thus parasite infection (Atieli *et al.*, 2009). In some urban areas and in developed countries, houses are designed with screens on the windows, eaves and doors to deter the entry of mosquitoes and other nuisance insects (Lindsay *et al.*, 2003). Several studies have shown that transmission of many vector borne diseases is facilitated by house designs (Lindsay *et al.*, 2002; Kumar *et al.*, 2004; Kirby *et al.*, 2008). Other than protection against malaria vectors, house modification offers protection against

nuisance bites and other mosquito borne diseases (Kumar *et al.*, 2004; Ogoma *et al.*, 2010).

2.4.3 Biological control

This is one of the most rapid developing areas in alternative vector control strategies. It serves to exploit organisms that are natural enemies of vectors to regulate vector population growth. Larvivorous fish (*Gambusia*, *Tilapia* and *Poecilia*) have been widely used in malaria control, they have been used as additional measures for control of *Anopheles* mosquitoes in rice irrigation systems (*Aedes* and *Culex* in ponds and sewage systems). Spore forming bacteria namely *Bacillus thuringiensis* and *Bacillus sphaericus* have been used effectively to control mosquito larvae (Afrane *et al.*, 2016).

2.5 The burden of insecticide resistance in Africa

Insecticide resistance is the selection of a heritable trait in an insect population that results in an insecticide no longer functioning as intended (McCaffery and Nauen, 2006). Insecticide resistance in *An. gambiae* and *An. arabiensis* has been reported in many parts of Africa. In West Africa, resistance to DDT and dieldrin was first reported in the 1950s (Brown, 1958), in South Africa resistance to dieldrin and DDT has been reported (Hargreaves *et al.*, 2000; Greenwood and Mutabingwa, 2002) and in Sudan, resistance to malathion, DDT, dieldrin and permethrin has been detected (Hemingway *et al.*, 2004). Resistance to DDT and pyrethroid in *An. funestus* has been detected in

Uganda (Verhaeghen *et al.*, 2006). While in Sudan, DDT resistance in *An. arabiensis* was first detected in 1965 then malathion resistance reported later on (Etang *et al.*, 2007). Current surveys have shown high levels of resistance to DDT and permethrin by *An. arabiensis* in Sudan (Matambo *et al.*, 2007). *Anopheles arabiensis* resistance to permethrin has also been reported in Tanzania (Matowo *et al.*, 2010; Appendix VI).

In western Kenya, Vulule *et al.* (1994) reported a decrease in *An. gambiae* susceptibility to pyrethroids following installation of ITNs. Studies in western Kenya indicate that resistance levels have increased marginally. Stump *et al.*, (2004) reported an increase in *kdr* alleles frequency in *An. gambiae* from western Kenya following the massive distribution of LLINs. Other studies have reported a dramatic increase in knock down resistance allele frequency in western Kenya from 1996-2010 coincident with the scale up of ITNs and a fixation of *kdr* L1014S alleles in *An. gambiae* populations but absent in *An. arabiensis* populations (Mathias *et al.*, 2011). Studies in central Kenya have also indicated that *An. arabiensis* is susceptible to most insecticide (Kamau and Vulule, 2006). Although insecticide-resistant genes have been observed widely in *An. gambiae* populations, currently no resistance has been observed in *An. arabiensis* species from Kenya (Baliraine *et al.*, 2009; Ochomo *et al.*, 2013).

2.6 Mechanisms of insecticide resistance in malaria vectors

The major mechanisms responsible for insecticide resistance include; target site mutation and metabolic resistance. Changes in the insecticide target site reduce the binding of the insecticide to the target whereas an increase in the insecticide metabolism lowers the amount of insecticide reaching the target site (Sharp *et al.*, 2007; Ridl *et al.*, 2008). Although these two mechanisms clearly play a major role in conferring insecticide resistance in mosquitoes. Physiological and behavioral changes in the mosquito population may also play a role in insecticide resistance.

2.6.1 Metabolic resistance

Metabolic resistance is the most common resistance mechanism that occurs in insects. This mechanism is based on the enzyme system that detoxifies any foreign substances in the insects. Three categories of enzymes that play a role in the detoxification of insecticides includes; monooxygenases, esterases and glutathione S-transferases (Hemingway and Ranson, 2000). These enzymes are usually elevated in insecticide resistant mosquitoes enabling them to metabolize or degrade insecticides before they are able to exert their toxic effects. Esterases enzymes hydrolyze ester bonds or sequester insecticides (Hemingway *et al.*, 2004).

2.6.2 Target site mutation

There are three major targets for insecticides in the insects which include; acetylcholinesterase (AChE), voltage-gated sodium channels and ligand-gated ion channels. Acetylcholinesterase breaks down the neurotransmitter acetylcholine in the nerve synapses (Martinez Torres *et al.*, 1998). Acetylcholinesterase is the target site for organophosphates and carbamates, which inhibit the function of AChE (Weill *et al.*, 2002). The sodium channels are the target site for DDT and pyrethroids, which give rise to so-called knockdown resistance (*kdr*), including *An.* species (Martinez Torres *et al.*, 1998; Soderlund and Knipple, 2003).

2.6.3 Behavioral changes

Behavioural changes are considered to be contributing factors that lead to the avoidance of lethal doses of an insecticide. Avoidance behavior, also known as excitorepellency, can be either natural (protective avoidance) or developed (behavioral resistance) (Muirhead-Thomson, 1960). There have been several reports of mosquitoes changing their behavior as a result of intensive indoor use of insecticides. Currently there is little data on behavioural avoidance traits which are genetic or adaptive responses (Bogh *et al.*, 1998). Genetic changes in malaria vector populations that shifted feeding or resting behaviour to minimize contact with insecticides indoors could have a dramatic impact on the efficiency of current malaria vector control interventions. There is need for control studies to quantify the extent of this behavioural change and assess

whether scaling up of ITNs and IRS could increase the importance of outdoor transmission of malaria and necessitate new tools to target malaria vectors that are exophagic and exophilic.

2.6.4 Cuticular resistance

Reduced uptake of insecticide often referred to as cuticular resistance is considered a minor mechanism of resistance. In malaria vectors where insecticides are typically delivered on bed nets or wall surfaces, uptake of the insecticides is primarily through the appendages. Hence an increase in the thickness of the tarsal cuticle, or reduction in its permeability for lipophilic insecticides could have a major impact on the bioavailability of the insecticides in vivo (Ranson *et al.*, 2011). Microarray experiments have identified two genes (*cplcg3* and *cplcg4*), encoding for cuticular proteins that are up regulated in pyrethroid resistant strains of *Anopheles* mosquitoes (Awolola *et al.*, 2009).

CHAPTER 3

3.0 GENERAL MATERIALS AND METHODS

3.1 Study Sites

The study was conducted in seven sites from Western Kenya namely Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula. Ahero (Latitude 00.17259°S, Longitude 034.91983°E, altitude 1162-1360m above the sea level), Chulaimbo (Latitude 00.03572° S, Longitude 034.62196°E, altitude 1328-1458m above the sea level) and Kisian (Latitude 00.02464° S, Longitude 033.60187°E, altitude 1280-1330m above the sea level) are located in Kisumu County (Low lands); Emutete (Longitude 34°64' E, Latitude 00°22' N, elevation 1,463 – 1,603 m above the sea level) and Emakakha (Longitude 34°64'E, Latitude 0°22'N, 1,463–1,604 m above sea level), are found in Vihiga County (highlands); Iguhu (Latitude 0°17' N, Longitude 34°74' E, and elevation 1,450–1,580 m above sea level) is located in Kakamega County (highland) and Kabula (Longitude 00.54057 N, Latitude 034.56410°E, altitude 1545m above the sea level) is located in Bungoma County (highland) (Appendix I).

Malaria transmission is perennial in the lowland sites of Ahero, Kisian and Chulaimbo. A mixture of *An. gambiae sensu strict (s.s.)* and *An. arabiensis* is expected in all lowland sites (Githeko *et al.*, 1996) except Ahero, where only *An. arabiensis* has been collected since the late '90s (Stump *et al.*, 2004). The highland sites of Emutete, Emakakha, Iguhu and Kabula show low seasonal

malaria transmission, with peaks at the end of the long (early April to early June) and short (October-November) rainy seasons and high year -to-year variation. The prevalent malaria vector in the highlands is *An. gambiae s.s.* (Githeko *et al.*, 1996).

Agricultural activity is particularly intense in Ahero and Kabula. In Ahero the principal crop harvested is rice where as in Kabula sugar cane is the main crop cultivated, the site and its environs being the home of the largest sugar cane factory in Kenya (Nzoia and Mumias Sugar companies). In Iguhu, Emutete and Emakaha there are numerous small holder sugar mills, and maize, millet and sorghum are also grown for subsistence. Emutete and Emakakha are characterized by large tea plantations, with smaller cultivation of maize, potatoes and bananas. Chulaimbo and Kisian have low cultivation of potatoes, bananas and maize. Crops like Maize, sweet potatoes bananas and garden vegetables are grown in all the sites, Apart from cash crops such as rice, cultivated intensively in Ahero, and sugar cane, harvested in Kabula, the other crops grown a similar in Ahero and Kabula include: mainly maize, sweet potatoes, bananas and garden vegetables.

The average annual rainfall for this region is approximately 2,000 mm (Mushinzimana *et al.*, 2006; Ndenga *et al.*, 2006) with long rainy seasons from April to June and short rainy seasons from October to November. Due to population pressure, natural swamps within valley bottoms in these three study areas are being reclaimed for crop cultivation by digging open water drains

(Ndenga *et al.*, 2006). Intergrated malaria control in these sites involves indoor residual spraying with pyrethroids, use of long lasting insecticidal nets treated with deltamethrin (Atieli *et al.*, 2010) and larval control using larvicides, filling in ponds, draining stagnant water and clearing of bushes (Ndenga *et al.*, 2006).

3.2 Sampling design for the study sites

Seven study sites were selected randomly from western Kenya, basing on the availability of larval habitats, malaria control activities present and reported insecticide resistance. Mosquito larval habitats were also selected randomly from the study sites, where the mosquito larvae was collected and transferred to the insectary at KEMRI Kisumu for rearing.

3.3 Collection of mosquito larvae

Anopheles mosquito larvae were collected from various aquatic habitats using mosquito dippers and hand pipettes. The samples were then transported to an insectary within KEMRI, Kisumu. They were placed in pans containing spring water and fed on a mixture of fish food and brewer's yeast that were changed every day. Upon pupation, individual pupae was transferred to cages and allowed to emerge as adults that were fed on 10% sucrose in cotton swabs (Appendix III).

CHAPTER FOUR

4.0 THE RESIDUAL EFFECT OF INSECTICIDES SPRAYED ON WALLS AND IMPREGNATED ON LONG-LASTING INSECTICIDE NETS ON MALARIA VECTORS

4.1 Introduction

Malaria is now considered to be a major problem particularly in sub-Saharan Africa where over 80% of malaria cases have been reported, despite global efforts to control the disease (WHO, 2012a). Long lasting insecticide nets (LLINs) and indoor residual spraying (IRS) are the key tools currently used for malaria vector control (WHO, 2012a). Factors influencing the efficacy of LLINs and IRS programmes for malaria control include; adherence to the recommended procedure for the application of the insecticide, resistance of the *Anopheles* mosquito population to the insecticide and the duration of the insecticides on the sprayed walls and insecticide treated nets (WHO, 2013a). There is growing concern as to whether LLINs and IRS are sufficient for effective control of *Anopheles gambiae* mosquito population in western Kenya, which is increasingly becoming resistant to the available Pyrethroids.

Insecticide resistance in malaria vectors has been widely studied in Africa, and it has been detected in all major malaria vectors with multiple and cross resistance being commonly found in malaria endemic regions (Santolamazza, *et al.*, 2008; Ranson *et al.*, 2009; Edi *et al.*, 2012; Coetzee and Koekemoer, 2013; Protopopoff *et al.*, 2013; Abdallah *et al.*, 2014; Reveron *et al.*, 2014;

Randriamaheriyoana *et al.*, 2015; Cisse *et al.*, 2015; Wanjala *et al.*, 2015; Mutunga *et al.*, 2015; Glunt *et al.*, 2015). Insecticides may lose their efficacy over time because of chemical degradation and availability of the insecticide on the surface sprayed or impregnated with the insecticide, which may be affected by the ability of the sprayed surface to absorb and retain the insecticide (Mulambalah *et al.*, 2010). Reduction of the efficacy of ITN/LLIN and IRS control programs eventually leads to an increase in malaria cases (N'Guessan *et al.*, 2007; Zhou *et al.*, 2013).

The time duration of insecticides on sprayed walls in IRS programs has been previously studied. For example, the duration of DDT on the sprayed walls was 6 to 12 months, where as that of lambda-cyhalothrin was found to be 3-4 months (WHOPEP, 2015). The loss of efficacy of the insecticide with time in LLINs is not clear, particularly in areas where mosquitoes are resistant to pyrethroids. The classes of LLINs are recommended by WHO include permethrin-incorporated net, deltamethrin-coated net and alphacypermethrin-coated (WHO, 2004, 2006). The duration of the insecticide on the nets should be 3 years, if the net is used well as recommended by the manufacturers. However, several studies have reported reduced efficacy, of the nets before three years elapse (Graham *et al.*, 2005; N'Guessan *et al.*, 2007; Lindblade *et al.*, 2008).

The recent spread of insecticide resistance across Africa has been linked to extensive use of pyrethroids for malaria control. For instance, resistance to

pyrethroid in *An. gambiae* has been reported in 27 sub-Saharan African countries, where as resistance in *Anopheles arabiensis* has been reported 14 sub-Saharan countries and in *An. funestus* in at least 4 countries (Ranson *et al.*, 2009). The high levels of knockdown resistance (*kdr*) allele frequency have also been observed in *An. gambiae* populations geographically spread across Africa (Santolamazza *et al.*, 2008; Ranson *et al.*, 2009; Mathias *et al.*, 2011; Yadouleton *et al.*, 2011, Dabire *et al.*, 2012; Koffi *et al.*, 2012). The scale up of ITN and IRS programs has been reported to select for higher insecticide resistance in *Anopheles* mosquito populations in western Kenya (Stump *et al.*, 2004). The major concern for effective malaria control is the efficacy of old LLINs nets in areas where mosquito populations are resistance to the insecticide used for the intervention, and the time intervals within which the houses should be sprayed again after one round of IRS for effective control of malaria vectors.

The objective of this study was to assess the residual effect of insecticides sprayed on walls and impregnated on long-lasting insecticide nets for malaria control in western Kenya, in the regions with moderate to high insecticide resistance in *Anopheles* mosquitoes. This information will help to plan the malaria vector control programs, such as how frequent the long lasting insecticide nets should be replaced and after how long the houses should be re-sprayed in IRS programs.

4.2 Materials and Methods

Lambdacyhalothrin (ICON) and Deltamethrin were the insecticides used for indoor residual spraying whereas deltamethrin was impregnated on long-lasting insecticide nets in malaria control programmes in western Kenya. Lambdacyhalothrin (ICON 10CS) and deltamethrin are pyrethroid insecticides and used for IRS control of malaria vectors. Lambdacyhalothrin is designed to last on the sprayed wall for up to six months whilst deltamethrin last for three months on walls and insecticides, impregnated on long lasting insecticide nets are designed to be effective for five years. To evaluate the persistence of Lambdacyhalothrin (ICON) and deltamethrin on sprayed walls, wall bioassays were conducted on sprayed mud slabs using mosquitoes from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu Kabula and Kisumu strain as a control. To evaluate the persistence of insecticides impregnated on LLINs two experiments were carried out, net cone bioassays using mosquitoes from Kabula and Emutete, and net chemical analysis of the nets.

4.2.1 Sample size determination

World health organization recommends that 120 -150 mosquitoes should be tested for any insecticide, with at least four replicates of 20 -25 mosquitoes per test and a minimum of 50 mosquitoes used as control. In wall bioassays, a sample of 125 mosquitoes was tested per site against each insecticide (25 mosquitoes, 5 replicates). For the net con bioassays, WHO recommends that three pieces of nets are obtained from each net, with at least 10 replicates of 10 mosquitoes. In this study 120 mosquitoes were tested on each (10 mosquitoes, 12 replicates) (WHO, 2013c).

4.2.2 Preparation of mud slabs and wall bioassays

The mud slabs were made using mud in petri dishes with circumference of 15 centimeters. Five houses were randomly selected in Emutete and Emakakha villages where the indoor residual spraying programs with lambdacyhalothrin and deltamethrin were conducted respectively. The mud slabs and filter papers were attached on the wall of the houses using nails. Eight mud slabs and filter papers were attached on the houses to be sprayed with lambdacyhalothrin (ICON) where as four mud slabs and filter papers were attached on the houses to be sprayed with deltamethrin. This was because lambdacyhalothrin (ICON) is designed to persist on the sprayed walls for six months and deltamethrin for three months (Table 2.1). One hundred grams of lambdacyhalothrin was diluted in 1litre of water, and then 62.5ml of diluted insecticide was further diluted in 10litres of water which was sprayed on the wall as recommended by

the manufacture. The insecticide was sprayed on the walls using Hudson X-pert compression sprayer (10 litres capacity), the same procedure was repeated for deltamethrin insecticide.

One mud slab and filter paper were taken from each house every month and taken to the laboratory in KEMRI, for bioassays. The bioassays were carried out for eight months in order to evaluate the residual effect of lambda-cyhalothrin (ICON) and for four months to evaluate the residual effect of deltamethrin using standard WHO cones. The cones were fitted tightly on the surface of each mud slab and filter paper (Figure 4.1). Twenty five female *An. gambiae* which were 2-5 days old, collected as described in section and 3.2 gently put into each cone. The mosquitoes were then exposed to the treated mud walls for 30 minutes. At the end of exposure time, the live mosquitoes were transferred into the clean paper cup with cotton wool pad soaked in 10% sugar solution and mortality was recorded after 24 hours recovery period. Laboratory-reared susceptible Kisumu mosquito strain was used as a control. One mud slab that had not been sprayed was used as a negative control. Every month mosquitoes 125 from each of the seven study sites were tested on five replicates of treated mud slabs. Tests were carried out at the temperature of $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $80\% \pm 10\%$ relative humidity during the exposure time and the subsequent recovery period, with a 12D:12N photoperiod.



Plate 4.1 Cones attached to the mud slabs for the bioassays

4.2.3 Collection of the long-lasting insecticide nets

Olyset LLINs, the commonly used insecticide treated net in Kenya, is made up of a single filament polyethylene, blended with 2% permethrin as an active ingredient at the concentration of 1000mg permethrin per m². Samples of 30 old LLINs were randomly collected from Emutete and Iguhu villages. Household heads were surveyed for the ages of the nets and given new nets. The ages of the nets were further checked by a questionnaire survey to the spouse of the household heads on the months that the nets were first used, and also checked with the health centre administrators on LLINs mass distribution years and months.

The collected nets were coded, packaged separately in to plastic bags and transported to KEMRI Kisumu laboratories where they were stored at 4°C to

be used for bioassays and chemical analysis. Six pieces of netting materials, 30cm x 30cm were cut, with two pieces from the roof panel, upper side panel and lower side panel from each net. Three pieces from each position were used for net cone bioassays to determine their insecticidal effects, and the remaining three pieces for insecticide concentration analysis. One new unused net was used as a positive control.

4.2.4 Net cone bioassays

Net bioassays were conducted on 30 cm x 30 cm pieces cut from three positions (roof panel, upper side, lower side) from each net sample; four standards cones were fixed with a plastic manifold onto each of the four netting pieces. Ten 2–5-day-old female *An. gambiae* mosquitoes were then released in each cone and exposed to the nets for 3 minutes. After exposure, the live mosquitoes were transferred from the cones in paper cups, provided with sugar solution for 24hrs. Knock-down was measured 60 minutes after exposure and mortality measured after 24 hrs. A negative control (new net) was included in each round of cone bioassay testing. Laboratory reared susceptible Kisumu mosquito strain was used as a control. Each net was tested with twelve replicates and 120 mosquitoes from each site (WHO, 2013c).

4.2.5 Chemical analysis of the Long Lasting Insecticide Nets (LLINs)

To test for the insecticide concentration in the LLINs, Three pieces of the nets (30cm x 30cm) were cut from the three positions of each sampled net (roof

panel, lower side and upper side) were placed in a glass test tube. The netting materials were then immersed in to an extraction solvent (4:1 hexane : chloroform solution) then vortexed for 1 minute. The netting materials were incubated at room temperature for 10 minutes and then filtered through 0.45 μm PTFE (polytetrafluoroethylene) membrane. The contents were diluted to a final volume of 10ml. An aliquot of the 10ml solution was analysed by gas chromatography (You, 2007). Molecular grade of permethrin and deltamethrin with known concentration (1mg/ml in 4hexane:1chloroform, stabilized with 0.1% acetic acid) was used as an internal standard to quantify the concentration of permethrin and deltamethrin. Three independent insecticide extractions and gas chromatography analysis were conducted for each net.

4.2.6 Data analysis

Observed mortality of mosquitoes from different sites was calculated using the formula (WHO, 2013 b);

$$\text{Observed mortality} = \frac{\text{Number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

If the mortality of the mosquitoes exposed to the control mud slab was $\geq 5\%$ and $\leq 20\%$ then the mortality was corrected using Abbott's formulae (Abbott, 1925);

$$\text{Corrected mortality} = \frac{(\% \text{ observed mortality} - \% \text{ control mortality})}{(100 - \% \text{ control mortality})} \times 100$$

If the control mortality was $\geq 20\%$ then the test were discarded.

Data on the mortality of mosquitoes in eight sites exposed to different treatments of lambda-cyhalothrin and deltamethrin was analyzed using one-way Analysis of Variance (ANOVA). Mortalities between two treatments (filter paper and mud wall) were analyzed using t-test. Data on the monthly mortality of mosquitoes from different sites was analyzed using one-way ANOVA. Pearson correlation was used to compare the relationship between the age of the net and the chemical content of the net. The mortalities of mosquitoes exposed to different ages of the net were analyzed using one-way ANOVA and the mosquito mortality in two mosquito populations (Kabula and Emutete) exposed to the LLINs was analyzed using a paired t-test. The entire mean mortality was separated using Tukey HSD test.

4.3 Results

4.3.1 Mortality of mosquitoes in the seven sites exposed to mud slabs sprayed with lambda-cyhalothrin

Kisumu strain mosquitoes (control) had the highest mean mortality but did not differ significantly from that of Ahero. The mean mortality of mosquitoes from Ahero did not differ significantly from those of Kisian, Chulaimbo, Emakakha, Emutete and Iguhu. Kabula mosquitoes had a significantly lower mean mortality ($F = 6.61$, $P = 0.0001$; Table 4.1).

Table 4.1: Mean percentage mortality (\pm SE) of mosquitoes from seven sites exposed to mud slabs sprayed with lambda-cyhalothrin

Site	N	Mean \pm SE
Kisumu (control)	125	100.00 \pm 0.00a
Ahero	125	92.00 \pm 4.24a
Kisian	125	89.25 \pm 6.45ba
Chulaimbo	125	87.30 \pm 4.5ba
Emutete	125	86.75 \pm 4.57ba
Emakakha	125	88.50 \pm 5.00ba
Iguhu	125	88.0 \pm 3.74ba
Kabula	125	80.0 \pm 3.56c

Mean values denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F = 6.61$, $P < 0.001$

4.3.2 Monthly mortality of mosquitoes exposed to mud slabs sprayed with lambdacyhalothrin

The mean mortality of mosquitoes immediately after spraying (start) was the highest but it was not significantly different from the mortality in the 1st, 2nd, 3rd and 4th months after spraying. The mortality dropped significantly in the 5th month, but did not differ significantly with the mortality in 6th and 7th months after spraying ($F = 8.60$, $P = 0.0001$; Table 4.3).

Table 4.2: Mean percentage mortality (\pm SE) of mosquitoes from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula exposed to mud slabs sprayed with lambdacyhalothrin within a period of 8 months

Time Duration (Months)	Mean \pm SE
Start	88.25 \pm 2.15a
1	84.00 \pm 2.72a
2	81.63 \pm 3.15a
3	79.25 \pm 3.37a
4	77.00 \pm 3.99 a
5	75.00 \pm 4.29b
6	72.88 \pm 4.29 b
7	70.88 \pm 4.65b

Mean values denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F = 2.66$, $P = 0.019$.

4.3.3 Monthly mortality of mosquitoes in the seven sites exposed to filter papers sprayed with lambda-cyhalothrin

The mean mortality of mosquitoes immediately after spraying (start) was the highest but it was not significantly different from the mortality in the 1st and 2nd months after spraying. Similarly, the mortality in the 2nd month did not differ significantly with the mortality in the 3rd and 4th months. The mean mortality decreased significantly in the 5th, 6th and 7th months ($F = 8.60$, $P = 0.0001$; Table 4.3).

Table 4.3: Mean percentage mortality (\pm SE) of mosquitoes from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula exposed to filter papers treated with lambda-cyhalothrin within a period of eight months

Time Duration (Months)	Mean \pm SE
Start	79.41 \pm 9.96a
1	75.48 \pm 9.50a
2	72.94 \pm 9.38ba
3	70.16 \pm 8.79b
4	63.48 \pm 11.12 b
5	61.52 \pm 10.81c
6	59.90 \pm 10.62c
7	58.52 \pm 10.47c

Mean values denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F = 8.60$, $P < 0.001$

4.3.4 Comparison between mortalities of mosquitoes from Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula exposed to mud slabs and filter papers sprayed with lambdacyhalothrin

The mean mortality of mosquitoes exposed to filter papers sprayed with lambdacyhalothrin was significantly lower than that of mud slabs sprayed with lambdacyhalothrin ($t = 2.61$, $P = 0.014$; Table 4.4).

Table 4.4: Mean percentage mortality (\pm SE) of mosquitoes between filter paper and mud slab treatments sprayed with lambdacyhalothrin

Treatments	Mean mortality \pm SE	t-value	P-value
Filter paper	67.68 \pm 1.3 a		
Mud slabs	78.61 \pm 1.7 b	2.61	0.014*

*P-value is significantly different tested at 95% CI.

4.3.5 Mortality of mosquitoes in the seven sites exposed to mud slabs sprayed with deltamethrin

Kisumu strain mosquitoes (control) had significantly higher mortality compared to the other study sites ($F = 4.00$, $P = 0.0001$). The mortality of mosquitoes from Ahero did not differ significantly from those of Kisian, Chulaimbo, Emakakha Emutete and Iguhu. Kabula mosquitoes had the lowest mortality which differed significantly from Ahero, Kisian, Chulaimbo, Emakakha, Emutete and Iguhu mosquitoes (Table 4.5).

Table 4.5: Mean percentage mortality (\pm SE) of mosquitoes exposed to mud slabs sprayed with deltamethrin in the seven sites

Site	N	Mean \pm SE
Kisumu (Control)	125	81.49 \pm 13.57a
Ahero	125	70.00 \pm 11.71b
Kisian	125	70.30 \pm 9.32b
Chulaimbo	125	68.31 \pm 9.26 b
Emutete	125	68.70 \pm 9.13b
Emakakha	125	69.97 \pm 9.47b
Iguhu	125	69.13 \pm 9.48 b
Kabula	125	59.23 \pm 9.84c

Mean values denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F = 4.00$, $P < 0.001$

4.3.6 Monthly mortality of mosquitoes exposed to Mud slab sprayed with deltamethrin

The mortality of mosquitoes exposed to mud slabs immediately after spraying was high but did not differ significantly from the mortality of mosquitoes in first and second months after spraying ($F=9.74$, $P=0.0001$). The mortality of mosquitoes in the third month after spraying was significantly higher than that of the first month but did not differ significantly from the mortality of mosquitoes in the second month after spraying (Table 4.6).

Table: 4.6: Mean percentage mortality (\pm SE) of mosquitoes exposed to exposed to mud slab sprayed with deltamethrin in the three months

Month	Mean \pm SE	F-value	P-value
Start	81.857 \pm 3.388a		
1	78.143 \pm 4.100a		
2	73.286 \pm 4.536ab		
3	70.71 \pm 4.716b	9.74	0.0001

Mean values in the same column denoted by similar letters are not significantly different at $P=0.05$. Mean separated using Tukeys HSD.

4.3.7 Mortality of mosquitoes exposed to filter paper treatment sprayed with deltamethrin in the three months

The mortality of mosquitoes exposed to mud slabs immediately after spraying (start) was high but did not differ significantly from the mortality of mosquitoes in first and second months after spraying. The mortality of mosquitoes in the third month after spraying was significantly lower than that of the first month but did not differ significantly from the mortality of mosquitoes in the second month after spraying (F=6.84, P=0.002; Table 4.7).

Table 4.7: Mean percentage mortality (\pm SE) of mosquitoes exposed to filter paper sprayed with deltamethrin in the three months

Month	Mean \pm SE	F-value	P-value
Start	88.857 \pm 4.375a		
1	85.714 \pm 4.386a		
2	81.286 \pm 4.821ab		
3	78.857 \pm 4.488b	6.84	0.002

Mean values in the same column denoted by similar letters are not significantly different at P= 0.05. Mean separated using Tukeys HSD

4.3.8 Comparison of mortalities of mosquitoes between mud slab and filter paper deltamethrin treatments

The mortality of mosquitoes exposed to filter paper treatment was significantly higher compared to the mortality of mosquitoes exposed to mud slab treatments ($t = 19.94$, $P = 0.0001$; Table 4.8).

Table 4.8: Mean percentage mortality (\pm SE) of mosquitoes between filter paper and mud slab treatments sprayed with deltamethrin

Treatments	Mean mortality \pm SE	t-value	P-value
Filter paper	83.68 \pm 5.81a		
Mud slabs	76.00 \pm 5.92b	19.94	0.0001*

*P-value is significantly different tested at 95% CI.

4.3.9 Net chemical content recorded in the long lasting insecticide nets of different ages

The new nets had the highest chemical content but it did not differ significantly from the mean chemical content of the 6 and 12 months old nets. The mean chemical content was significantly lower in the 36 months old nets but did not differ significantly from that of 18, 24 and 30 months old nets ($F= 7.95$, $P < 0.001$; Table 4.9).

Table 4.9: Mean net chemical (permethrin) content $\mu\text{g/ml}$ ($\pm\text{SE}$) and the ages of the nets

Age of the nets (months)	Mean \pm SE
0	0.140 \pm 0.00a
6	0.128 \pm 0.041a
12	0.136 \pm 0.039a
18	0.084 \pm 0.015b
24	0.068 \pm 0.013b
30	0.068 \pm 0.013b
36	0.056 \pm 0.011 b

Mean values denoted by similar letters are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F= 7.95$, $P < 0.001$.

4.3.9.1 Correlation between the age of the nets and net chemical content

(Permethrin)

There was a significant negative correlation between net chemical content and the age of the nets ($r = -0.938$, $P = 0.002$). The net chemical content declined with the age of the net (Figure 4.1).

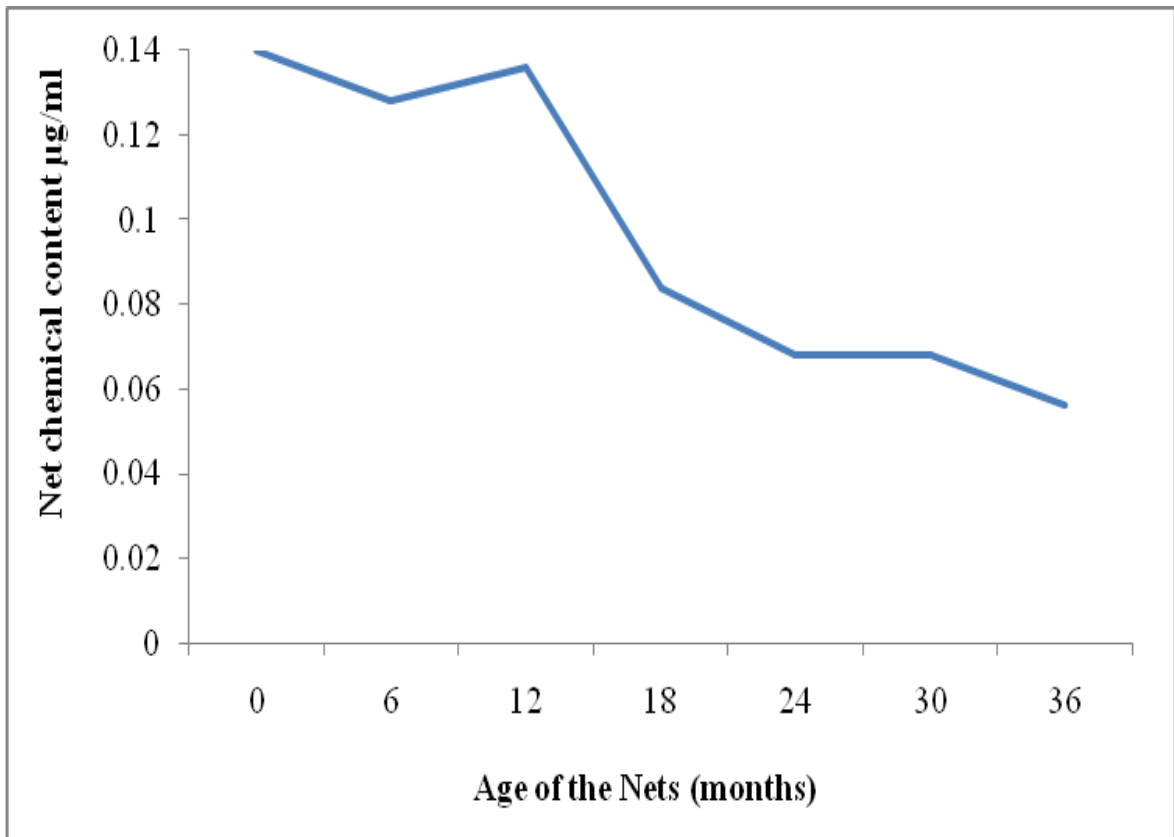


Figure 4.1 Change in the levels of chemicals recorded in different ages of the net

4.3.10 Mosquito mortalities exposed to different ages of the nets

The mean mortality of Emutete mosquitoes exposed to new nets was significantly higher than that of those exposed to six months old nets (Table 4.10). The mortality of mosquitoes reduced significantly when exposed to 12 months old nets which did not differ significantly from the mortality of mosquitoes exposed to 18 months old nets. The mortality of the mosquitoes exposed to 36 months old nets was significantly lower compared to that of the new nets as shown in table (Table 4.10).

The mean mortality of mosquitoes from Kabula was significantly high when exposed to the new nets but decreased significantly in the older nets (Table 4.10). There was a significant difference between the mortality of mosquitoes exposed to six months old, 12 months old, 18 months old but there was no significant difference between the mortality of mosquitoes exposed to 30 months old nets and 36 months old nets (Table 4.10). Kabula Mosquitoes had a significantly lower mortality compared to Emutete mosquitoes when exposed to all age groups of the nets ($F= 58.93$, $F = 74.77$ $P <0.001$; Table 4.10)

Table 4.10: Mean mortality percentage (\pm SE) of mosquitoes from Emutete and Kabula exposed to different age of the nets

Age of the nets (Months)	N	Mean mortality \pm SE (Kisumu strain)	Mean mortality \pm SE (Emutete)	Mean mortality \pm SE (Kabula)	t-value	P- value
0	120	100	85.0 \pm 0.00a	78.0 \pm 0.00a	-	-
6	120	100	72.4 \pm 3.50b	70.0 \pm 2.236b	1.16	0.310
12	120	100	67.2 \pm 0.84c	63.8 \pm 2.86c	2.312	0.082
18	120	100	66.2 \pm 1.30c	58.4 \pm 2.41d	11.76	0.0001*
24	120	100	61.6 \pm 1.34d	54.0 \pm 1.58e	7.76	0.001*
30	120	100	60.0 \pm 2.50d	50.0 \pm 1.48f	6.29	0.003*
36	120	100	54.8 \pm 0.84e	48.8 \pm 1.92f	5.26	0.006*

Mean values denoted by similar letters in same row are not significantly different at $P \leq 0.05$. Mean separated using Tukey HSD. $F = 58.93$ and $F = 74.77$ respectively, $P < 0.001$.

*Indicates a mean difference in mosquito mortality in the two sites test using paired t-test at 95% Confidence interval.

4.4 Discussion

Results of this study reported a marked variation in the mortality of mosquitoes exposed to different insecticides, sprayed surfaces, study sites, time duration of the sprayed surfaces, chemical content of the nets and the age of the nets.

When exposed to mud slabs treated with lambda-cyhalothrin, the mean mortality of mosquitoes from Ahero was the highest where as that of Kabula mosquitoes was the lowest but did not differ significantly to that of Iguhu, Emutete, Emakakha Chulaimbo and Kisian. A similar trend was observed when the mosquitoes were exposed to mud slabs treated with deltamethrin. The difference in the mortality rates of mosquitoes from different sites indicates the presence of insecticide resistance in some areas like Kabula which recorded the lowest mortality in all treatments. The difference can also be attributed to the geographical and topographical features of the study sites that have a direct effect on the survival and longevity of mosquitoes (Githeko *et al.*, 2006; Wanjala *et al.*, 2011). This observation coincides with previous similar studies that reported the presence of insecticide resistance in *Anopheles gambiae* mosquitoes from Bungoma and high susceptibility in Ahero mosquitoes (Stump *et al.*, 2004; Mathias *et al.*, 2011; Ochomo *et al.*, 2013; Ochomo *et al.*, 2014). The difference in the mortality of mosquitoes with site can also be explain by the difference in the agricultural activity and usage of pesticides which has been linked to the selection of insecticide resistance (Ranson *et al.*, 2000; Wanjala *et al.*, 2015).

Results of this study also showed that the activity of lambda-cyhalothrin decreased significantly within the first three months after spraying, whereas the activity of deltamethrin declining significantly one month after spraying, becoming ineffective earlier than the time they would normally be due for re-spraying. According to World Health Organization Pesticide Evaluation Scheme (WHOPES) recommendations, an ideal insecticide should have a minimum effect of $\geq 80\%$ mosquito mortality at 24 hours post exposure on the sprayed surface for 30 minutes (WHO, 2003). Lambda-cyhalothrin has been formulated to last on the sprayed walls for 6 months, whereas deltamethrin is designed to persist on the sprayed walls for 3 months (WHO, 1990). Previous studies in Tanzania, reported that Lambda-cyhalothrin was effective after seven months of spraying on the wall recording 100% mortality of *An. gambiae* s. l. (Curtis *et al.*, 1998). In other countries like Vietnam, Lambda-cyhalothrin (ICON) persisted on wood for up to four months, on bamboo for five months, and on bricks for three months in bioassays against *An. dirus* (WHOPES, 2007). In Benin, trial by WHOPES reported that capsules suspensions and wet powder of Lambda-cyhalothrin formulations with 30 mg/m² lasted for up to two months, where as in other trials in India the same formulations lasted for up to four to six months on the sprayed walls (WHOPES, 2007). Therefore, basing on the mortality of mosquitoes, the factors influencing the efficacy and persistence of insecticides on sprayed surfaces include; the type of the sprayed surface, the dosage of the insecticide and the age of spray deposits (Giga and

Canhao, 1991; Villarreal, *et al.*, 1995; Curtis *et al.*, 1998; Rozilawati *et al.*, 2005; WHOPEs, 2007; Raghavendra *et al.*, 2011). This study provides important evidence for programmatic decision making. Lambda-cyhalothrin and deltamethrin have been widely used for indoor residual spraying programmes to control malaria vectors in Kenya. The duration of insecticides on the sprayed walls was largely considered to be 6 months according to the manufacturers for proper functioning of IRS programmes. However, this perception and practice is not ideal, since findings of the study revealed a much shorter residual effect of 3-4 months on mud walls which constituted 80% of the houses in the study areas.

Results of this study showed significant low mortality rates of mosquitoes when exposed to mud slab treatments compared to filter paper treatments. This could be due to the ability of mud wall surface to absorb and retain the insecticides after spraying. Previous studies in Kenya, Tanzania and Vietnam also found that the types of walls affects the duration of insecticides sprayed on the wall (Villarreal *et al.*, 1995; Curtis *et al.*, 1998; WHOPEs, 2007; Mulambalah *et al.*, 2010; Raghavendra *et al.*, 2011). In order to use an appropriate insecticide for indoor residual spraying, this factor should be considered because many rural areas in tropical Africa have a variety of walls ranging from mud walls, brick walls to cemented walls. Similar studies have also reported a wide variation in residual efficacy of lambda-cyhalothrin across different types of wall surfaces (Santos *et al.*, 2007; Mutagahywa *et al.*, 2015).

The results of this study also confirmed that there was a significant degradation of the insecticide (deltamethrin) on LLINs 18 months after usage as indicated by net chemical analysis. Long lasting insecticide nets have been designed to retain the insecticide for 5 years (WHO 2006). The early degradation of insecticide on the LLINs can be explained by external factors such as temperature, frequent washing and hanging in the sun and smoke coming from the houses where the nets are used. Similar studies have reported that the effectiveness of LLINs can reduce with the number of times the net is washed, condition of the nets physical and insecticide resistance in mosquito vectors (Dabire *et al.*, 2006; Atieli *et al.*, 2010). Other studies have also reported that permethrin content of nets used in the field can be rapidly lost in the presence of high temperatures and exposure to ultra violet light which causes degradation of permethrin (Gimnig *et al.*, 2000; Sreehari *et al.*, 2009).

The mortality of mosquitoes exposed to different ages of the net decreased with the ages of the nets, older nets showed lower mortality of mosquitoes compared to new nets. The mortality of Kabula mosquitoes exposed to different ages of the nets was significantly low compared to Emutete mosquitoes in 18-36 month old nets (Table 4.10). This suggests that prolonged use of LLINs may reduce its effectiveness due to both insecticidal decay and insecticide resistance. These results coincide with a recent study in Western Kenya which reported that resistant *An. gambiae* species were

resistance to excito-repellency effects of the Long Lasting Insecticide Nets compared to susceptible mosquitoes (Kawada *et al.*, 2011). This poses a great challenge to the efficacy of LLINs based malaria control programs with the current spread of insecticide resistance in Western Kenya.

The findings from this study indicate that insecticide resistance directly affects the insecticide based malaria control programmes. The LLINs that were tested (Olyset®) showed reduced efficacy of the insecticide impregnated on the nets, and residual insecticide of IRS with lambda-cyhalothrin (ICON 10CS) and deltamethrin showing reduced efficacy of the insecticide within the first 3-4 months and one month respectively. These findings suggest that similar residual efficacy studies should be recommended to investigate if insecticides for IRS provide sufficient protection, especially when new insecticides or formulations are introduced. Based on this findings, implementation of two rounds of IRS with lambda-cyhalothrin (ICON 10CS) should be considered where mud walls comprise a significant proportion in malaria endemic areas. The LLINs should also be replaced after 3 years in endemic areas for effective malaria control.

CHAPTER FIVE

5 STATUS OF INSECTICIDE RESISTANCE OF MALARIA VECTORS IN WESTERN KENYA

5.1 Introduction

Pyrethroid insecticides have been extensively used both on insecticide treated nets and indoor residual spraying (IRS) for malaria control programmes where malaria is endemic across Africa (WHO, 2013a). Currently, insecticide based malaria control depends on one class of insecticides, the pyrethroids, which is the only class recommended for use on the long lasting insecticide treated nets and it has also been widely deployed in IRS programmes in Africa (WHOPES, 2015). If any of these interventions are used consistently, they can dramatically reduce cases of malaria and associated deaths (Brooke *et al.*, 2013; Zhou *et al.*, 2014). Successful reduction of mortality and morbidity associated with malaria after use of these tools in many countries across Africa, has led to the possibility of finally eliminating malaria in Africa (Roberts *et al.*, 2007). However, the emerging insecticide resistance to the majority of existing insecticides has become a threat to malaria vector control efforts.

Apart from pyrethroids, other classes of insecticides are recommended for indoor residual spraying. Organophosphates have widely been used in indoor residual spraying programmes in Fenithrothion, Malathion and Priphos-methyl formulations in some countries. This group of insecticides is very effective, although it has a short duration on the sprayed surfaces (2-3months when used

for IRS) (WHO, 1990). They act by inhibiting acetylcholinesterase, thus preventing the breakdown of the neurotransmitter acetylcholine which leads to the overstimulation of neuromuscular functions and subsequent death of the vector (WHO, 2011).

Carbamates have also been utilized in IRS programmes for vector control as Bendiocarb and Propoxur formulations. They have a similar mode of action to that of organophosphates, and like organophosphates, are also highly effective, but last for a period of 2-3 months on the sprayed walls (WHO, 2011).

There are two major mechanisms of insecticide resistance; the first involves alteration in the target site of the insecticide, which leads to the reduction of the insecticide's binding capability, thus increasing the rate at which the insecticide is metabolized. This in turn lowers the amount of insecticide reaching the target site in the insect (Ranson *et al.*, 2011). Alteration in the target site is characterized by knock-down resistance (*kdr*) to pyrethroids and DDT caused by mutation in the voltage-gated sodium channel (VGSC) (Martinez Toress *et al.*, 1998). Knock down resistance at the amino acid position L1014F-*kdr* is predominantly found in *An. gambiae* from West Africa where as L1014S-*kdr* mutation is predominantly found in *Anopheles gambiae* from East Africa (Ranson *et al.*, 2000). Alteration of target site in *An. gambiae* single amino acid substitution of glycine to serine at position 119 in the catalytic domain of the acetylcholinesterase (AChE) gene confers resistance to both organophosphates and carbamates (Weil *et al.*, 2004).

The second mechanism of resistance involves metabolic resistance which occurs when there is elevation in metabolic enzyme activities, this leads to detoxification of insecticides before it gets to the target site (Hemingway *et al.*, 2004). The cytochrome P450s are the primary enzyme family responsible for detoxifying pyrethroids in insects (Stevenson *et al.*, 2011). Monooxygenases enzymes have been found in *Anopheles gambiae* (Ranson *et al.*, 2000) and, as in other insects, only a few enzymes can detoxify insecticides.

Previous studies have reported an increase in pyrethroid resistance in East Africa; Ethiopia, Tanzania, Sudan, Uganda and Kenya (Mbogo *et al.*, 2003; Verhaeghen *et al.*, 2006; Ramphul *et al.*, 2009; Verhaeghen *et al.*, 2010; Morgan *et al.*, 2010; Matowo *et al.*, 2010; Okia *et al.*, 2013). In Kenya, several studies have reported an increase of pyrethroid resistance in malaria vectors over the past decades (Stump *et al.*, 2004; Kamau *et al.*, 2006; Ochomo *et al.*, 2010; Bonizzoni *et al.*, 2012; Ochomo *et al.*, 2012; Wanjala *et al.*, 2015). With the wide spread resistance to pyrethroids and DDT, carbamate and organophosphate classes of insecticides are the possible alternatives that can be considered for malaria control. The question as to whether insecticide resistance has a major impact on malaria vector control has not been clearly addressed. There is need to profile the insecticide susceptibility to the available alternative insecticides such as organophosphates and carbamates for malaria control. This study aimed at assessing the status of *Anopheles gambiae*

resistance to pyrethroid carbamates and organophosphates in the highlands and low lands of western Kenya.

5.2 Materials and Methods

Four experiments were carried out and they were as follows: WHO tube bioassays, biochemical assays, PCR for species identification and Real Time PCR for *KDR* genotyping.

5.2.1 Sample size determination

World health organization recommends that 120 -150 mosquitoes should be tested for any insecticide, with at least four replicates of 20 -25 mosquitoes per test and a minimum of 50 mosquitoes used as control. In this study, 200 mosquitoes were tested from each site against every insecticide (WHO, 2013b).

5.2.2 World Health Organization tube bioassays

This assay was used to test the presence of phenotypic insecticide resistance in mosquitoes. Two to five days old female mosquitoes collected from seven study sites (Kabula, Iguhu, Emutete, Emakakha, Ahero, Chulaimbo and Kisian) were used to test for the susceptibility to the following insecticides; Lambdacyhalothrin (dose of 0.05%), Deltamethrin (dose of 4%), Permethrin (dose of 0.75%), DDT (dose of 0.05%), Malathion (dose of 5%); and Bendiocarb (dose of 0.1%). Paraffin oil treated filter papers without insecticides were used as control. Kisumu strain, a reference mosquito reared

at KEMRI was the control. Ten Mosquitoes were put in the exposure tubes for 60 minutes and then transferred back to the holding tubes (Plate 5.1). They were then maintained in the holding tubes for 24 hours (the recovery period) and supplied with a pad of cotton-wool soaked in 10% sugar solution. Mortalities were recorded immediately after 24 hrs and the status of susceptibility of mosquitoes classified according to the WHO recommended standard (WHO, 2013b). Two hundred mosquitoes were tested in each site, for every insecticide, 8 replicates were used and 2 replicates of control each of 20 mosquitoes. Tests were carried out at the temperature of $26^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and $80\% \pm 10\%$ relative humidity during the exposure time and the subsequent recovery period, with a 12D:12N photoperiod. The knocked down and resistant mosquitoes from this bioassay were separately kept in RNA later solution (a solution used for preservation of mosquitoes) at -20°C for species identification and genotyping.



Plate 5.1 WHO bioassays tubes

5.2.3 Biochemical assays

Biochemical assays were used to test for the presence of metabolic resistance. Three groups of enzymes namely; nonspecific esterases, mono-oxygenases and glutathione s- transferases are believed to be responsible for detoxification of insecticides in mosquitoes (Hemingway and Ranson, 2000). The assays were performed on mosquitoes collected from the seven study sites (Ahero, Kisian, Chulaimbo, Emutete, Emakakha, Iguhu and Kabula) after exposure to WHO insecticide impregnated filter papers using the protocol described by WHO (2013 b).

To quantify esterase activity, fresh mosquitoes from the assays were crushed separately in 100 μ l of 0.05M potassium phosphate at the 7.2 pH and 900ml of buffer added in a well of microtitre plates. One hundred microlitre aliquote of β -naphthyl acetate (56mg/100mL acetone with 90mL buffer) was added to each 100 μ l of mosquito homogenate and incubated at 24⁰c for 10 minutes. After incubation, 100 μ l aliquot of dianisidine (100mg/100mL water) was then added. Optical density of the samples was read with a plate reader at 540nm.

To quantify the activity of monooxygenases enzyme, 100 μ l of mosquito homogenate was transferred in to wells of microtitre plates and 200 μ l of 3, 3' 5'- tetramethyl-benzidine dihydrochloride hydrate (50mg/25mL methanol/75ml 0.25M Na acetate buffer) was added. A volume of 25 μ l of 3% hydrogen

peroxide was then added to each well and incubated for 5 minutes. Optical density of the sample was read at 650nm.

For quantification of glutathione-S-transferase (GST) activity, 100 μ l of mosquito homogenate was transferred to the wells of microtitre plates, and 100 μ l of reduced glutathione (61mg/100ml, KPO₄ buffer) was added per well. One hundred microlitre of 1chloro-2, 4-dinitrobenzene (cDNB) (20mg/10ml acetone 90ml KPO₄ buffer) was added and absorbance read immediately at 340nm. Optical density of the sample was read and recorded again at 5 minutes and the difference in the absorbance between the two times per sample was determined.

The total proteins of the mosquito samples were determined as described by Brogdon *et al.*, (1988). A 20 μ l aliquot of mosquito homogenate was transferred into a well of microtitre plates and 80 μ l Potassium Phosphate (KPO₄) buffer was added. A volume of 200 μ l of protein dye reagent was added and optical densities read immediately at 620nm for all enzyme assays. Each sample was tested in triplicates.

5.2.4 Deoxyribonucleic acid extraction

The legs of each mosquito were used for DNA extraction. The mosquito legs were placed in a 500 μ l eppendorf tube. A total of 50 μ l of potassium phosphate solution were added and placed in a water bath for at 65°C for 20-30minutes. After incubation, 14 μ l of potassium acetate was added and mixed by vortexing, the mixture was then cooled in ice water for 30minutes. The

neutralized tissue was then centrifuged at 14,000rpm for 10 mins. The supernatant was poured in a sterile eppendorf tube then 200 µl of ethanol was added. The sample was centrifuged, Ethanol poured of, then it was left to dry completely. The extracted DNA was stored at 4°C or used immediately for PCR (Collins *et al.*, 1987).

5.2.5 Species identification using polymerase chain reaction

Species-specific PCR assay (Wilkin *et al.*, 2006) was used to identify the species of knocked down and resistance mosquitos' samples, which had been previously, preserved after the wall bioassays, net bioassays and WHO tube bioassay. The DNA templates extracted from mosquitoes and complex rDNA intergene space gene were amplified using specific primers below;

AR for *An. arabiensis* F GCTGGCGAGTTGTAGAGATGCG

R GTGTTAAGTGTCCCTTCTCCGTC

GA for *An. gambiae* F GCTGGCGAGTTGTAGAGATGCG

R GCTTACTGGTTTGGTCGGCATGT

The PCR reaction constituted of a reaction volume of 25 µl consisting of; distilled water - 19.8µl, dNTPs -1.6 µl, primers 1 µl, MgCl₂ -0.3 µl, Taq DNA polymerase – 0.3µl and buffer 2 µl. Species identification PCR cycling consisted of the following conditions; denaturation at 95°C for 5minutes followed by 30 cycles of of 95 °C for 30seconds, annealing at 58 °C for 30seconds and extension at 72 °C for 30 seconds then a final extension at 72°C for 5minutes.

5.2.5.1 Electrophoresis

After PCR the plate was removed from the machine, the oil at the bottom blotted out and the plate sealer removed carefully to avoid spillage. 3 µl of the loading dye (bromophenol blue) was added into the bottom of the mineral oil using fresh tips (one for each well) making sure the tips went right below the mineral oil. The gel was loaded with 15 µl loading dye (dip tip to the bottom of the well to avoid picking the mineral oil). The lid of the electrophoresis tank was replaced and run from negative to positive 15 -30 minutes and stopped when the dye was almost at the end. The power was switched off. The gel was placed on a plastic tray and visualized in a dark room. After removing the gel from the electrophoresis chamber, the gel was placed on the UV source slab, the main light switched off and UV source switched and scanned. After visualization the camera was placed squarely on the gel and a photograph taken. The lower paper was pulled then the upper paper was gradually and uniformly pulled out to develop the film as it came out. It was then left to rest for 30-60seconds after which the photograph was peeled out and visualized. The gel was put back onto a tray, the UV source wiped with paper towel and the read gel discarded for incineration. Then the data was recorded (Scott *et al.*, 1993).

5.2.6 Genotyping using Real-Time PCR

Real Time PCR assay was conducted to determine the presence of knock-down resistance (*kdr*) allele amino acid position 1014 of the voltage-gated sodium

channel, in the mosquito samples preserved after the bioassays described above (wall bioassays, net bioassays and WHO tube bioassays). Extraction of DNA was performed as described in section 5.2.4.

Samples were genotyped using probes for the wild-type (5'-CTTACGACTAAATTTC-3'), labeled with HEX) and L1014S (5'-ACGACTGAATTTC-3'), labeled with 6-FAM) alleles. An RT-PCR reaction was conducted using StratageneMxPro 3000 machine in a 96-well format. Each reaction included 5.0 µl of 2x Taqman, RT-PCR master mix (Applied Biosystems), 0.2µM *kdr* forward primer (5'-GCTGCGAGTTGTAGAGATGCG-3'), 0.2µM *kdr* reverse primer (5'-GCTTACTGGTTTGGTCGGCATGT-3'), the wild-type and L1014S probes at respective concentrations of 0.2 µM and 0.15 µM, ~ 50 ng DNA template, and sterile water in a final volume of 10 µl. Each 96-well plate included positive controls for all three genotypes in triplicate along with a no-template negative control. PCR conditions included an initial melting step at 95°C for 10 minutes followed by 45 cycles of 95°C for 25 seconds and 64°C for 1 minute. Reaction curves for each set of reactions was visualized using StratageneMxPro QPCR s (Bass *et al.*, 2007; Mathias *et al.*, 2011).

5.2.7 Data Analysis

Observed mortality of mosquitoes from different sites was calculated using the formula (WHO, 2013 b);

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total number of mosquitoes}} \times 100$$

Total sample size

If the mortality of the mosquitoes exposed to the control filter paper was $\geq 5\%$ and $\leq 20\%$ then the mortality was corrected using Abbott formulae (Abbott, 1925);

$$\text{Corrected mortality} = \frac{(\% \text{ observed mortality} - \% \text{ control mortality})}{(100 - \% \text{ control mortality})} \times 100$$

If the control mortality was $\geq 20\%$ then the test were discarded.

The status of susceptibility of the mosquito population was classified basing on WHO criteria (98-100% mortality indicates susceptibility, 90-97% mortality suggests possibility of resistance that needs to be confirmed, and $<90\%$ mortality suggests resistance) (WHO, 2013b). The mortality of mosquitoes was compared between different sites and between different insecticides using ANOVA. The fold increase in the enzymatic activity was calculated by dividing the optical density of the resistant mosquitoes by that of susceptible mosquitoes from the same site. Heterozygous and homozygous mutation rates of *kdr* gene were calculated. To determine if the *Kdr* genotypes were under selection, Hardy-Weinberg equilibrium test for *kdr* genotypes was performed. Chi square test was used to determine the significance of the departure from Hardy-Weinberg equilibrium.

5.3 Results

5.3.1 Mortality of mosquitoes from the seven study sites exposed to

Malathion

Mosquitoes from all the study sites were highly susceptible to Malathion and recorded 100% mortality (Table 5.1)

Table 5.1: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to Malathion

Insecticide	Site	N	Mean \pm SE
Malathion	Kisumu Strain (control)	200	100.0 \pm 0.00a
	Ahero	200	100.0 \pm 0.00a
	Kisian	200	100.0 \pm 0.00a
	Chulaimbo	200	100.0 \pm 0.00a
	Emutete	200	100.0 \pm 0.00a
	Emakakha	200	100.0 \pm 0.00a
	Iguhu	200	100.0 \pm 0.00a
	Kabula	200	100.0 \pm 0.00a

Mean value denoted by the similar letters in a column are not significantly different at $P \leq 0.05$

5.3.2 Mortality of mosquitoes from the seven study sites exposed to

Bendiocarb

Kisumu Strain, Ahero, Emakakha and Emutete were highly susceptible to Bendiocarb with 100% mortality but did not differ significantly from Kisian and Chulaimbo mosquitoes which had 99% and 98% mortality respectively. Iguhu (87%) and Kabula (84%) mosquito populations had a significantly lower mortality compared Ahero, Kisian, Chulaimbo, Emutete and Emakakha and also differed significantly ($F = 92.89$, $P = 0.0001$; Table 5.2).

Table 5.2: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to Bendiocarb

Insecticide	Site	Sample Size (N)	Mean \pm SE
Bendiocarb	Kisumu Strain (control)	200	100.0 \pm 0.00a
	Ahero	200	100.0 \pm 0.00a
	Kisian	200	99.11 \pm 0.61a
	Chulaimbo	200	98.83 \pm 1.17a
	Emutete	200	100.0 \pm 0.00a
	Emakakha	200	100.00 \pm 0.00a
	Iguhu	200	86.90 \pm 0.88b
	Kabula	200	83.83 \pm 0.95c

Mean values denoted by similar letters in a column are not significantly different at $P \leq 0.05$

5.3.3 Mortality of mosquitoes from the seven study sites exposed to DDT

All mosquito populations showed remarkable resistance to DDT except Kisumu strain that showed 100% mortality. Ahero mosquito populations had significantly higher mortality (73%) compared to the other sites ($F = 16.70$, $P = 0.0001$). There were no significant differences between the mortality of the mosquitoes from Emakakha, Emutete, Iguhu, Kisian, and Chulaimbo. The lowest mortality was recorded in Kabula mosquito population which was significantly different from Kisian mosquitoes but did not differ from Emakakha, Emutete, Iguhu and Chulaimbo mosquitoes (Table 5.3).

Table 5.3: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to DDT

Insecticide	Site	Sample Size (N)	Mean \pm SE
DDT	Kisumu strain (control)	200	100.00 \pm 0.00a
	Ahero	200	73.33 \pm 2.49b
	Kisian	200	62.00 \pm 2.66c
	Chulaimbo	200	59.20 \pm 1.16cd
	Emutete	200	52.00 \pm 1.07d
	Emakakha	200	57.67 \pm 1.94cd
	Iguhu	200	56.50 \pm 0.71cd
	Kabula	200	50.50 \pm 2.14d

Mean values denoted by similar letters in a column are not significantly different at $P \leq 0.05$

5.3.4 Mortality of mosquitoes from the seven study sites exposed to Lambdacyhalothrin

Mosquitoes from all sites were resistant to Lambdacyhalothrin (mortality <90%) except Kisumu strain that showed 100% mortality. Ahero mosquitoes had a significantly higher mortality compared to other sites, followed by Kisian mosquitoes which were significantly different from Emakakha mosquitoes ($F = 62.05$, $P = 0.0001$). The mortality of Chulaimbo and Iguhu mosquitoes did not differ significantly. Kabula mosquitoes had the lowest mortality but did not differ significantly from Emutete and Chulaimbo mosquitoes (Table 5.4).

Table 5.4: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to Lambdacyhalothrin

Insecticide	Site	Sample Size (N)	Mean \pm SE
Lamdacyhalothrin	Kisumu strain (control)	200	100.00 \pm 0.00a
	Ahero	200	87.16 \pm 0.60b
	Kisian	200	81.14 \pm 0.51c
	Chulaimbo	200	72.68 \pm 1.05ef
	Emutete	200	70.17 \pm 0.70f
	Emakakha	200	77.83 \pm 0.60d
	Iguhu	200	74.25 \pm 1.15e
	Kabula	200	68.17 \pm 0.60f

Mean values denoted by similar letters in a column are not significantly different at $P \leq 0.05$

5.3.5 Mortality of mosquitoes from the seven study sites exposed to

Deltamethrin

When exposed to diagnostic dosage of Deltamethrin, Kisumu strain had a significantly high mortality compared to other sites, followed by the mortality of mosquitoes from Kisian mosquitoes, which did not differ significantly from the mortality of Ahero and Iguhu mosquitoes. There was no significant difference in the mortality of Emutete, Emakakha and Chulaimbo mosquitoes. The mortality of Kabula mosquitoes was significantly lower compared to all the other sites ($F = 55.65$, $P = 0.0001$). Mosquitoes from all sites were resistant to Deltamethrin except Kisumu strain that was used as a control (Table 5.5).

Table 5.5: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to Deltamethrin

Insecticide	Site	Sample Size (N)	Mean \pm SE
Deltamethrin	Kisumu strain (control)	200	100.00 \pm 0.00a
	Ahero	200	84.67 \pm 0.42b
	Kisian	200	85.17 \pm 0.70b
	Chulaimbo	200	74.60 \pm 0.51c
	Emutete	200	74.20 \pm 1.16c
	Emakakha	200	77.75 \pm 1.32c
	Iguhu	200	82.29 \pm 0.87b
	Kabula	200	62.89 \pm 1.43d

Mean values denoted by similar letters in a column are not significantly different at $P \leq 0.05$

5.3.6 Mortality of mosquitoes in the seven study sites exposed to

Permethrin

When exposed to Permethrin, the mortality of mosquitoes from, Kisumu strain had significantly higher mortality compared to the other sites. The mortality of mosquitoes from Ahero was not significantly differed from the mortality of Kisian, Chulaimbo and Emakakha mosquitoes. The mortality of Iguhu mosquitoes was not significantly different from that of Emutete mosquitoes. The mortality of Kabula mosquitoes was significantly lower compared to the other sites ($F = 30.10$, $P = 0.0001$). Mosquitoes from all the study sites except Kisumu strain were resistant to Permethrin (Table 5.6).

Table 5.6: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites exposed to Permethrin

Insecticide	Site	N	Mean \pm SE
Permethrin	Kisumu strain (control)	200	100.00 \pm 0.00a
	Ahero	200	83.17 \pm 2.27b
	Kisian	200	75.50 \pm 1.19bc
	Chulaimbo	200	79.25 \pm 3.52b
	Emutete	200	68.20 \pm 0.37c
	Emakakha	200	77.33 \pm 0.33b
	Iguhu	200	72.00 \pm 0.69c
	Kabula	200	52.40 \pm 2.98d

Mean values denoted by similar letters in a column are not significantly different at $P \leq 0.05$

5.3.7 Mortality of mosquitoes from all the study sites exposed to six insecticides

The highest mortality occurred when mosquitoes were exposed to Malathion but it did not differ significantly from Bendiocarb. These two insecticides caused significantly higher mortality compared to that of Lambdacyhalothrin, Permethrin and Deltamethrin which showed no significant difference. When exposed to DDT, mosquitoes gave the lowest mortality but did not differ significantly from that of permethrin, ($F = 12.21$, $P = 0.0001$; Table 5.7).

Table 5.7: Mean percentage mortality (\pm SE) of mosquitoes from seven study sites six insecticides

Insecticides	Mean \pm SE
Malathion	100.00 \pm 0.00a
Bendiocarb	96.13 \pm 6.62a
Lambdacyhalothrin	78.88 \pm 10.51b
Deltamethrin	80.25 \pm 10.74b
Permethrin	76.00 \pm 13.33ab
DDT	63.75 \pm 16.24c

Mean values denoted by similar letters are not significantly different at $P \leq$

0.05. Mean separated using Tukey HSD. $F = 12.21$, $P < 0.001$.

5.3.8 Metabolic activity of Monooxygenases (P450)

There was an increase in the monooxygenases enzymatic activity in resistant mosquitoes exposed to Pyrethroids (Permethrin, lambdacyhalothrin, Deltamethrin) and DDT. There was a 7 fold increase in the monooxygenases enzymatic activity in Kabula mosquito populations exposed to lambdacyhalothrin, 2.5 in Emakakha, 1.8 in Emutete populations and 2 in Iguhu populations but there was no increase in the monooxygenases activity in Kisian and Chulaimbo resistant mosquitoes exposed to lambdacyhalothrin (Figure 5.1).

There was a 4 fold and 2.5 fold increase in Kabula mosquito populations exposed to permethrin and deltamethrin respectively, 3.8 fold and 3.6 fold in Emakakha populations, 3 fold and 2 fold in Emutete populations 2 fold and 2 fold in Iguhu populations and lastly 2 fold and 2 fold increase in Kisian populations. The monooxygenase enzyme activity increased in resistant mosquito populations exposed to DDT in Kabula mosquito populations where there was a 2.5 fold increase, in Chulaimbo 1.8 fold increase, 1.7 fold increase in Emakakha, Emutete, Iguhu and Kisian mosquito populations (Figure 5.1).

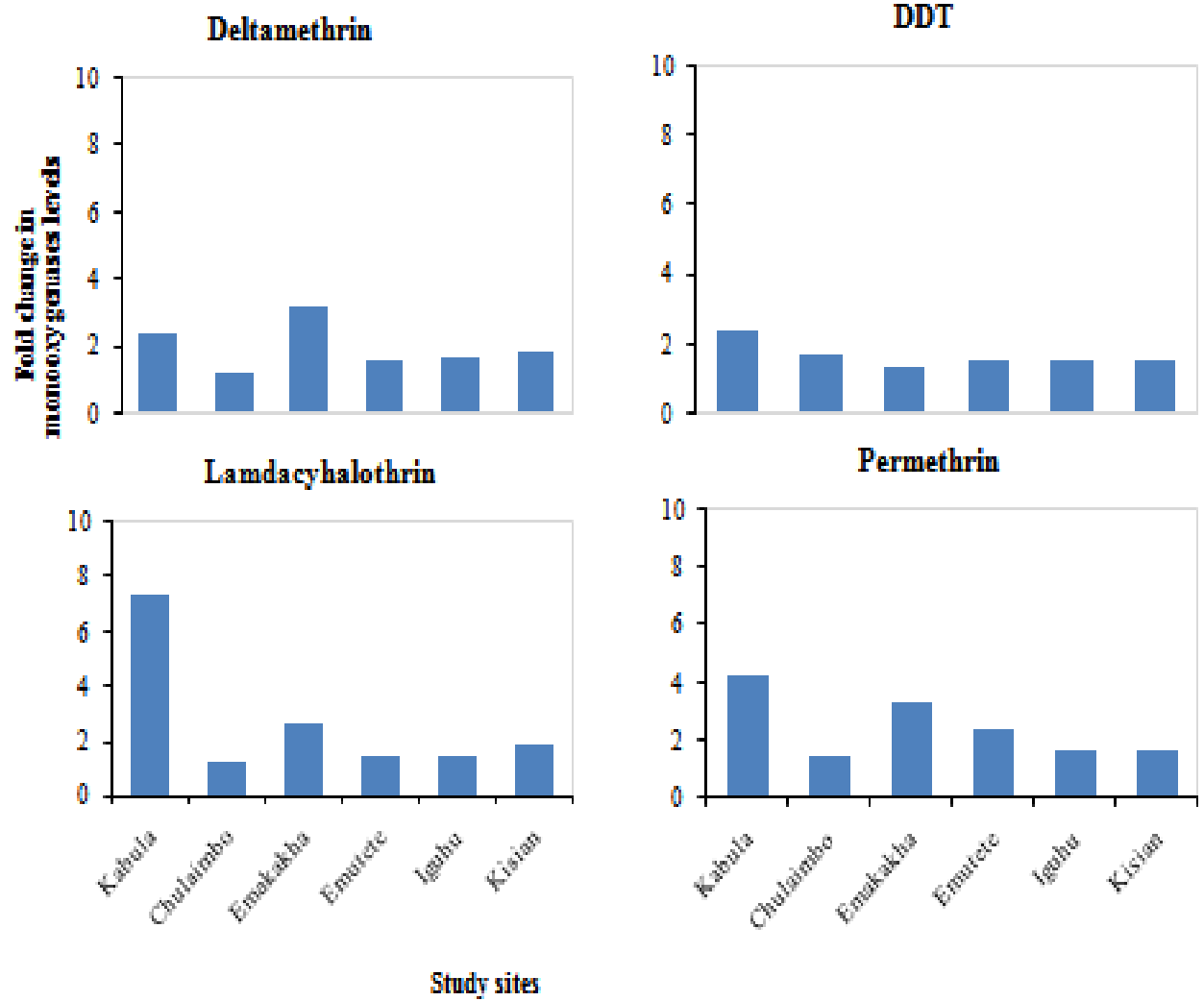


Figure 5.1: Fold change in the monoxygenases (P450) levels in resistant mosquitoes against susceptible mosquitoes from the same study site.

5.3.9 Metabolic activity of esterases

There was also an increase in esterases activity in resistant mosquitoes exposed to pyrethroids (permethrin, lambda-cyhalothrin and deltamethrin) and DDT. There was a 1.1 fold, 1.2 fold and 1.2 fold increase in Kabula mosquito populations exposed to Lambda-cyhalothrin, permethrin, and deltamethrin respectively; 1.2, 1.8 and 2 fold increase in Chulaimbo mosquito populations, 1.5, 1.3 and 1.5 fold increase in Emakakha populations, 1.5, 1 and 1.6 fold increase in Emutete populations, 1.4, 1.7 and 1.6 in Iguhu populations and lastly 1.5, 1.6 and 1.5 in Kisian mosquito populations. There was also an increase in esterases activity in resistant mosquitoes exposed to DDT, 1.2 fold increase in Kabula, Emutete, Iguhu, and Emutete mosquito populations, 1.5 fold increase in Chulaimbo populations, 1 fold increase in Kisian and 0.7 fold increase in Emakakha mosquito populations (Figure 5.2).

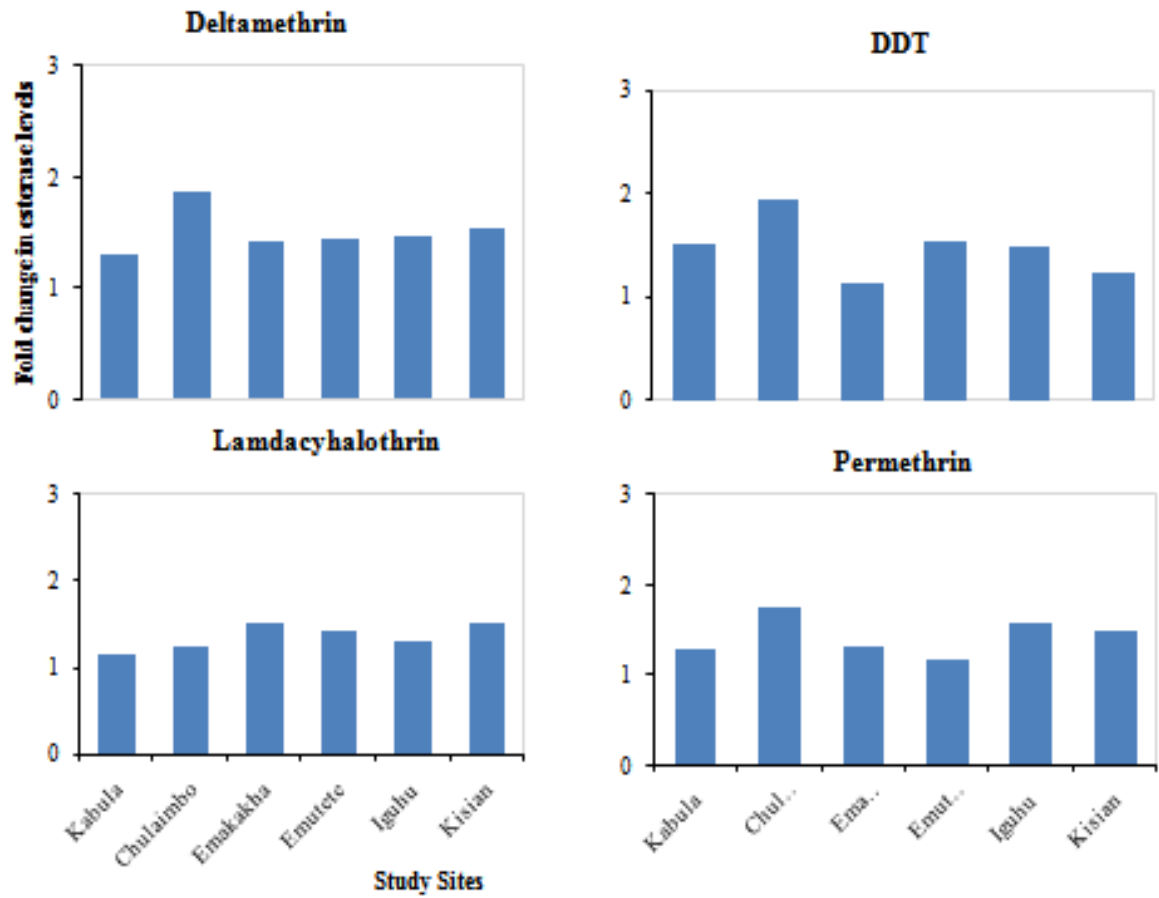


Figure 5.2: Fold changes in esterase levels in resistant mosquitoes against susceptible mosquitoes from the same study site.

5.3.10 Metabolic activity of Glutathione-s- transferases

There was 1 fold change in the GST activity in the resistant mosquitoes that were exposed to pyrethroids and DDT in all the study sites (Fig 5.3).

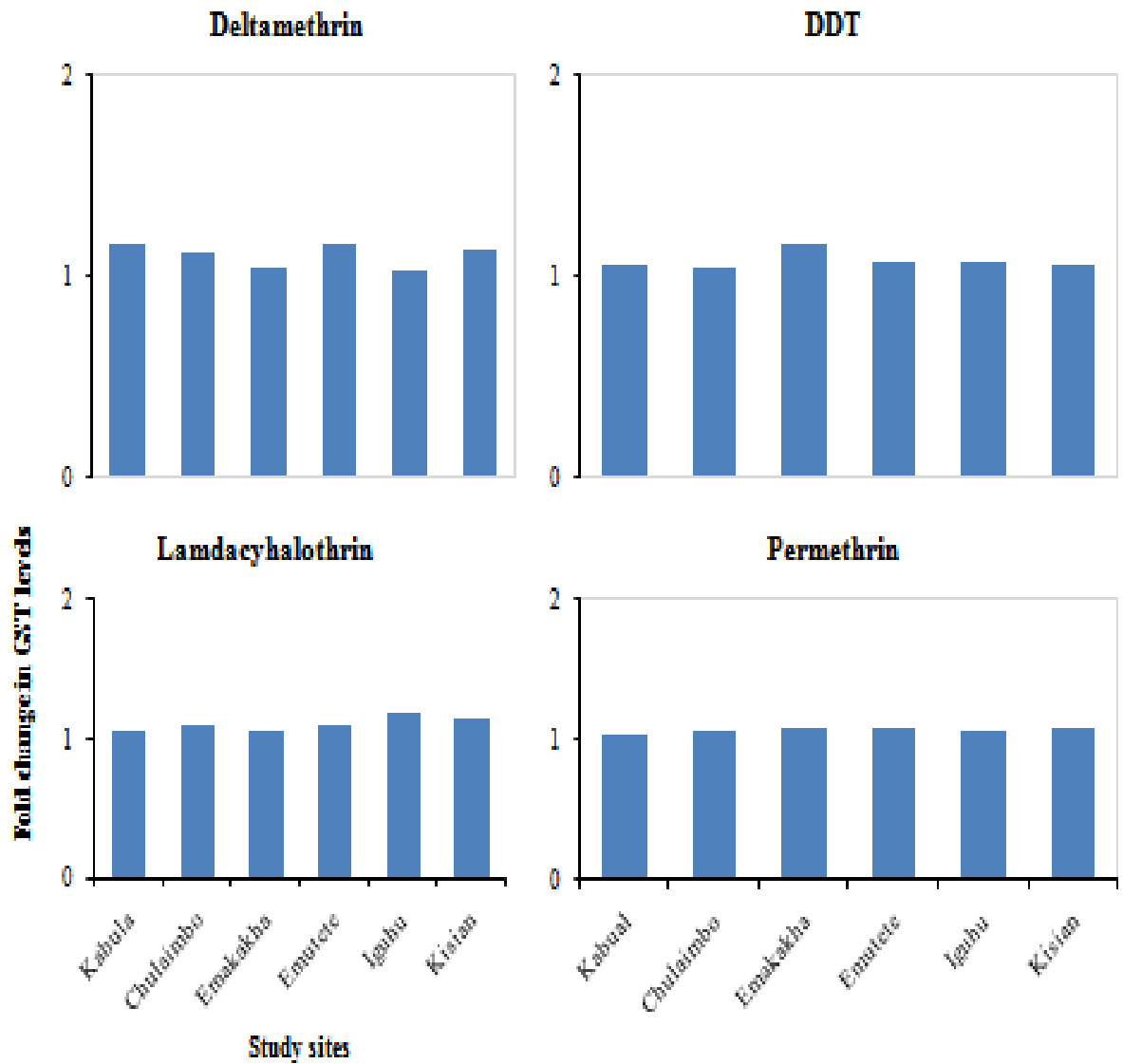


Figure 5.3: Fold changes in GST levels in resistant mosquitoes against susceptible mosquitoes from the same study site

5.3.11 Species identification

Polymerase chain reaction analysis showed that *An. gambiae* was the predominant species in Chulaimbo (68.6%), Emutete (95.2%), Emakakha (92.6%), Iguhu (89.2%) and Kabula (94.3%), where as *An. arabiensis* was predominant in Kisian (66.8%) and Ahero (88.4%) (Table 5.8; Appendix V).

Table 5.8 Species composition at different study sites

Study site	N	<i>An. arabiensis</i> (%)	<i>An. gambiae</i> s.s. (%)	Not determined (%)
Ahero	66	88.4	5.8	5.8
Kisian	235	66.8	30.4	2.8
Chulaimbo	110	28.4	68.6	3.0
Emutete	210	3.8	95.2	2.0
Emakakha	71	2.5	92.6	4.9
Ighu	310	7.8	89.2	3.0
Kabula	70	2.7	94.3	3.0

5.3.12 Knock down resistance allele Genotyping

Homozygous *kdr* mutations of L1014S was high in *An. gambiae* s.s. dominant populations, whereas L1014S mutations were relatively low in *An. arabiensis* dominant populations. The frequency of *kdr* allele was significantly high in Chulaimbo and Emakakha, followed by Emutete, Iguhu and Kabula. The mosquitoes from Kisian had the lowest frequency of *kdr* allele in the *An. gambiae* mosquitoes. In *An. arabiensis* mosquitoes, the frequency of the *kdr* allele was high in Chulaimbo mosquitoes and Bungoma mosquitoes followed by Ahero, Iguhu and lastly Kisian. There was no mutation in the *An. arabiensis* mosquitoes from Emakakha. *Kdr* genotyping failed in 2.8% of the samples. In addition, in *An. gambiae* most of the mutations were homozygous. Hardy-Weinberg analysis showed that, out of the four *An. arabiensis* populations tested for Hardy-Weinberg equilibrium, only one population (Chulaimbo) significantly deviated, and the deviation came as a result of heterozygosity deficiency. In *An. gambiae*, five out of the six populations tested showed significant deviation from Hardy-Weinberg equilibrium, all caused by heterozygosity deficiency (Table 5.9).

Table 5.9 Genotype and allele frequencies of *kdr* at the seven study sites in western Kenya

Study site	<i>An. gambiae</i>						
	N	LL	LS	SS	Frequency	χ^2	P-value
Ahero	13	13	0	0	0.0	-	-
Kisian	60	30	5	25	41.7	35.45	<0.0001
Chulaimbo	66	4	2	60	90.9	12.73	<0.0001
Emutete	97	8	7	82	91.7	48.24	<0.0001
Emakakha	67	3	8	56	83.5	1.28	0.25
Iguhu	118	12	8	98	83.1	52.38	<0.0001
Kabula	63	3	7	53	84.1	20.76	<0.0001

Study site	<i>An. arabiensis</i>						
	N	LL	LS	SS	Frequency	χ^2	P-value
Ahero	53	43	10	0	4.0	0.08	0.77
Kisian	45	43	2	0	1.2	0.02	0.94
Chulaimbo	25	12	0	10	40.1	24.00	<0.0001
Emutete	10	0	0	0	0	-	-
Emakakha	15	1	0	0	0.0	-	-
Iguhu	20	16	1	3	15.0	0.01	0.80
Kabula	5	2	0	0	0	-	-

N is sample size, LL (wild type), LS (heterozygote mutation), SS represents homozygote mutation, and Frequency is the mutation allele frequency (%).

Symbol ‘-‘ stands for not done. χ^2 and P-value are the results of Hardy-Weinberg equilibrium test.

5.4 Discussion

Results of this study indicate that mosquitoes were highly susceptible to Malathion recording a mortality of 100% in all the study sites. The mortality of mosquitoes from the five study sites (Ahero, Kisian, Chulaimbo, Emutete and Emakaha) was high when exposed to Bendiocarb, but resistance was observed in two sites, Iguhu and Kabula when they were exposed to the same insecticide. This suggests that mosquitoes in western Kenya have not yet developed resistance to Malathion and Bendiocarb. Mosquitoes from all the study sites showed marked resistance to DDT, recording the highest mortality of 78% in Ahero mosquitoes. When exposed to pyrethroids (lambdacyhalothrin, permethrin and deltamethrin), insecticide resistance was observed in all mosquito populations with the highest mortality of 87% observed in Ahero populations exposed to Lambdacyhalothrin. The difference in the mortality rates within different insecticide can be explained by the difference in the mode of actions, Malathion and Bendiocarb inhibit cholinesterase where as pyrethroids and DDT are modulators of voltage gated sodium channels (WHO, 2011). The observed resistance of mosquitoes to pyrethroids could be as a result of over use of these insecticides in Kenya for control of malaria, which has led to cross resistance in DDT because the two classes of insecticides have a similar mode of action (Ranson *et al.*, 2011).

The results of this study concurs with previous studies by Chouaibou *et al.* (2008) who reported that *An. gambiae* populations in northern Cameroon were

susceptible to carbamates and organophosphates but highly resistant to organochlorines and pyrethroids. However, they do not agree with the findings of Matowo *et al.* (2014) who reported that *An. arabiensis* mosquito populations in Tanzania were highly susceptible to Organochlorines (DDT), Organophosphate and Carbamates but highly resistant to pyrethroids. The insecticide resistance to pyrethroids that was observed in western Kenya could be linked to massive scale up of pyrethroid insecticide based vector control in Kenya and extensive use of this group of pesticide to control agricultural pests (Mathias *et al.*, 2011, Wanjala *et al.*, 2015).

Previous studies in Kenya reported that the localized use of insecticide treated nets in Kisumu increased the permethrin tolerance of *An. gambiae* populations (Vulule *et al.*, 1994, 1999). Stump *et al.* (2004) also observed an increase in the level of mosquito resistance to pyrethroids in western Kenya as a result of massive scale up of ITN distribution and usage. In Moshi, Tanzania, a field trial by Mosha *et al.* (2008) reported that while commonly used ITNs killed relatively few host-seeking *An. arabiensis*, the nets continued to provide personal protection through the strong excito-repellent activity of permethrin (Mosha *et al.*, 2008). Kawada *et al.* (2011) reported that resistant *An. gambiae* populations from Kenya were tolerant to excito-repellent activity of LLINs. However, LLINs have been reported to be still effective in malaria control, and the effect of insecticide resistance to malaria control has not been documented (Ochomo *et al.*, 2013).

Results of this study indicated a significant difference in mortality of mosquitoes from different sites when exposed to Bendiocarb, Pyrethroids and DDT. Ahero Mosquitoes recorded the highest mortality followed by Kisian, where as the mean mortality of mosquitoes from Emutete, Emakakha and Iguhu did not differ significantly when exposed to the insecticides. Kabula Mosquito populations showed the lowest mortality when exposed to all the insecticide except Malathion. This suggests that topographical features can have an effect on the mosquito survival and longevity thus affecting their response to insecticides (Afrane *et al.*, 2006; Githeko *et al.*, 2006, Wanjala *et al.*, 2011). The marked resistance observed in Kabula mosquito populations can be linked to the spread of the resistance alleles from Uganda since this region borders Uganda where insecticide resistance to pyrethroids is wide spread (Verhaeghen *et al.*, 2006, Morgan *et al.*, 2010). The same observation was made by Ochomo *et al.* (2012) who reported resistance in Bungoma mosquitoes and high susceptibility in Ahero mosquitoes.

This study also reported the elevation of monooxygenases and esterases enzyme activity in resistant *An. gambiae* mosquito populations from Kabula, Iguhu, Emutete, Emakakha and Chulaimbo and Kisian exposed to Permethrin DDT but no elevation of monooxygenases and esterases in *An. arabiensis* mosquitoes from Ahero. There was no elevation of GSTs in all mosquito populations. This suggests that, the observed resistance to permethrin and DDT in these areas could be caused by elevation of the enzyme activity of

monooxygenases and esterases but not the GSTs. For instance, mosquitoes from Kabula had the lowest mortality when exposed to pyrethroids and DDT, there was the highest fold change (2.5 fold) in the monooxygenase enzyme activity in the same mosquito population when they were exposed to DDT. The lack of elevation in monooxygenases and esterase enzyme activity in Ahero mosquitoes, could explain why the mosquitoes had the highest mortality when exposed to the same insecticides. In a similar study, Matowo *et al.* (2014) elucidated the observed pyrethroids and DDT resistance to the elevation of metabolic enzyme activities in the mosquito populations. The results from this study are similar to previous studies by Ochomo *et al.* (2012) that reported an elevation in esterases and monooxygenases enzyme activities in *An. gambiae* permethrin resistant populations from Bungoma and Budalangi. Vulule *et al.* (1994, 1996, 1999) reported an elevation in both β -esterase and oxidase enzyme expression in *An. gambiae* permethrin resistant populations, but there was no elevation in GST enzymes levels in the *An. gambiae* s.s. Furthermore, there was no evidence of elevated monooxygenases enzyme activities in *An. arabiensis* populations from Ahero in this study, which had previously been covered by a pyrethroid based IRS and LLINs. This contradicts the previous reports that massive scale up of insecticide based vector control methods could be selecting for resistance in malaria vectors (Stump *et al.*, 2004).

There was a marked phenotypic resistance to deltamethrin and lambda-cyhalothrin in this study, but no measurable elevated expression

monooxygenases and esterases in resistant mosquitoes that had been exposed to deltamethrin and lambda-cyhalothrin. This suggests that mechanisms of resistance to Deltamethrin and Lambda-cyhalothrin could be target site resistance as a result of the presence of the *kdr* alleles, whereas resistance against permethrin and DDT may involve target site resistance and metabolic resistance as a result of the elevated expression of these β -esterase and monooxygenase enzymes (Ranson *et al.*, 2011).

Results of species identification indicated that the percentage of *An. gambiae* was the highest in Kabula, Iguhu, Emutete, Emakakha, and Chulaimbo whereas the percentage of *An. arabiensis* was higher in Ahero and Kisian. The absence of *An. gambiae s.s* in Ahero could be due to the species shift with decrease of abundance of *An. gambiae* towards the more zoophilic sibling species *An. arabiensis* following scaling-up of ITNs as reported earlier by Mathias *et al.* (2011). Similar studies in the same sites have reported the abundance of *An. gambiae* in Bungoma, Emutete and Iguhu (Martinez Torres *et al.*, 1998; Atieli *et al.*, 2010) and *An. funestus* in Kisian and Chulaimbo (Bayoh *et al.*, 2010).

The findings from this study also showed that the frequency of *kdr* allele was high in *An. gambiae* from Kabula, Emutete and Emakakha mosquito populations. This could be due to extensive insecticide based vector control activities in this regions especially indoor residual spraying with deltamethrin and lambda-cyhalothrin, and use of long lasting insecticide nets. Knock down

resistance (*kdr*) mutations in *An. gambiae* s.s. has been well documented (Kawada *et al.*, 2011; Ochomo *et al.*, 2012; Okumu *et al.*, 2012; Okumu *et al.*, 2013). Compared to *An. gambiae* s.s., *kdr* mutations in *An. arabiensis* was low in the study areas (mainly Ahero and Kisian), although WHO bioassay test did show that *An. arabiensis* were resistant to DDT and pyrethroid insecticides. This suggests that other insecticide resistant mechanisms could be present in these mosquitoes. However, previous studies by Kawada *et al.* (2011) reported a high frequency and wide distribution of 1014S in *An. arabiensis*, which is in contrast to the findings from this study. In Ahero, rice is the predominant crop and pesticides have been frequently used for pest control even before the scaling-up of ITNs, but the *kdr* mutation at L1014S rate was found to be low (Table 5.9). The agricultural use of pesticide types are different from the insecticides commonly used for IRS and ITNs in the area, this mixed use of multiple types of insecticides may have delayed the development of resistance in *An. arabiensis*.

CHAPTER SIX

6 GENERATIONAL AND BIOCHEMICAL CHANGES INVOLVED DURING THE SELECTION PROCESS OF PYRETHROID RESISTANT *An. gambiae* AND *An. arabiensis* POPULATIONS

6.1 Introduction

Three major vectors of malaria in Western Kenya; *Anopheles gambiae*, *An. arabiensis* and *An. funestus* (Githeko *et al.*, 1996; Bayoh *et al.*, 2010). Their resistance to insecticides based on WHO susceptibility tests has been widely reported in Africa (Hemingway, 1993; Ranson *et al.*, 2000; Verhaeghen *et al.*, 2006; Matowo *et al.*, 2010; Ochomo *et al.*, 2014; Matowo *et al.*, 2014; Wanjala *et al.*, 2015).

Two primary mechanisms of insecticide resistance have been described in malaria vectors. The first one involves insecticide detoxification based on increased activity of monooxygenases and esterases in *Anopheles gambiae* and *Anopheles arabiensis* which has previously been reported in Kenya and neighbouring Tanzania (Vulule *et al.*, 1999; Matowo *et al.*, 2010; Ochomo *et al.*, 2013) and elevation in glutathione-s-transferase activity that has been observed in *Anopheles gambiae* populations in South Africa in *Anopheles arabiensis* (Hangreaves *et al.*, 2003). The second mechanism is target site alteration that has been shown to confer resistance to DDT and pyrethroid insecticides in *Anopheles gambiae* in most countries (Martinez-Torres *et al.*, 1998).

Pyrethroids act on the nervous system, targeting the voltage-gated sodium ion channels of the insects. Molecular characterization show that mutations in the sodium ion channel leads to pyrethroid resistance in many species of insects (Knipple *et al.*, 1994; Williamson *et al.*, 1996; Martinez-Torres *et al.*, 1998). Pyrethroid resistance brought about by the mutation of amino acid change from leucine to serine within the S6 hydrophobic transmembrane segment has been reported in *Anopheles gambiae* found in East Africa region; Uganda, Kenya, Tanzania and Sudan (Stump *et al.*, 2004; Verhaegehen *et al.*, 2006; Matambo *et al.*, 2007; Edi *et al.*, 2012; Ochomo *et al.*, 2013; Wanjala *et al.*, 2015). The resistance associated mutation in the same codon resulting in the amino acid change from leucine – Phenylalanine has previously been reported in West Africa populations of *Anopheles gambiae* (Martinez-Toress *et al.*, 1998).

Surveys in western Kenya have shown the presence of high levels of pyrethroid resistance in *Anopheles gambiae* mosquito populations but high susceptibility to organophosphates and carbamates (Stump *et al.*, 2004; Mathias *et al.*, 2005; Ochomo *et al.*, 2012; Bonizzoni *et al.*, 2012; Ochomo *et al.*, 2013; Wanjala *et al.* 2015). In this study, the genetic and metabolic changes that occur during the selection process of resistant *Anopheles gambiae* and *Anopheles arabiensis* from western Kenya were investigated.

6.2 Materials and Methods

To investigate the metabolic and generational changes that occur during the selection of pyrethroid resistant *Anopheles gambiae* and *Anopheles arabiensis* populations, three experiments were carried out in different mosquito generations. They were; WHO tube bioassays, biochemical assays and Real Time PCR for *kdr* genotyping.

6.2.1 Collection of mosquito samples

The mosquitoes that were used in this study were collected from two study sites; Ahero and Kabula (In Ahero, *An. arabiensis* is the predominant species whereas *An. gambiae* is the predominant species in Kabula) as described in section 3.2. The mosquito samples were then transferred to an insectary within KEMRI, Kisumu where they were reared to adult stage.

6.2.2 Selection of pyrethroid resistant *An. gambiae* and *An. arabiensis*

Newly-emerged mosquitoes were kept for a minimum of 2 days where they were fed on 10% sugar solution in cotton swabs. Cohorts of 50 male and 50 female mosquitoes per experiment were exposed to 0.05% deltamethrin treated papers in WHO tube bioassays for 1 hour (WHO, 2013 b). Knockdown was recorded after 1 hr and final mortality was recorded 24 hrs post exposure, during which time a 10% sugar solution in cotton swabs was made available to survivors kept in paper cups. Male and female survivors were pooled and maintained under standard insectary conditions (25°C, 80% RH) to produce

the next generation. This process was repeated for the subsequent generations of mosquitoes until there was no resistant mosquito after exposure. Susceptible mosquitoes from every cohort were preserved and used for biochemical tests and for molecular characterization.

6.2.3 Biochemical assays for testing the biochemical changes involved during the selection of pyrethroid resistant *An. gambiae* and *An. arabiensis* populations

The first to second generation of mosquitoes from Ahero and first to fourth generations of mosquitoes from Kabula was assayed to determine the levels of detoxifying enzyme activity for monooxygenases, non specific esterases and glutathione S-transferase. The samples were assayed using the 96-well microtitre plate as described in the section 5.2.3.

6.2.4 Genotyping to test for the genetic changes that occur during the selection for resistant *An. Gambiae* and *An. arabiensis* populations

A sample of 30 mosquitoes in every generation from both resistant and susceptible Ahero and Kabula mosquito populations based on their response to WHO assays using 4% deltamethrin were assayed for *kdr* mutation using Real-time PCR technique as described in the section 5.2.6.

6.2.5 Data analysis

To establish the variation in mortality of mosquitoes for different generations, a One-way analysis of variance (ANOVA) was conducted. The increase in the

enzymatic activity in different generations was calculated by dividing the optical density of the resistant mosquitoes by that of susceptible mosquitoes from the same site. Analysis of variance was used to determine the difference in the *kdr* allele frequency within different generations of mosquitoes.

6.3 Results

6.3.1 Phenotypic changes that occurred during the selection process of pyrethroid resistant *An. gambiae* and *An. arabiensis* populations as indicated by WHO tube bioassays

Successive selection for deltamethrin resistance showed a steady increase in mortality after exposure to deltamethrin. The F-5 and F-3 generation had 100% mortality for Kabula and Ahero mosquito populations respectively. The mean mortality of mosquitoes from Kabula was significantly higher in the fifth generation, followed by that of the fourth generation which did not differ significantly from that of the third generation. This was significantly higher compared to the mean mortality of mosquitoes in the second and first generations which also differed significantly ($F = 42.49$, $P = 0.0001$).

In Ahero mosquitoes, the mean mortality of mosquitoes in the third generation was significantly higher compare to the second and first generation which did not differ significantly ($F = 33.05$, $P = 0.0001$; Table 6.1).

Table 6.1: Mean percentage mortality (\pm SE) of mosquitoes of different generations in Kabula and Ahero

Generation	Mean \pmSE (Kabula – <i>An. gambiae</i>)	Mean \pmSE (Ahero – <i>An. arabiensis</i>)
First generation	65.13 \pm 5.70a	89.00 \pm 2.28 a
Second generation	75.00 \pm 4.52b	90.17 \pm 2.79 a
Third generation	90.00 \pm 2.94c	100.00 \pm 0.00 b
Forth generation	91.00 \pm 2.16c	
Fifth generation	100.00 \pm 0.00d	

Means denoted by similar letters in a column are not significantly different at $P \leq 0.05$

6.3.2 Changes in the monooxygenase levels that occurred during the selection process of pyrethroid susceptible *An. gambiae* and *An. arabiensis* populations

There was a 2.8 fold change of monooxygenases levels in the first generation of Kabula mosquitoes that slightly decreased to 2.7 in the second generation then reduced to 1.7 and 1.3 in the 3rd and fourth generations respectively. In Ahero mosquitoes, there was 1.1 increases in the monooxygenase levels both in the first and second generations (Figure 6.1).

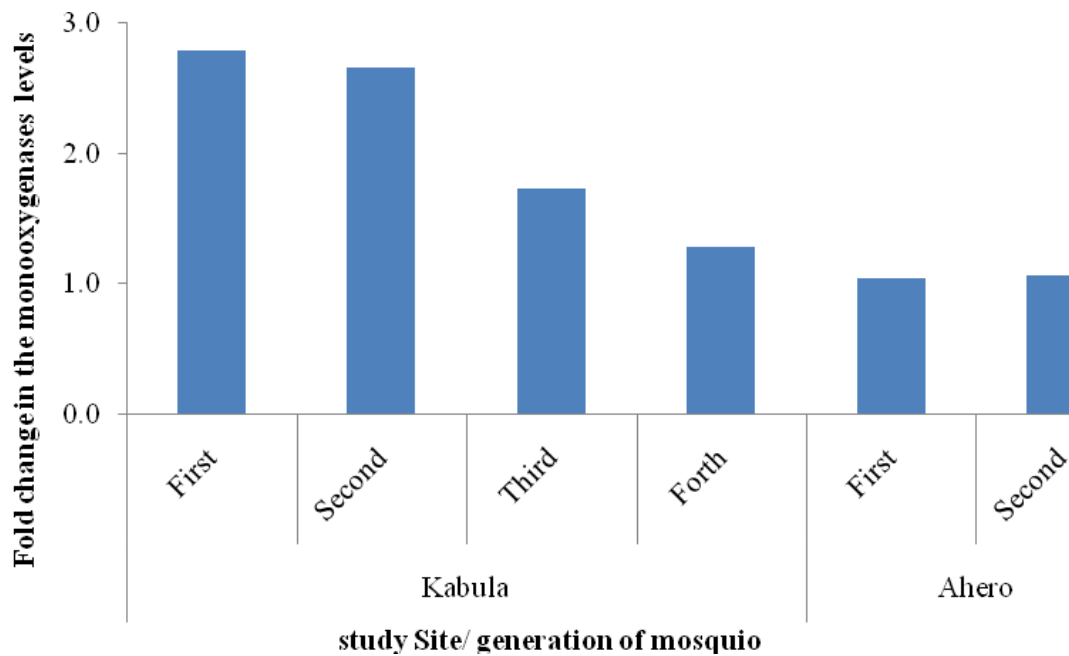


Figure 6.1: Change in the monooxygenase levels in the mosquitoes in different mosquito generations from Kabula and Ahero

6.3.3 Changes in the esterase levels that occurred during the selection process of pyrethroid susceptible *An. gambiae* and *An. arabiensis* populations

There was a 1.2 fold change of esterase enzymatic activity levels in the first generation of Kabula mosquitoes that slightly decreased to 1.1 in the second generation then reduced to 1.0 in the third and fourth generations. In Ahero mosquitoes, there was 1 fold increase in the esterase enzyme activity in the first and second generation (Figure 6.2).

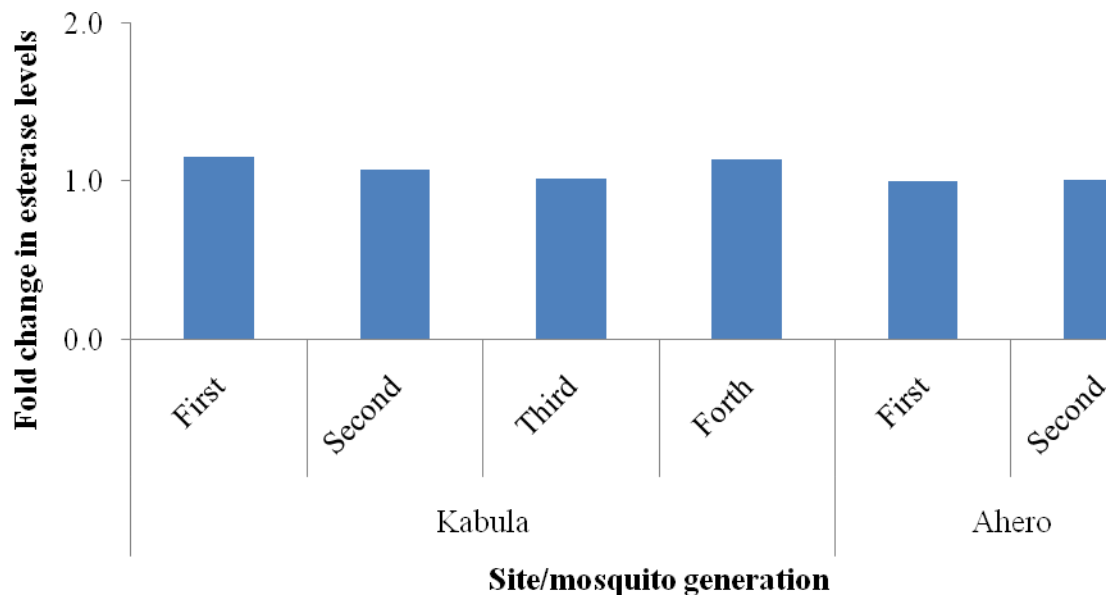


Figure 6.2: Fold change in the esterase levels in the mosquitoes in different mosquito generations from Kabula and Ahero

6.3.4 Changes in the glutathione S-transferase levels that occurred during the selection process of pyrethroid susceptible *An. gambiae* and *An. arabiensis* populations

There was a 1.1 fold change GSTs levels in the first generation of Kabula mosquitoes that slightly decreased to 1.06 in the second generation then went down to 1.03 and 1.02 in the third and fourth generations respectively. In Ahero mosquitoes, there was 1.05 fold increases in the Glutathione S-transferase enzyme activity in the first which reduced to 1.04 in the second generation (Figure 6.3).

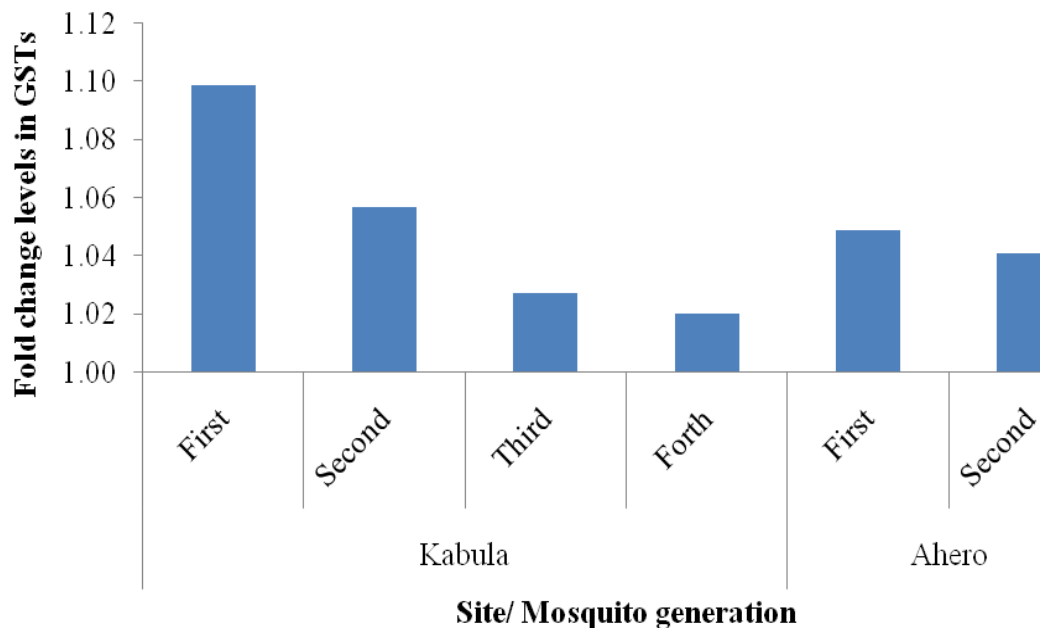


Figure 6.3: Change in the GSTs levels in the mosquitoes in different mosquito generations from Kabula and Ahero

6.3.5 Generational changes that occurred during the selection process of pyrethroid resistant *An. gambiae* and *An. arabiensis* populations

The PCR-based methods used to ascertain the *kdr* genotype of different mosquito progenies indicated that the frequency of the homozygous SS (L1014S allele) was significantly high in the first generation of Kabula *An. gambiae* populations (56.7%) but reduced in the subsequent generations. The *kdr* frequency in the second generation (50%) was significantly different from that of the third (10%) and fourth generations (10%), ($F = 38.09$, $P = 0.0001$). There were no *kdr* alleles found in all generations of Ahero mosquito populations (Table 6.2).

Table 6.2: The frequency of *kdr* allele % (\pm SE) in different generations of Kabula and Ahero mosquito populations

Site	Generation	N	LL	LS	SS	<i>Kdr</i> Frequency (%) (\pm SE)
Kabula	First generation	30	7	6	17	56.7 \pm 6.89a
	Second generation	30	10	5	15	50.0 \pm 5.49b
	Third generation	30	17	10	3	10.0 \pm 2.1c
	Forth generation	30	15	12	3	10.0 \pm 2.1c
Ahero	First generation	30	30	0	0	0.00
	Second generation	30	30	0	0	0.00

Mean values in the same column denoted by the same letters are not significantly different

LL represents wild type, LS represents heterozygote mutation, SS represents homozygote mutation, and Frequency is the mutation allele frequency (%).

6.4 Discussion

Results of part of the this study indicated that both *An. gambiae* and *An. arabiensis* populations' susceptibility to deltamethrin increased to reach 100% mortality at F-5 and F-3 generations for Kabula and Ahero mosquito populations respectively. This suggests that when mosquitoes were exposed to deltamethrin they became weaker in the subsequent generations. Previous studies link selection for resistance to over-use of insecticide-based vector control. However that was not the case in this study, indicating that for mosquitoes to develop resistance to a particular insecticide, they must be exposed to traces of the insecticides over time at the larval stage. These results contradict previous studies by Matowo *et al.* (2007), who raised SENN DDT resistant colony of *An. gambiae* and by the sixteenth generation mortality was 12.1%. Lines and Nassor. (1991) demonstrated a similar decline in DDT resistance with age in the *An. gambiae*. However, it has been shown those blood meals and possibly mating, impact on the ability of females to survive exposure to insecticides (Hunt *et al.*, 2005).

Kabula mosquitoes showed some resistance in the first, second and third generations, but eventually became susceptible in the fourth and fifth generations, where as Ahero populations were completely susceptible to deltamethrin in the third generation. Other studies reported that when a DDT-selected or permethrin-selected strain was used, mortality for both insecticides dropped to approximately 40% for DDT and < 20% for permethrin

(Hemingway, 1983). Surveys of natural populations of *An. arabiensis* throughout Sudan show high levels of insecticide resistance to all classes of insecticides except carbamates in Geriza and Central States (which includes Sennar) (Abdalla *et al.*, 2014).

Biochemical tests showed elevation of monooxygenase and esterase levels in the first and second generations of Kabula mosquitoes, which dropped in the third and fourth generations. There was no marked elevation in GST levels in all Kabula generations. There was also elevation of monooxygenase and esterase levels and second generations of Ahero mosquitoes which did not differ within the generations. This suggests that elevation in monooxygenases and esterases confers resistance to pyrethroids in *An. gambiae* species from western Kenya. Considering the elevation of the enzyme activity was only observed in resistant mosquitoes from the first generation. Similar studies in western Kenya have shown elevation in monooxygenases and esterases in permethrin resistant *An. gambiae* but no elevation in the same enzymes in susceptible *An. rabiensis* species (Ochomo *et al.*, 2012, 2013). Biochemical analysis of Sudan *An. arabiensis* by Hemingway (1983) did not confirm the bioassay and synergistic results implicating carboxylesterases as the mechanism responsible for the resistance. The findings from this study also indicated no elevation in GSTs in all generations, which contradicts previous studies by Matowo *et al.* (2010) that showed elevated GST enzyme levels in both males and females exposed to permethrin.

Results of this study reported a decrease in the frequency of *kdr* resistant alleles in the subsequent generations after exposure to deltamethrin. This suggests that there was a change in the genetic makeup of the mosquitoes with the frequency of *kdr* allele that confers resistance in mosquitoes reducing and even disappearing in the fifth generation of mosquitoes. Rapid selection for resistance is usually associated with phenotypically recessive mechanisms in which most exposure survivors are homozygous for the resistant loci. In this way, resistance alleles are driven to a high frequency very quickly.

The *kdr* mutation described by Martinez-Torres *et al.* (1998) in *An. gambiae* has been shown to be recessive in expression. However, this study did not indicate an increase in *kdr* gene frequency in the susceptible second generation, again suggesting that *kdr* played a role in resistance to pyrethroids. Other studies in Kenya by Ochomo *et al.* (2011), showed the presence of high frequency of *kdr* gene in resistant *An. gambiae* but no *kdr* was observed in susceptible *An. arabiensis*. Kawada *et al.* (2011) reported a high frequency of *kdr* in *An. gambiae* from western Kenya. A study on West African *An. gambiae* suggested that a large proportion of homozygous *kdr* RR females were killed by prolonged exposure to insecticides (Chandre *et al.*, 2000).

The *kdr* mutation has been shown to be closely associated with pyrethroid resistance in several *An. gambiae* populations (Martinez-Torres *et al.*, 1998; Brooke *et al.*, 1999; Chandre *et al.*, 1999; Diabate *et al.*, 2004; Awolola *et al.*, 2009; Fanello *et al.*, 2003, Ochomo *et al.*, 2013). However, neither of the *kdr*

mutations reported in *An. arabiensis* from four different African countries (Diabate *et al.*, 2004; Stump *et al.*, 2004; Kulkarni *et al.*, 2006; Verhaeghen *et al.*, 2006, Kawada *et al.*, 2011) has been positively correlated with the pyrethroid resistance phenotype as detected by insecticide bioassay. Diabate *et al.* (2004) identified three nucleotides upstream of the Leu →Phe *kdr* mutation which differentiates *An. gambiae* from *An. arabiensis* and which associates with the *kdr* mutation in *An. arabiensis*. They propose that this mutation has arisen independently in *An. arabiensis* as opposed to inheritance through introgression with *An. gambiae*. Stump *et al.* (2004), on the other hand, suggest introgression as one of the explanations for the occurrence of the *kdr* allele in a single individual in Kenya.

CHAPTER SEVEN

7.0 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General discussion

In this study, mosquitoes showed resistance to deltamethrin and lambda-cyhalothrin used for indoor residual spraying and impregnated on LLINs as revealed by wall bioassays and net cone bioassays. This was further confirmed by WHO tube bioassays, genotyping and biochemical assays. Mosquito resistance to pyrethroids has been reported in various parts of western Kenya by various studies, although few studies have reported mosquito susceptibility to other classes of insecticides like organophosphates and carbamates.

Although *Anopheles gambiae* mosquitoes from the study sites showed resistance to pyrethroids and organochlorines, they were susceptible to organophosphates and carbamates. The frequency of *kdr* allele approached fixation in *Anopheles gambiae* population but was absent in *Anopheles arabiensis* populations. Ochomo *et al.* (2012) reported similar results of high frequency of *kdr* allele in Bungoma *An. gambiae* population but absence of *kdr* allele in *An. arabiensis* in Ahero. Malaria control in these areas relies on the use of LLINs and IRS with pyrethroids. Stump *et al.* (2004) reported an increase in the level of insecticide resistance in Kenya as a result of ITN programs.

Although there was evidence of phenotypic resistance to deltamethrin and lambda-cyhalothrin, there was no measurable elevated expression of metabolic enzymes in surviving mosquitoes that had been exposed to deltamethrin and lambda-cyhalothrin. Therefore the mechanisms of resistance to deltamethrin and lambda-cyhalothrin could be target site resistance as a result of the presence of the *kdr* alleles, whereas resistance against permethrin and DDT may involve target site resistance and metabolic resistance as a result of the elevated expression of these β -esterase and oxidase enzymes. Therefore, the increase in frequency of the 1014S *kdr* allele in western Kenya populations reported in a previous study (Mathias *et al.*, 2011) and confirmed in the current study must be a relatively recent event that has escalated over the decade beginning in 2001.

This study is very important in guiding malaria control policy in Africa. The increased use of pyrethroid insecticides has selected for resistance in the major African malaria vector *An. gambiae*, *An. arabiensis*, and *An. funestus* (Kawada *et al.*, 2011; Ochomo *et al.*, 2012; Okumu *et al.*, 2012; Okumu *et al.*, 2013). In addition to being geographically widespread, *kdr* mutations have reached extremely high frequency levels (>80%) throughout Africa. The scale up of ITN and IRS programs will select for higher insecticide resistance (Mathias *et al.*, 2011). This represents the most important challenges in malaria control in Africa.

Malaria control in Africa relies heavily on the use of LLINs and IRS with pyrethroids. World Health Organization currently recommends that only pyrethroid insecticides should be used for impregnating bed net. Since *Anopheles gambiae* was resistant to this insecticide at all sites in western Kenya, it poses a great challenge on the effectiveness of ITN in malaria control. Although, WHO stated that all four types of insecticides may be used for IRS, considering the mosquito resistance detected, only organophosphate insecticides may be recommended in order to optimize control efficacy and to contain the insecticide resistance (WHO, 2012b).

7.2 Conclusions

- i. Lambdacyhalothrin and deltamethrin sprayed on the walls showed reduced efficacy within the first four and one month respectively and there was also reduced efficacy of the insecticidal effects of LLINs on mosquitoes 18 months after usage.
- ii. There was high phenotypic resistance to pyrethroids and DDT in *An. gambiae* populations, but high susceptibility to carbamates and organophosphates.
- iii. Both elevation in the activity of monooxygenases and esterases and the presence of *kdr* allele underlies phenotypic resistance.
- iv. There were phenotypic, genotypic and metabolic changes within different mosquito generations.

7.3 Recommendations

- i. New nets should be redistributed every three years as recommended by WHO while maintaining high levels of LLINs coverage and usage.
- ii. Indoor residual spraying with lambdacyhalothrin and deltamethrin should be done after every four months and two months respectively for effective control of malaria vectors.
- iii. Different classes of insecticides e.g Organophosphates should be used for indoor residual spraying especially in areas where there is high coverage of LLINs to prevent the development of insecticide resistance in mosquitoes.

7.4 Suggestions for further studies

- i. Further studies should be done to confirm whether the observed resistance could result in LLINs and IRS malaria control interventions failure.
- ii. There is need for base line surveys in Kenya, in order to make informed decisions on the choice of insecticides to be used for vector control operations.

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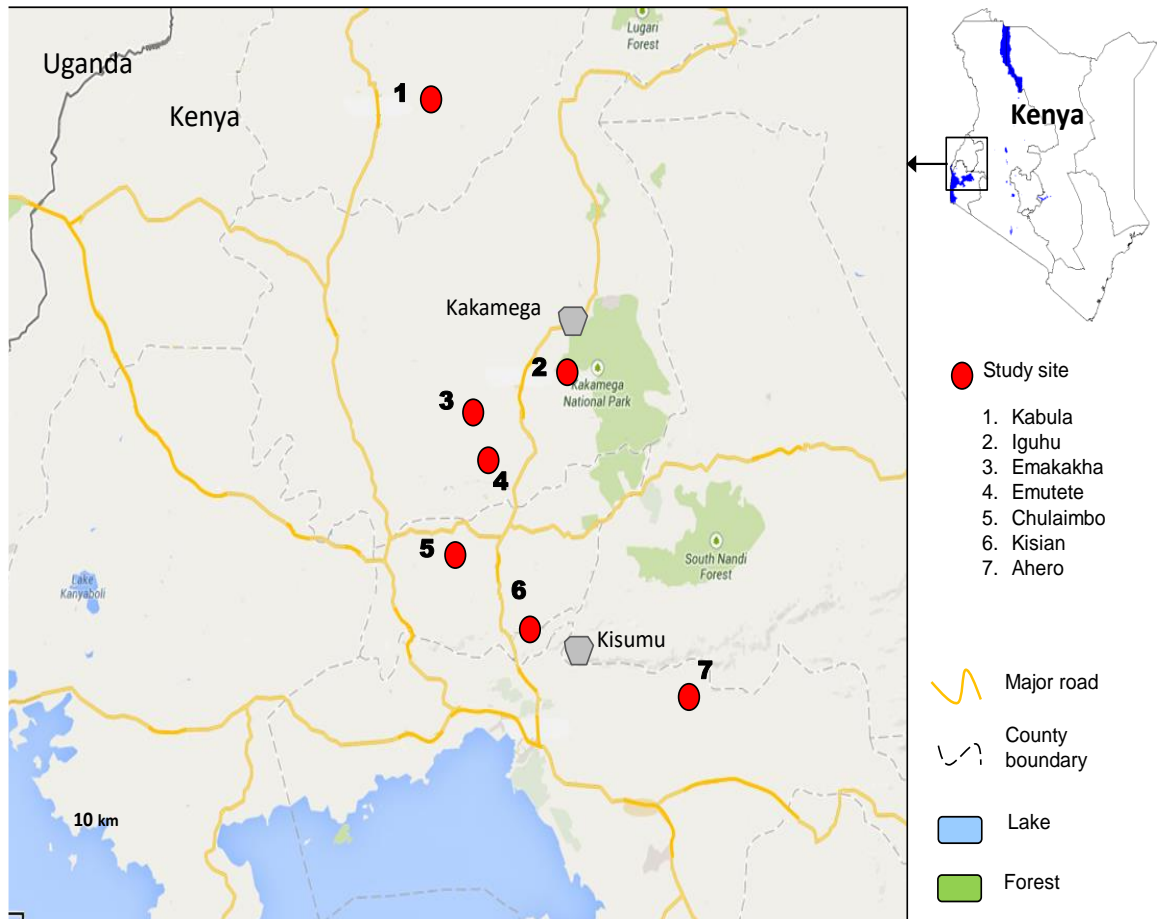
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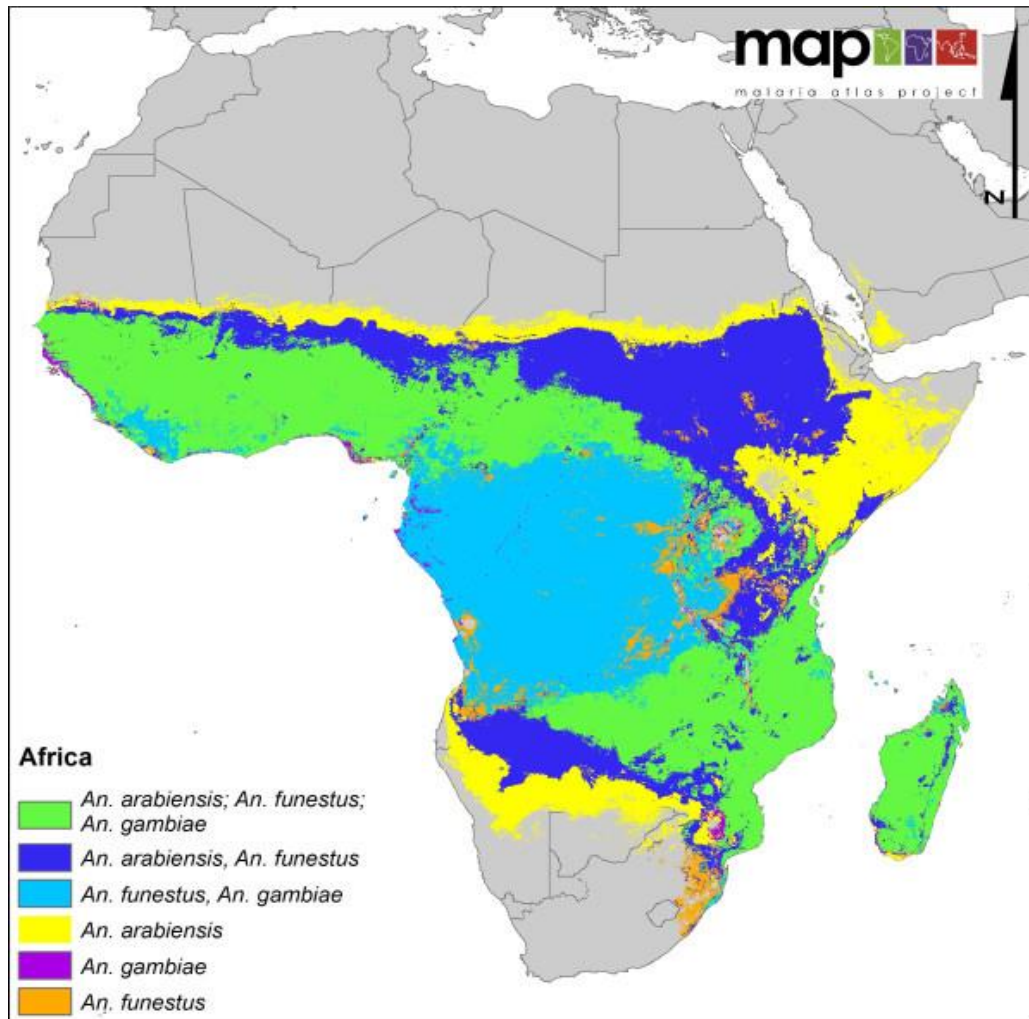
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APPENDICES

APPENDIX I: A map of the study sites



(Wanjala et al., 2015)

APPENDIX II: A map of malaria vectors distribution in Africa

(Sinka *et al.*, 2012)

APPENDIX III: Mosquito larvae collections

a) Larval habitats



b) Larval collection using dippers



c) Larval rearing trays



d) Adult mosquito cages



APPENDIX IV: Questionnaire for collection of long lasting insecticide from the field

1. Name of the Village.....

Date.....

2. Type of the net.....

...

3. Name of the household head

Age.....

4. How many members of the family do you have?

.....

5. Do you have a mosquito net?

Yes

No

6. If yes, how many mosquito nets do you have?

.....

7. Where did you get the mosquito net from?

.....

a. Hospital

b. Bought from the supermarket.

8. When did you get the mosquito nets?

.....

9. When did you start using it?

.....

10. Where do you hang the mosquito nets in the house?

.....

11. Who uses the mosquito net in the house?.....

- a. Parents
- b. Children
- c. Everybody

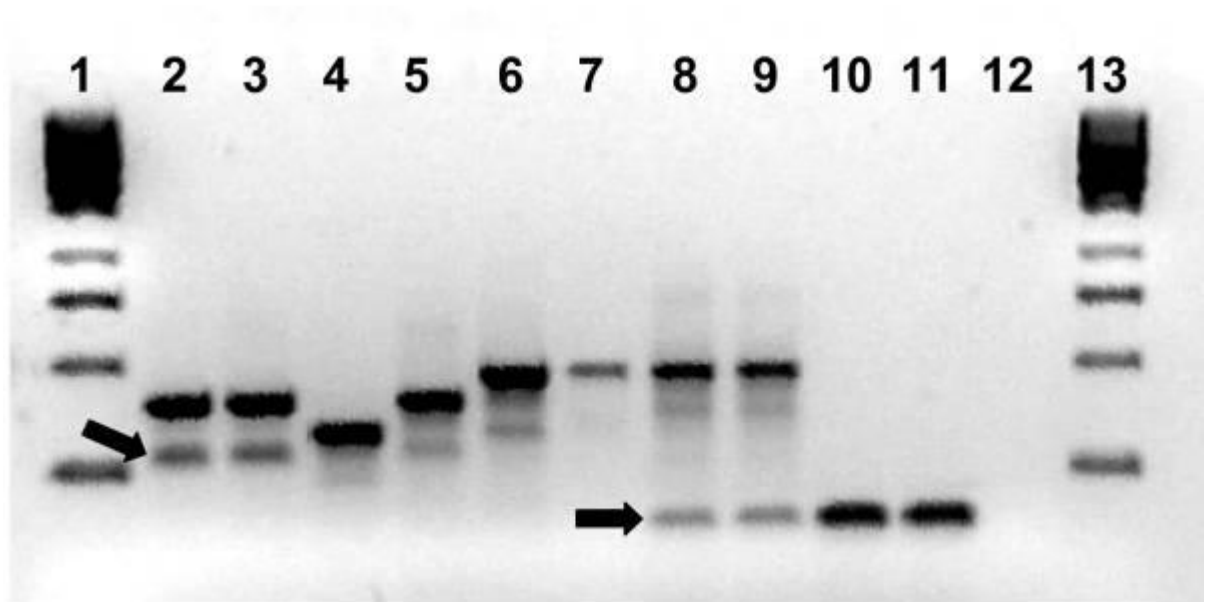
12. How often do you wash the mosquito nets?.....

13. How do you hang the mosquito nets after washing?

.....

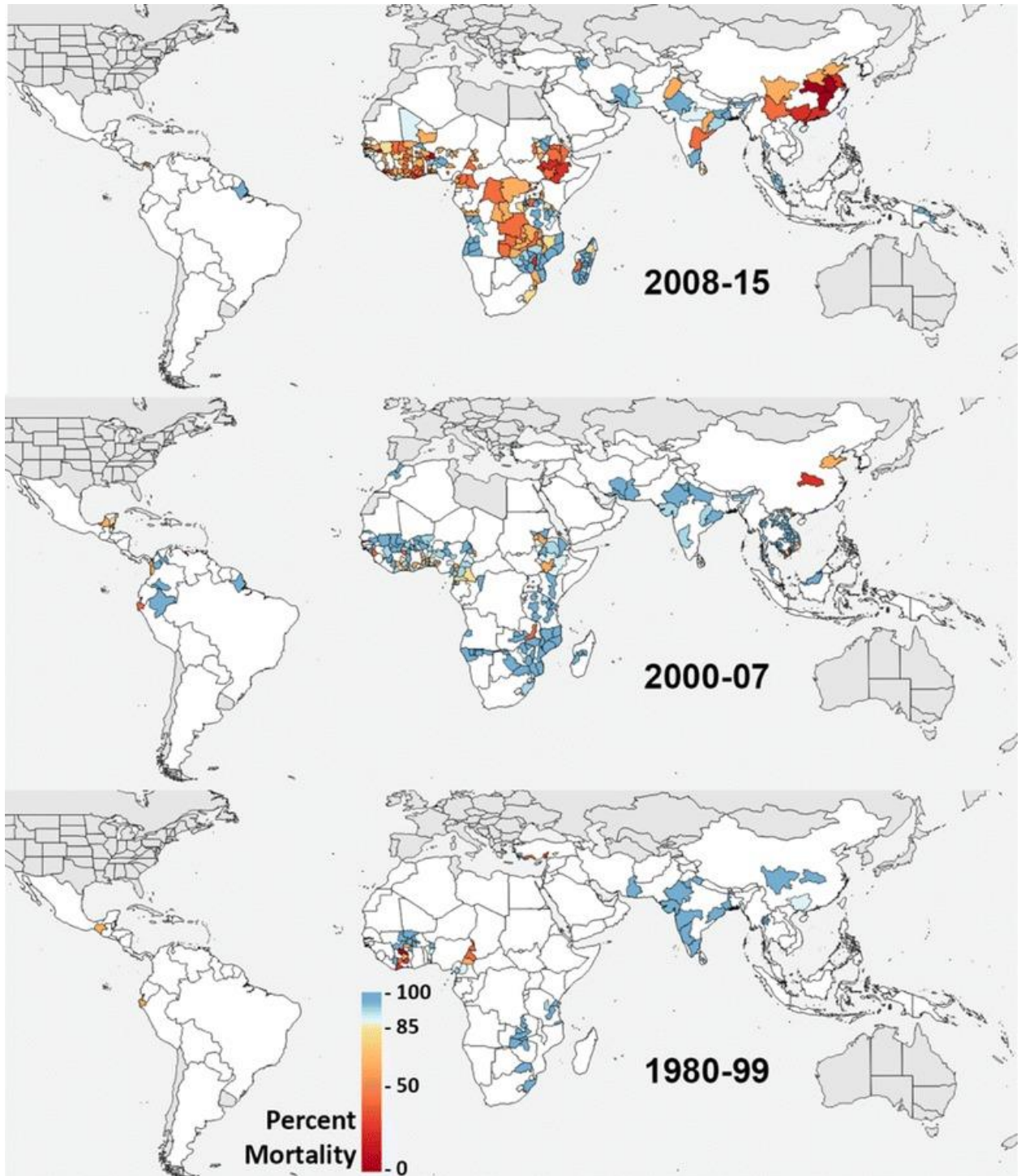
(After the interview, the old mosquito net was collected and replaced with new ones, and the physical condition of the net recorded, then the nets were taken to KEMRI Kisian laboratories for further analysis).

APPENDIX V: DNA amplification for identification of *An. gambiae* and *An. arabiensis* species.



The arrows highlight the presence of non-specific bands. Lanes contain (1,13) 1 kb DNA marker, (2,3,5) *An. gambiae* s.s., (4, 8, 9) *An. arabiensis*, (6,7,10,11).

**APPENDIX VI: Trends in pyrethroid resistance for *Anopheles* species
between 1980 and 2015**



(Coleman *et al.*, 2017)

APPENDIX VII: Publications

1. **Christine L.Wanjala**, Guofa Zhou , Jernard Mbugi, Jemimah Simbauni, Yaw A. Afrane, Edna Ototo, Maxiwel Gesuge, Harrisone Atieli, Andrew K. Githeko, Guiyan Yan Insecticidal decay effects of Long-Lasting Insecticide nets and Indoor Residual Spraying on *Anopheles gambiae* sl. in Western Kenya. *Parasites & Vectors* 2015, **8**:588
2. **Wanjala CL**, Mbugi JP, Ototo E, Gesuge M, Afrane YA, Githeko AK, Atieli HE, Zhou G and Yan G: Pyrethroid and DDT-resistance and organophosphate susceptibility among *Anopheles* mosquito Species Western Kenya. *Emerging Infectious Diseases* 2015 **21**:12.
3. Ednah N Ototo, Jenard P Mbugi, **Christine L Wanjala**, Guofa Zhou, Andrew K Githeko **and** Guiyun Yan. Surveillance of malaria vector population density and biting behaviour in western Kenya. *Malaria Journal* 2015, **14**:244