EFFECT OF KNOCKDOWN RESISTANCE ON *PLASMODIUM FALCIPARUM* SPOROZOITE RATES IN MALARIA VECTORS IN WESTERN KENYA

BY

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ABSTRACT

Malaria, a vector borne disease is still a public health problem in sub-Saharan Africa. In western Kenya, malaria is endemic with the predominant vectors being *An. gambiae s.s, An. arabiensis* and *An. funestus*. Pyrethroid-based long lasting insecticidal nets and indoor residual spraying are currently major effective tools employed to control malaria vectors in endemic areas in western Kenya. However, they are currently threatened by insecticide resistance. Malaria vectors exhibit varied geographical distributions and *An. gambiae s.s, An. arabiensis* and *An. funestus* are the primary vectors in Busia, Ahero and Bondo respectively. Knockdown resistance has been documented as a mechanism of resistance against pyrethroids. Recently, both L1014S and L1014F mutations associated with knockdown resistance have been reported in western Kenya. Whether these mechanisms correlate with sporozoite rates in *Anopheles gambiae s.l* mosquitoes in western Kenya is unknown. The overall aim of this study was to investigate whether knockdown resistance had impact on malaria transmission. Specifically, this study investigated rate of deltamethrin phenotypic resistance; *P. falciparum* sporozoite rate and *Anopheles* densities between wild-caught female *An. gambiae s. l* and *An. funestus* mosquitoes; determined whether *kdr* genotypes (L1014S and L1014F) frequency has association with *P. falciparum* sporozoite rate in *An. gambiae s.s* and *An. arabiensis*; compared the knockdown resistance allele frequency in larval raised and wild-caught female *An. gambiae s. l.* Samples were collected from Ahero, Bondo and Busia in western Kenya. In the laboratory-based experimental design, larval raised adults and wild caught *Anopheles* were exposed to 0.05% deltamethrin using the WHO tube assays to test for susceptibility. Conventional PCR and real-time PCR were used for species identification and *kdr* genotyping respectively. Sporozoite rates were determined using ELISA in all wild-caught female mosquitoes. Of 4,360 wild-caught *Anopheles* sampled, 32.8% were *An. funestus* and the rest were *An. gambiae s.l.* A total of 2,186 larvae raised adults *An. gambiae s.l.* were also used for the study. Independent t-test showed that *An. funestus* were more phenotypically resistant compare to *An. gambiae s.l.* ($P<0.001$). Independent t-test also showed that wild-caught adult mosquitoes were more phenotypically resistant than larval raised ones ($P<0.001$). Independent t-test showed that *An. funestus* had higher sporozoite infectivity rate than *An. gambiae s.l* ($P<0.001$). Two-way classification chi-square test of association revealed that there was a significant association between *kdr* east (L1014S) and sporozoite rate in *An. gambiae s.s* ($X^2_1=5.49$, $P=0.032$). It also showed that the L1014S and L1014F allele frequencies in larvae raised and wild *An. gambiae s.l* were not statistically significant. The finding of a higher level of resistance in wild versus larval raised adults could be alluded to other mutations and mechanisms of resistance. It also suggests that wild mosquitoes should be considered in assessment of phenotypic resistance. The observation of high sporozoite rates in *An. funestus* in Bondo and Busia compared to *An. gambiae s.l* indicates that this re-emerged species could be driving malaria transmission in the sub-Counties. The significant association between *kdr* east (L1014S) and sporozoite rate in *An. gambiae s.s*, suggest the epidemiological importance of that marker; it could drive malaria transmission. The insignificant difference observed in kdr (L1014S and L1014F) frequencies in wild and larval raised adults which did not reflect in phenotypic resistance suggest that other mechanisms of insecticide resistance might contribute to resistance in wild mosquitoes. The findings of this study will help in insecticide resistance monitoring and assessment programmes.

CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria is ranked as the fourth highest cause of death worldwide (WHO, 2016). Children under five years and pregnant women are mainly vulnerable leading to an approximately 70% of malaria deaths (WHO, 2016). Africa regions continue to have the heaviest malaria burden with 92% of global malaria-related deaths in recent past were reported in the region (WHO, 2016). Millions of people are still not accessing the services they require to prevent and treat malaria (WHO, 2016). Parasitic malaria infection of red blood cells kills approximately 2000 people per day, most of whom are children in Africa (White *et al.*, 2013).

Malaria transmission in western Kenya is very high compared to other part of the country with the three main vectors; *An. gambiae s.s*, *An. arabiensis* and *An. funestus* being among the most predominant vectors in Busia, Ahero and Bondo respectively (President's Malaria Initiative, 2016). The main strategies currently employed to control malaria vectors in malaria endemic areas heavily rely on reducing vector-human contact using long-lasting insecticide nets (LLINs) and indoor residual spraying (IRS) (Hemingway, 2014; Okara *et al.*, 2010). In Kenya, LLINs and IRS are the major control strategies (Chen *et al.*, 2008). The use of these strategies has increased exponentially for the past decade as part of the movement towards global coverage of all population at risk hence saving hundreds of thousands of lives (WHO, 2012). Pyrethroids such as deltamethrin, permethrin, cyfluthrin are the main classes of insecticides currently used for malaria vector control (Ranson & Lissenden, 2016) and had an impact on the global decline in reduction of malaria transmission between 2000-2015 (Bhatt *et al.*, 2015). Insecticide resistance in malaria vector control has been widely reported in

many malaria endemic regions particularly in Africa (Mnzava *et al.*, 2015) though resistance data on *An. funestus* population were reported to be limited (Ranson and Lissenden, 2016).

The resurgence of malaria vectors and the disease in western Kenya were partially attributed to reduced efficacy of insecticide treated nets (ITN) and insecticide resistance (Hamel *et al.*, 2011; Zhou *et al.*, 2011). Pesticides use for crop farming and livestock pest control have been reported to have had selective pressure on mosquito vectors by leaching into their breeding habitats suggesting that pre-exposure of immature mosquitoes or larvae to pesticides could lead to selection pressure to resistance (Yadouleton *et al.*, 2010). Indoor resting mosquitoes could also develop resistance to insecticides use in impregnation of bednets and IRS programs as they interact with LLINs during host seeking (Karunamoorthi & Sabesan, 2013). Earlier studies in western Kenya such as Ahero, Busia and Bondo reported high resistance to pyrethroids although most of these studies used *An. gambiae s.l* larvae collected from the fields to determine insecticide resistance (Ochomo *et al.*, 2012; Ochomo *et al.*, 2014; Wanjala *et al.*, 2015). Hence it was unknown whether wild adults mosquitoes resting indoors could be more resistant to pyrethroids than larvae sampled from within and around agricultural fields.

The main types of insecticide resistance mechanism are mutations in the target sites genes and metabolic alterations at the level of the activity of detoxification of proteins (Hemingway and Ranson, 2000) Knockdown resistance is a type of target site resistance mechanism caused by mutation in the voltage-gated sodium channel either by substitution of leucine (TTA) with phenylalanine (TTT) (Martinez-Torres *et al.*, 1998) commonly referred to as West African *kdr* mutation (L1014F) or substitution of leucine (TTA) with serine (TCA) (Ranson *et al.*, 2000) commonly referred to as East African *kdr* mutation (L1014S) at codon 1014. Mutations of the genes in the sodium ion channel, the target site of pyrethroids confer resistance to these insecticides (WHO, 2013). Knockdown resistance is one of the most widely known and well-studied mechanisms of insecticide resistance (Ranson *et al.*,

2011). However, the extent to which knockdown resistance mutation impact malaria transmission has not been elucidated.

Sporozoite rate of *P. falciparum* is one of the major components of vectorial competence as it indicate the ability of the *Anopheles* vector to transmit malaria (Garrett-Jones, 1964; Okwa *et al.*, 2007). Malaria infection of *P. falciparum* is the primary causes of malaria transmission in western Kenya (Hamel *et al.*, 2011). A study carried out in western Kenya to determine *An. gambiae s.s* and *An. arabiensis* susceptibility to insecticides documented moderate level resistance to pyrethroids but L1014S *kdr* was reported to have "approached fixation" in *An. gambiae s.s* (Mathias *et al.*, 2011). The L1014F *kdr* was first identified in western Kenya in 2012 in both *An. gambiae s.s* and *An. arabiensis* populations (Ochomo *et al.*, 2015). Further studies revealed that insecticide resistance in malaria vectors was emerging in Busia, Bondo and Ahero which could vary from time to time and within other small geographical areas in western Kenya (Ochomo *et al.*, 2014). The studies carried out however did not assess whether *Anopheles* having L1014S and L1014F mutations had higher sporozoite rate which could be linked to malaria transmission. Moreover, the variability in sporozoite rate, mosquito density between *An. gambiae s.l* and *An. funestus*; whether there is similarity in *kdr* (L1014S and L1014F) frequencies in larvae raised adults and wild-caught adult *An. gambiae s.l* mosquitoes is unknown.

1.2 Statement of the Problem

Malaria is a common public health problem in tropical and sub-tropical countries. Malaria vector resistance to insecticides used in vector control poses a great challenge to resistance management strategies outlined by World Health Organization (WHO) in the Global Plan for Insecticide Resistance Management (GPIRM). Malaria vector resistance to pyrethroids was partly attributed to application of agricultural pesticides which contaminate mosquito breeding habitats. Adults mosquitoes could also develop resistance to pyrethroids due to interaction with LLINs impregnated with pyrethroids and insecticides use in IRS program. Thus, it was unclear whether adults mosquitoes resting indoors could be more phenotypically resistant to deltamethrin than larvae raised ones. Even though *kdr* west (L1014F) has been implicated in insecticide resistance compromising vector control in *An. gambiae s.s*, the impact of knockdown resistance mutations (L1014S and L1014F) on sporozoite rate which could be linked to malaria transmission in holoendemic region such as western Kenya is unclear. *An. funestus* and *An. gambiae s.l* are major vectors identified to be responsible for malaria transmission in western Kenya. However, it is unknown which of the two (*An. gambiae s.l* or *An. funestus*) is more competent in terms of higher sporozoite rate and vector density and could therefore be driving malaria transmission in the region. Furthermore, studies have shown insecticide resistance to pyrethriods in malaria endemic areas with western Kenya not exempted, but it is unknown whether there is variation *kdr* alleles (L1014S and L1014F) frequency in field larval raised *An. gambiae s.l* and wild caught adult *An. gambiae s.l* mosquitoes.

1.3 Research Objectives

1.3.1 General Objective

To assess whether knockdown resistance had effect on *Plasmodium falciparum* sporozoite rates in malaria vectors sampled from western Kenya

1.3.2 Specific Objectives

1. To determine rate of deltamethrin phenotypic resistance among *An. gambiae s.l* and *An. funestus* mosquitoes sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya.

2. To determine *P. falciparum* sporozoite rate and malaria vector densities between wild *An. gambiae s. l* and *An. funestus* mosquitoes sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya

3. To determine whether kdr genotypes (L1014S and L1014F) frequency had association with *P. falciparum* sporozoite rate in *An. gambiae s.s* and *An. arabiensis* sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya

4. To determine whether *An. gambiae s.s.* and *An. arabiensis* larval raised mosquitoes had higher kdr allele frequency than wild *An. gambiae s.s* and *An. arabiensis* collected from households in Busia, Bondo and Ahero.

1.4 Null Hypothesis

1. The rate of deltamethrin phenotypic resistance is not similar among *An. gambiae s.l* and *An. funestus* mosquitoes sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya.

2. There is no difference in *P. falciparum* sporozoite rate and malaria vector densities between wild *An. gambiae s.l* and *An. funestus* mosquitoes sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya

3. The *kdr* genotypes (L1014S and L1014F) frequency has no association with *P. falciparum* sporozoite rate in *An. gambiae s.s* and *An. arabiensis* sampled from Busia, Bondo and Ahero in malaria holoendemic region of western Kenya

4. *An. gambiae s.s* and *An. arabiensis* larval raised mosquitoes do not have higher *kdr* frequency than wild *An. gambiae s.s* and *An. arabiensis* collected from households in Busia, Bondo and Ahero.

1.5 Justification of the study

Malaria transmission is a burden to public health in many endemic countries especially during high transmission season. Increase in insecticides resistance in major vectors such as *An. gambiae s.s. An. arabiensis* and *An. funestus* as a result of prolonged exposure to insecticides have been reported. There is the need to determine phenotypic resistance in both larval and wild mosquitoes in order to consider which one is more suitable for monitoring insecticide resistance. Knockdown resistance mutations (L1014S and L1014F) were identified in western Kenya but there is no conclusive evidence to support that these mutations (L1014S and L1014F) correlate with *P. falciparum* sporozoite rate in *An. gambiae s.l.* Studies carried out in endemic areas in Africa seem to be contradicting. Hence there is no conclusion that *kdr* resistant vectors could transmit malaria more or less than non-resistant vectors.

Also, assessing sporozoite rate in major malaria vectors (*An. gambiae s.s, An. arabiensis* and *An. funestus*) in western Kenya could provide useful data on malaria epidemiological outcomes.

1.6 Significance of the Study

The significant association between *kdr* east (L1014S) and sporozoite rate in *An. gambiae s.s* suggests the epidemiological importance of that marker. The significant difference in *P. falciparum* sporozoite rate in *An. gambiae s.l* and *An. funestus* could serve as a useful reference data to agencies such as President Malaria Initiative, Ministry of Health and researchers in order to scale up malaria vectors control strategies or employ a combination of vector control tools to reduce malaria transmission. Furthermore, significant difference on the level of phenotypic resistance between larval raised mosquitoes versus wild caught mosquitoes could be useful in insecticide resistance monitoring and assessment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria Epidemiology

Malaria is the most devastating protozoan disease of human beings; it is transmitted by *Anopheles* mosquitoes (White *et al*., 2013). There are about 400 different species of *Anopheles* mosquitoes, but only 30 of these are vectors of major importance in malaria transmission (WHO, 2016). It is a complex disease caused by five *Plasmodial* species *Plasmodium falciparum, Plasmodium ovale, Plasmodium vivax, Plasmodium malariae and Plasmodium knowlesi* (Raghavendra *et al*., 2011). Among these species *P. falciparum* malaria remains one of the most important pathogen-specific causes of human mortality (Collins *et al*., 2000) and 99% of the majority of mortalities in 2015 were attributed to *P. falciparum* malaria (WHO, 2016). Malaria is ranked as the major killer of children, mainly in Sub-Sahara Africa, taking the life of a child every two minutes. In 2015, 91 countries and territories worldwide had on-going malaria transmission but global burdens of mortality are dominated by countries in Africa (WHO, 2016).

Malaria is estimated to have cost nearly US$300 million on average annual expenditure for management and control alone for the past one and half decade; US$ 6.4 billion per year was globally estimated to be mobilized by 2020 to fully fund the fight against malaria (Malaria, 2012). The emergence and resurgence of vector-borne diseases like malaria in the 21st century is still a major threat to human health, causing more than million deaths and considerable mortality and morbidity globally. High population growth across the globe which led to extensive deforestation, irrigation, unplanned urbanization has been identified to influence the proliferation of vector-borne disease such as malaria (Karunamoorthi & Sabesan, 2013). The resurgence of malaria endemic in tropical countries

was also ascribed to antimalarial drug resistance, insecticide resistance in control vectors, funding reduction and cessation and relaxation of control efforts (Cohen *et al.*, 2012).

In western Kenya, *P. falciparum* is the most dangerous parasite of malaria and major cause of mortality and morbidity, accounting for over 99% of all malaria infection in the country (Ojakaa *et al.*, 2011;Zhou *et al.*, 2011). It was estimated that 28 million people reside in malaria endemic areas with approximately 11 million people living in areas where malaria parasite prevalence is estimated to be greater than 20% (President's Malaria Initiative, 2016). Endemic areas include Busia, Bondo, Ahero and Lake Victoria basin in western Kenya where transmission is severe throughout the year with *P. falciparum* prevalence greater than 20% and higher entomological inoculation rate (Fig. 2.1). For the past decades vector control through the use of insecticides has been targeted as the main weapon and it is still the most effective weapon in malaria control programmes (Karunamoorthi, 2011).

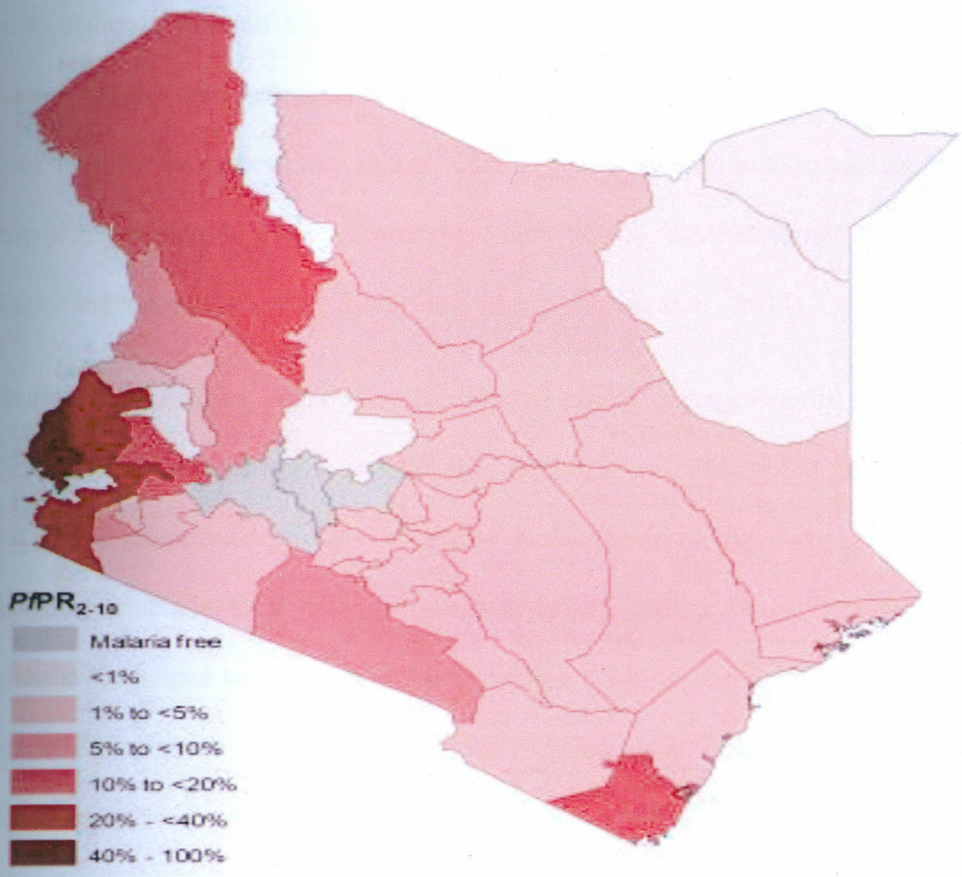

Figure 2.1: Map of Kenya indicating population-adjusted *P. falciparum* prevalence by County (Noor *et al.*, 2012)

2.2 Malaria Vector Control

Vector control is defined as measures of any kind directed against a vector of disease and intended to limit its ability to transmit the disease (Karunamoorthi, 2011). Vector control is one of the most effective strategies currently available in malaria control programmes (Cohen *et al.*, 2012). The options available for present day vector control efforts mainly include insecticide-treated materials, biological control agents, insect growth regulators (IGRs), environmental management, and personal protection methods against mosquito vectors (Raghavendra *et al.*, 2011).

Undoubtedly, the ongoing malaria vector control efforts depend heavily on insecticide treated nets (ITNs) including long-lasting ITNs and IRS (Hemingway, 2014). Indoor residual spray is largely responsible for spectacular reduction in disease incidence during the early twentieth century, including elimination of malaria from many countries (Wakabi, 2007). Hence International malaria control programme has helped to deploy about 289 million ITNS to sub-Sahara Africa which had increased the use of ITNs and IRS in large scale than ever before in malaria endemic countries. Consequently, numerous successes were achieved through the use of ITNs and IRS (Karunamoorthi & Sabesan, 2013). Indoor residual spraying is widely used in areas of seasonal transmission including epidemic-prone areas and increasingly being used in more malaria-endemic areas (Malaria, 2012). Insecticide treated nets (ITNs) impregnated with pyrethroids, that were introduced do not only decrease the man–mosquito contact by deterrence or excito-irritability but also kill the mosquito with its residual insecticidal activity. The common insecticide used in vector control are pyrethroids (Malaria, 2012; WHO, 2013). Pyrethroids have become relatively inexpensive, provide quick knockdown and are relatively safe compounds to use near humans to control common house frequenting mosquitoes (Chareonviriyaphap *et al.*, 2013).

Although vector control in many countries still use insecticides in the absence of viable alternatives, few development of control strategies using ovitraps, space spray, biological control agents among others were established but used in limited scale. Recent introduction of safer and reliable vector control agents such as insect growth regulators, biocontrol agents, natural plants products and environmental management methods have also yielded positive results and are yet to be implemented on large scale for vector control (Raghavendra *et al.*, 2011).

2.3 General Introduction to Insecticide Resistance

According to WHO, (1957) insecticide resistance is defined as "the developed ability in a strain of insects to tolerate doses of toxicant which would prove lethal to majority of individuals in a normal population of the same species". A population is described as resistant to an insecticide if a mortality rate less than 90% is observed in the tests (WHO, 2013). Insecticide resistance has been observed in over 50 *Anopheles* species (Diptera: culicidae) responsible for the transmission of malaria to human worldwide (Hemingway and Ranson, 2000). Insecticide resistance occurs through physiological and behavioral changes on a population level that are set in motion by repeated environmental exposures to insecticide or other toxins over time (Reid & McKenzie, 2016).

In Africa, the first insecticide resistance in *An. gambiae* was noticed in Bobo Dioulasso, Burkina Faso in the year 1967 (Metcalf, 1989). Later, (DDT) resistance was found in neighboring countries; Cote d'Ivoire, Nigeria and Mali and East and Central Africa countries (Carnevale *et al.*, 2009). Dichlorodiphenyltrichloroethane (DDT) was more or less abandoned worldwide after 1980s and was replaced by organophosphate, pyrethroid and carbamate (Metcalf, 1989). However, as resistance still persists, vector control was negatively affected mainly in Africa, Latin America and India through the use of agricultural pesticides. In the late 1970s, pyrethroids were introduced and increasingly used in

1990s till today (Corbel & N'Guessan, 2013). Since the pyrethroids were employed for vector control, cases of rapid resistance were reported in malaria vectors worldwide including *An. albimanus*, *An. darlingi*, *An. culicifacies*, *An. stephensi*, *An. gambiae*, *An. funestus*, *An. arabiensis* and *An minimus* (Corbel & N'Guessan, 2013).

Insecticide resistance necessitates that all malaria-endemic areas should continuously monitor local vectors so as to ascertain the impact of control mechanisms. Now evidence of accumulation of malaria vector resistance to commonly used insecticides has been reported in several malaria endemic countries worldwide, including Côte d'Ivoire, South Africa, Burkina Faso, Ghana, Equatorial Guinea, Angola, Gabon, Benin, Ethiopia and Congo–Brazzaville (Karunamoorthi & Sabesan, 2013). The development of insecticide resistance in insect pests and disease vectors is increasing on an alarming scale worldwide (Fig. 2.2). However, patterns of resistance is not uniform across all areas, with some vector populations having low instances of resistance in spite of long-term use of chemicals to control them (Chareonviriyaphap *et al.*, 2013).

The two main mechanisms of resistance; target site mutation and metabolic-based mechanisms have been reported in *An. gambiae s.s.* from West Africa (Hemingway, 2014). These have resulted in elevated levels of resistance rising up to 1000-fold and the emergence of cross resistance (involving resistance to different classes of insecticides) to additional resistance (resistance to new class of insecticide) (Edi *et al.*, 2012). Insecticide resistance has been documented in areas covered with LLINs (Haji *et al.*, 2013). In agricultural areas, *An. gambiae s.s.* were found breeding in wells used for irrigation (Klinkenberg *et al.*, 2008). *An. gambiae s.s.* and *An. arabiensis* can also be found in temporarily rain pools, hoof prints around edges of dams, pans and rice fields (Coetzee *et al.*, 2013). Pyrethroids resistance of vector mosquitoes is one of the key barriers against effective vector control (Kawada *et al.*, 2011). High levels of resistance to pyrethroid in *An. gambiae s.s.* (Protopopoff *et al.*,

2013; Toé *et al.*, 2014) and *An arabiensis* (Haji *et al.*, 2013) has been observed. Previously, insecticide resistance is known to be centered on Western and Central Africa but a recent study pointed out that resistance has spread dramatically throughout malaria endemic areas in Africa and beyond (Nkya *et al.*, 2014). Surveys carried out in western Kenya including Busia, Bondo and Ahero have documented emergence of genotypic, phenotypic and metabolic insecticide resistance (Chen *et al.*, 2008; H. Kawada *et al.*, 2011; Mathias *et al.*, 2011; Ochomo *et al.*, 2014). These could have negative effect in the use of insecticide treated nets for vector control programmes.

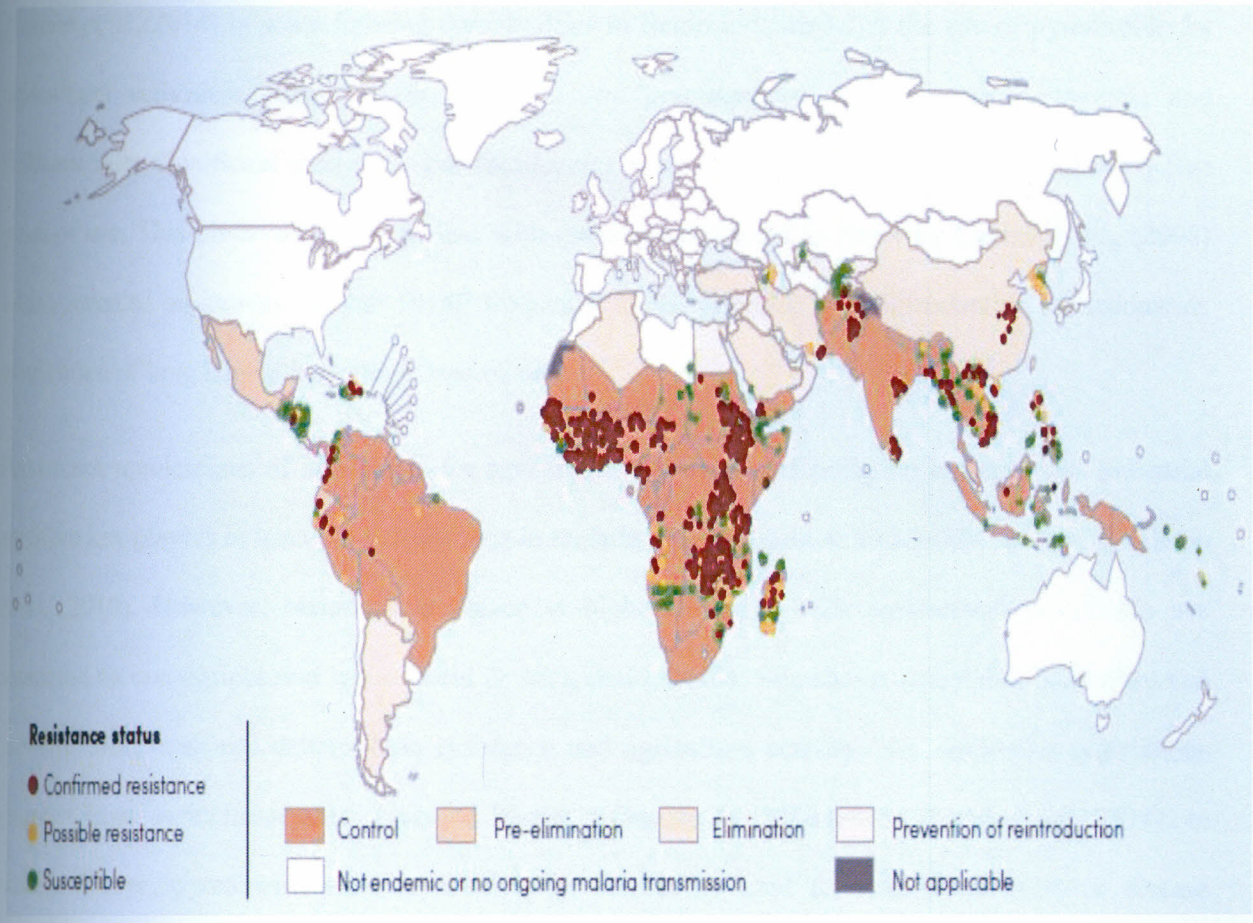

Figure 2.2: Malaria Vector resistance to insecticides across various endemic areas worldwide (WHO, 2015)

significant influence on selection pressure for mosquito vectors. Studies have shown that the preferential breeding sites for malaria vectors are within and around agricultural areas prone to the use of insecticides for pest control (Muriu *et al*., 2008; Vanek *et al*., 2006). For instance, *An. gambiae s.l* was found to be more resistant to pyrethroids (delthamethrin and permethrin) in areas of regular use of pesticides but susceptible in areas of low or no use of pesticides (Diabate *et al*., 2002). Thus, there is a risk of insecticide resistance development in malaria vectors due to pesticides exposure. Studies have shown that agricultural use of pyrethroids for crop protection and livestock pest control had led to development of resistance in mosquitoes (Diabate *et al*., 2002; Dongus *et al*., 2009; Overgaard, 2006). Endophilic vectors prefer resting indoors after host seeking for blood meal hence they interact with LLINs which could predispose them to develop resistance (Takken, 2002). Despite exposure to insecticides at different stages of *Anopheles* development, it is unknown whether larvae collected from within and around agricultural fields could be more resistant to deltamethrin than wild adults resting indoors.

2.5 Malaria Vector Density in Kenya

Over 140 *Anopheles* species have been documented across Africa of which at least 8 of this number were considered to be malaria vectors. *Anopheles* mosquitoes belong to phylum Arthropoda, class Insecta, order Diptera, sub-order Nematocera, family Culicidae, sub-family Culicinae, genus *Anopheles*, and sub-genus Cellia (Gillies & Coetzee, 1987). *Anopheles gambiae sensu stricto* also known as *Anopheles gambiae* and *Anopheles arabiensis* are noted as effective vectors of human malaria (White, 1974). *Anopheles merus, Anopheles melas, Anopheles quadriannulatus, and Anopheles bwambae* have also been identified as members of *Anopheles* complex. Other species such as *Anopheles nili, Anopheles moucheti* and *Anopheles funestus* (members of *Funestus* group) though do not form part of *Anopheles* complex have also been documented as important players in malaria

transmission in Africa (Gillies & Coetzee, 1987). To be among the most important vectors of human malaria, these vectors were identified by virtue of their competence as vectors, average sporozoite rates, preference for feeding on humans and abundance (Okara *et al.*, 2010).

In Kenya, *An. gambiae, An. arabiensis* and *An. funestus* are the most widely distributed vectors across most provinces, transmission intensities and urban-rural areas and are said to be the most important species for malaria transmission (Kawada *et al.*, 2011). However, *An. arabiensis, An. gambiae* and *An. funestus* are more ubiquitous along the coastal and western regions, close to Lake Victoria and in the lake habitats (Minakawa *et al.*, 2012). *Anopheles arabiensis* is also found in the arid areas of North West in Turkana areas whereas few species of *An. gambiae* were also found in more central regions of the country (Okara *et al.*, 2010). The spatial occurrence of the three most dominant vectors vary between different classifications of transmission intensity. For example, *An. gambiae* was documented more often in areas with highest transmission intensity, with less frequent reports at sites in very low transmission (Okara *et al.*, 2010). In western Kenya, *An. arabiensis* and *An. funestus* are known to be a predominant vector in Ahero/Nyando area due to rice farming which provide good environment for breeding (Ochomo *et al.*, 2012) but Teso/Busia region is known for the presence of *An. gambiae s.s* as the main vector with *An. arabiensis* and *An. funestus* being minor vectors (Ochomo *et al.*, 2014). In Bondo sub-County, the distribution of bed nets was linked to rapid decrease in *An. funestus* population (Bayoh *et al.*, 2010) but subsequent study had shown that *An. funestus* had re-emerged in Bondo sub-County (McCann *et al.*, 2014). There was also a shift from *An. gambiae s.s* population to *An. arabiensis* (Bayoh *et al.*, 2010). Hence the density of major vectors in western Kenya was unclear due to re-emergence and shifting from one species to another.

2.6 Insecticide Resistance versus *P. falciparum* Sporozoite Rates in Malaria Vectors

Plasmodium falciparum malaria is a tropical disease in Africa transmitted by *Anopheles* mosquitoes (Kelly-Hope & McKenzie, 2009). Female *Anopheline* mosquitoes transmit malaria during a blood feed by inoculating microscopic motile sporozoites, which seek out and invade hepatocytes and then multiply (White *et al.*, 2013). Study conducted in Chad to assess circumsporozoite rate in *An. arabiensis* and *An. gambiae s.s.* revealed high vector infection rate at site with extensive use of agrochemicals (Foster *et al.*, 2016). However, there was significant increase in insecticide resistance but no *kdr* resistance allele was detected in *An. arabiensis* populations (Foster *et al.*, 2016). A similar study carried out in Zambia documented absence of sporozoite positive mosquitoes among *An. gambiae s.l* and *An. funestus* that were resistant to IRS pyrethroid (Chanda *et al.*, 2011). This could have influence on malaria transmission in those areas. In Bioko Island of Equitorial Guinea, prior to implementation of IRS, *P. falciparum* sporozoite positivity in *An. gambiae* was reported to be 6% but later reduced to 0.4% immediately after spraying the area and later it rebounded partially to 3.1% (Hemingway *et al.*, 2013). The overall effect of *kdr* status on sporozoite positivity was highly significant. Lower sporozoite positivity in both M and S form was observed in *An. gambiae*. The study also found that pyrethroid-resistant *kdr* homozygotes could transmit less malaria than the susceptible counterparts. The study however, concluded that the *kdr* status could not reduce the bio-efficacy of pyrethroid IRS (Hemingway *et al.*, 2013). Studies on possible impact of *kdr* (L1014S and L1014F) on vector competence which leads to malaria transmission seem to be conflicting. In Benin, the presence of *kdr* (L1014F) mutation did not have an effect on *P. falciparum* infection rate in *An. gambiae s.s* (Ossegrave *et al.*, 2012). In contrast, previous study carried out in Senegal suggested that *kdr* had an effect on increased malaria transmission (Trape *et al.*, 2011). Alout *et al.*, (2013) also documented that *An. gambiae s.s* having *kdr* mutation had high sporozoite rates which could influence malaria

transmission. A similar study conducted in Tanzania also reported significant association of one marker, vgsc-1014S with *P. falciparum* sporozoite rate in *An. gambiae s.s.* with the *P. falciparum* sporozoite rate being 4.2% (Kabula *et al.*, 2016). In western Kenya, it was unknown whether resistant *Anopheles* having *kdr* resistance genes could exhibit more vectorial competence in terms of higher *P. falciparum* sporozoite rate which would be linked to parasite transmission.

Globally, insecticide resistance data on *An. funestus* is limited though resistance had been reported in some malaria endemic areas (Ranson & Lissenden, 2016). *Anopheles funestus* has no markers for *kdr* (L1014S and L1014F) hence the main mechanism identified for pyrethriods resistance is over-expression of oxidation enzymes such as CYP6P9a and CYP6P9b (Brooke *et al.*, 2001; Djouaka *et al.*, 2011). Increased in malaria transmission was attributed to high level of insecticide resistance in *An. funestus* population in Southern Africa (Hargreaves *et al.*, 2000). In Bondo region of western Kenya, high level of *An. funestus* resistance to pyrethroids (mortality rate of 60%) and *P. falciparum* sporozoite infectivity rate of 4.45% were reported in 2010 and 2011 (McCann *et al.*, 2014). The prevalence of *P. falciparum* in ITNs and IRS areas could be attributed to increasing levels of insecticide resistance which could lead to malaria control failures (Chanda *et al.*, 2011). *Plasmodium falciparum* malaria is a major public health problem in western Kenya, but whether *An. gambiae s.l* or *An. funestus* had higher sporozoite rate and could be the major vector driving malaria transmission in the region was unknown.

2.7 The Mechanisms of Malaria Vector Resistance to Insecticides

The main mechanisms that have been proven and conclusively documented to cause insecticide resistance in mosquitoes are target site insensitivity, known as knockdown resistance (*kdr*) and metabolic resistance due to elevated levels of detoxifying enzymes such as P450 monooxygnases, gluthathione-S-transferases (GTS) and carboxyesterases (Protopopoff *et al.*, 2013; Ranson *et al.*, 2000). Other types of mechanisms of resistance include reduced penetration and behavioural avoidance. Fig. 2.3 indicates behavioral and physiological changes associated with insecticide resistance in susceptible vector and the resistant vector.

2.8. Knockdown Resistance Mechanism

Knockdown resistance (*kdr*) is caused by a series of genes involving a mutation in the sodium ion channel, the target site of pyrethroids and Dichloro-diphenyl-trichloroethane (DDT), and conferring resistance to these insecticides (WHO, 2013). These mutations induce amino acid substitutions leading to alterations in the structural and chemical attributes of target proteins, rendering them less susceptible to insecticide binding (Brooke & Koekemoer, 2010).

Knockdown resistance is the best understood type of resistance mechanism and molecular diagnostics to detect this resistance mechanism are now incorporated into insecticide resistance monitoring and surveillance strategies in malaria control programmes (Corbel & N'Guessan, 2013). Single amino acid mutation in the IIS6 membrane-spanning region of the voltage-gated sodium channel gene confers with the target site DDT-pyrethroid resistance in *An. gambiae s.l* (Karunamoorthi & Sabesan, 2013). Knockdown resistance associated with pyrethroid resistance in malaria vectors involves either substitution of leucine residue found at codon 1014 with phenylalanine (1014F), first documented in West Africa (Martinez-Torres *et al.*, 1998) or substitution of leucine with serine (1014S) in the

voltage-Gated Sodium Channel (VGSC) documented in East Africa (Ranson *et al.*, 2000). These two mutations have been held responsible for cross resistance against DDT and pyrethroid insecticides (Protopopoff *et al.*, 2008). Resistance to pyrethroids rely heavily on *kdr* mechanisms, although other mechanisms such as cuticle alteration; where the cuticle is changed into lesser permeable thus reducing the entry rate on amount of insecticide have also been observed (Protopopoff *et al.*, 2008).

Knockdown resistance was earlier thought to have evolved in S-form but has eventually developed from the S-form to M-form. It was suggested that this happened due to hybrid formation and later introgression. The commonly known and widespread form in sub-Sahara Africa is the S-form. In Eastern part of Africa, the S-form has been documented but studies suggested that the *kdr* allele is currently spreading in M-form (Cuamba *et al.*, 2006). Once *kdr* allele frequency reaches a certain threshold and its combined with a drastic decrease in vector densities by insecticides, an exponential increase in the resistant form is observed in a short period of time (Protopopoff *et al.*, 2008).

Study conducted by Okia *et al.*, (2013) revealed that *kdr* mutation L1014S frequency was moderate (34%-37%) in *An. gambiae s.s.* but no L1014F was identified in *An. arabiensis* though all the *An. arabiensis* used for the study were wild type. The frequency of *kdr* mutation L1014S observed in *An. gambiae s.s.* population were found to be lower than previous studies which reported 86% (Ramphul *et al.*, 2009) and 47% (Verhaeghen *et al.*, 2010) in the year 2008 and 2006 respectively. It was unclear why there was rapid reversion to wild type, the susceptible genotype. However, genotypic frequencies of all the population investigated do not meet expectations of Hardy-Weinberg principle but the study suggested that the population of *An. gambiae* was undergoing selective pressure (Okia *et al.*, 2013). In areas under ITNs coverage and areas not under ITNs coverage, significant increase in *kdr* frequency was documented; approaching 60% in less than three years (Protopopoff *et al.*, 2008). Moreover, the study pointed out that *kdr* mutation identified in the population was not significantly different from

houses having at least one ITN and those in houses with no ITNs. The significant increase in *kdr* frequency was ascribed to low migration of *Anopheles* population and selection overcome gene flow (Protopopoff *et al.*, 2008). Meanwhile, Wondji *et al.*, (2005) noted that areas with extensive use of long-lasting insecticide treated nets, have relatively high level of gene flow between *An. arabiensis* populations 300km apart.

The leucine-serine mutation was observed in a single *An. arabiensis* population in western Kenya (Stump *et al.*, 2004) but subsequent study by Mathias *et al.*, (2011) reported absence of L1014S in *An. arabiensis*. However, previous study revealed *kdr* east mutation at the same amino acid position causing leucine-serine substitution (L1014S *kdr* allele) which was associated with permethrin resistance in *An. gambiae s.l* (Ranson *et al.*, 2000). In an areas treated with ITNs in Kenya, the frequency of the 1014S *kdr* allele doubled from the year 1987 to 2001 but these increments was not observed outside these areas (Stump *et al.*, 2004). In Bioko Island of Equatorial Guinea, the failure of IRS pyrethroids was linked to high frequency of *kdr* L1014F in *An. gambiae s.s* population (Sharp *et al.*, 2007). This mutation (L1014F *kdr* allele) was first reported in western Kenya, in 2012 though it was present in low frequencies (Ochomo *et al.*, 2015). This exponential increase in *kdr* allele could be however attributed to the use of ITN which confer selection pressure for *kdr* mutation (Fanello *et al.*, 2003). In Ahero, Busia and Bondo of western Kenya, L1014S and L1014F mutations associated with pyrethroid resistance in *An. gambiae s.l* were documented but it was unclear whether the frequency of these genes in field larvae raised mosquitoes is similar to adult sampled via indoor resting collection due to differential exposure to insecticides.

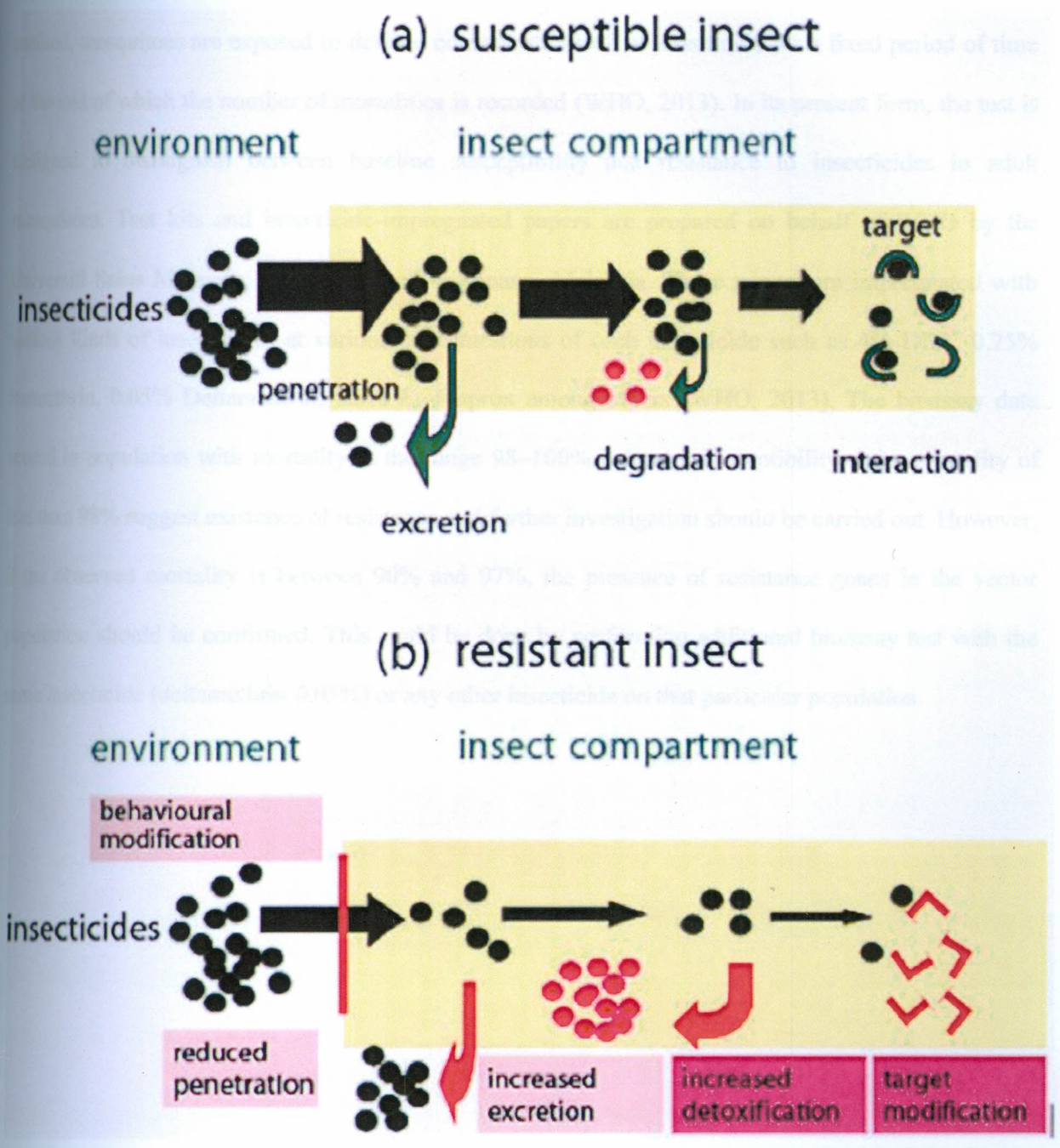

Figure 2.3: Scheme of potential behavioral and physiological changes associated with insecticide resistance in malaria vectors (a) Susceptible insect/vectors (b) Resistant insect/vectors (Lapied *et al.*, 2009).

2.9 WHO Tube Bioassay

This is a standard method recommended by WHO for testing for insecticide resistance. Using this method, mosquitoes are exposed to defined concentrations of an insecticide for a fixed period of time at the end of which the number of mortalities is recorded (WHO, 2013). In its present form, the test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes. Test kits and insecticide-impregnated papers are prepared on behalf of WHO by the Universiti Sains Malaysia, which is based in Penang, Malaysia. These papers are impregnated with various kinds of insecticides at various concentrations of each insecticide such as 4% DDT, 0.75% Permethrin, 0.05% Deltamethrin, 0.5% Etofenprox among others (WHO, 2013). The bioassay data scored in population with mortality in the range 98–100% indicates susceptibility and a mortality of less than 98% suggest existence of resistance and further investigation should be carried out. However, if the observed mortality is between 90% and 97%, the presence of resistance genes in the vector population should be confirmed. This could be done by performing additional bioassay test with the same insecticide (deltamethrin 0.05%) or any other insecticide on that particular population.

CHAPTER THREE

MATRIALS AND METHODS

3.1 Study Areas

Wild mosquitoes and larva were collected from three sites, Busia County (0°06'N, 33°58'E; 1147 Elevation 3764ft), Ahero (00°11'S, 34°55'E; 1155 Elevation 3788ft) in Kisumu County and Bondo located in Siaya County in western Kenya (Fig. 3.1). Busia County is bordered by Kakamega County to the South, Teso to the south West. The main occupation of the people in Busia County is predominantly farming as they depend on extensive production of tobacco, sugarcane, grain cereals including maize, bananas, onions and vegetable (Jaetzold *et al*., 2005). *An. gambiae s.s* is the main malaria vector though *An. arabiensis* and *An. funestus s.l.* are also available in lower proportions (Ochomo *et al*., 2014).

Ahero is located about 20 kilometers East of Kisumu. The area is a marked variation in altitude in Nyando where Ahero is located. The areas along the Nyando plateau have altitude as low as 1100 m above sea level and is very prone to flooding, with other areas as high as 1540 m above sea level. The main occupation of the people in Ahero is farming. Ahero is a rice producing area due to local topography, annual flooding regimes and irrigation. Since rice farming is a major economic activity, it provides habitats conducive for the breeding of *An. arabiensis* mosquitoes, which promotes malaria transmission (Ochomo *et al*., 2014).

Bondo is located in Siaya County with a population of about 309,190 (KNBS, 2010). It borders Siaya to the north, Gem district to the east and Lake Victoria to the South. It has altitude rising from 1,140m in the eastern part to 1,400m above sea level in the western part. The two main rivers, Nzoia and Yala

traverse the district and they enter Lake Victoria through the Yala Swamp. The western part of the district is drier and the eastern part is wetter towards higher altitudes. Relative humidity is high and mean evaporation is between 1800m to 2000m per annum. The major public health challenge in the district is *P. falciparum* malaria (Hamel *et al*., 2011). In children under five years, malaria prevalence is about 30%. There was a great reduction in *An. funestus* mosquitoes following implementation of ITNs distribution programme in the early 2000s in certain areas of the county. There was also a great shift from *An gambiae s.s* mosquito to a greater extent *An. arabiensis* population (Bayoh *et al*., 2010) in Bondo sub-County. But a study conducted later revealed that *An. funestus* had re-merged in Asembo, Bondo sub-County (McCann *et al*., 2014).

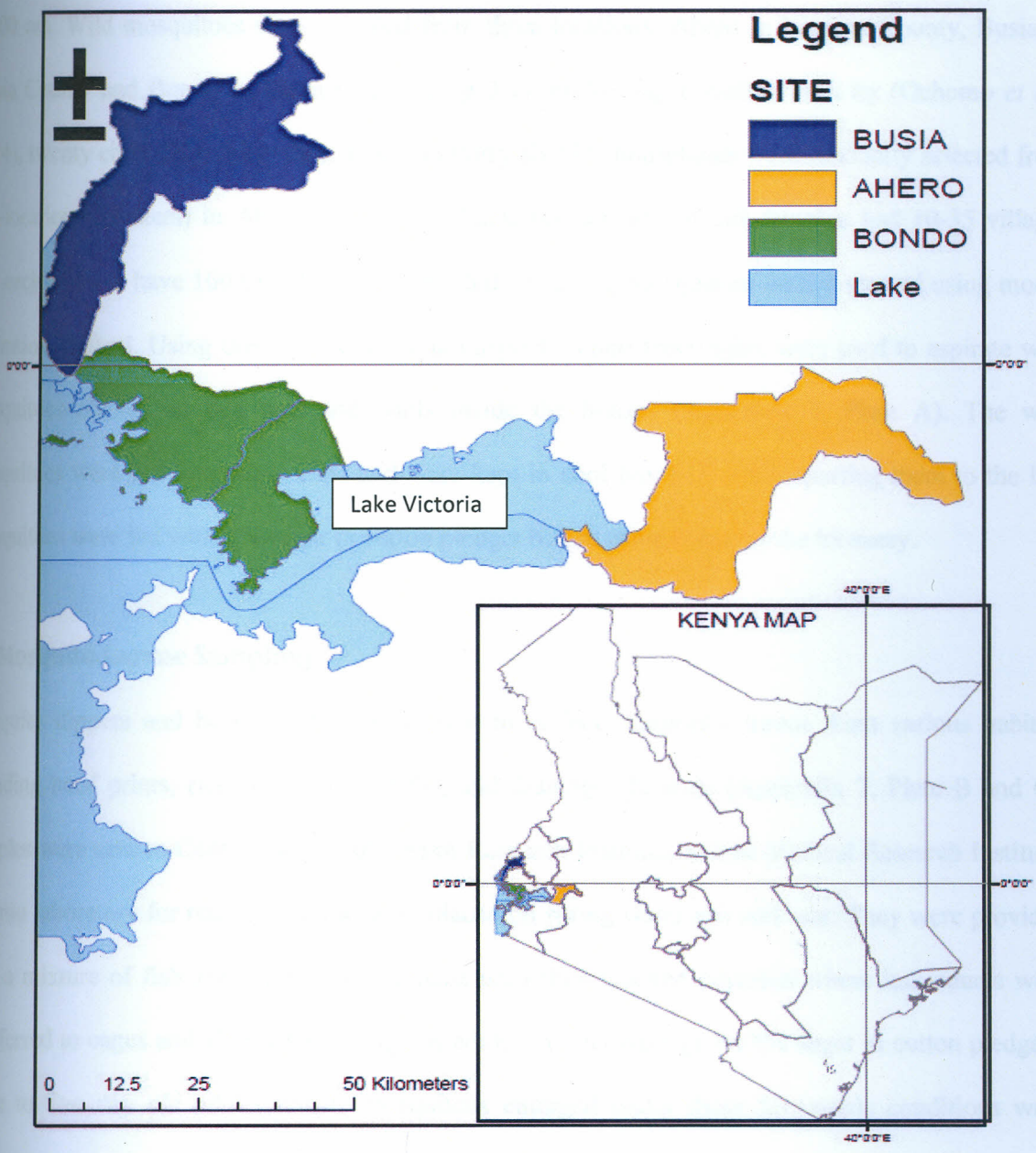

Figure 3.1: Map indicating the study areas; Ahero, Busia and Bondo.

3.2 Wild Mosquito Sampling

Field sample collection was done from December to January, 2016 between early hours of 6:00 am to 11:00 am. Wild mosquitoes were sampled from three locations; Ahero in Kisumu County, Busia in Busia County and Bondo in Siaya County (Fig 3.1). Following a method used by (Ochomo *et al.*, 2014), twenty eight (28), thirty three (33) and thirty six (36) households were randomly selected from sub-locations (clusters) in Ahero, Bondo and Busia respectively. A sub-location had 10-35 villages and each of them have 100 to 120 households. Indoor resting mosquitoes were collected using mouth aspiration method. Using this technique, mouth aspirators and flash lights were used to aspirate wild mosquitoes resting on bed nets and walls inside the houses (Appendix 7, Plate A). The wild mosquitoes were put into paper cups and were kept in cool box before transporting them to the lab. Mosquitoes were fed with 5% sugar in cotton pledget before using them for the bioassay.

3.3 Mosquito Larvae Sampling

Mosquito dippers and hand pipettes were used to collect *Anopheles* larvae from various habitats including hoof prints, rice fields, mud paths, and drainage channels (Appendix 7, Plate B and C). Samples were sent to Centre for Global Health Research Institute, Kenya Medical Research Institute, Kisumu laboratory for rearing. Larvae were placed on spring water pan and rear. They were provided with a mixture of fish feed and brewer's yeast until they reached pupation where individuals were transferred to cages and allowed to emerge as adults. Adults were given 5% sugar in cotton pledgets. Three to five day old adults female mosquitoes emerged under these favourable conditions were exposed to deltamethrin (0.05%) using WHO tube bioassay test kits to determine phenotypic resistance.

3.4 Morphological Identification of *An. gambiae s.l* and *An. funestus*

Anopheline mosquitoes were identified following morphological features outlined by Gillies & Coetzee, (1987) with ×40 magnification using light microscope. The dark spot at the upper margins of the wings is peculiar to all *Anopheles*. The palpis are known to have segmented into three and elongated. It was observed that *An. funestus* had a pale spot on second dark area and in between the two dark spots on vein 6 is a light spot. There were no presence of fringes on vein 6 and also absence of speckles on the legs in *An. funestus*. *An. gambiae s.l* on the other hand, had speckles on the legs with tarsi 1-4 having noticeable pale bands.

3.5 Testing for Phenotypic Resistance using WHO-Tube Bioassay Method

The bioassay was performed following a method developed by WHO for assessing insecticide resistance (WHO, 2013). A total of 2,186 field collected larvae raised *An. gambiae s.l*, 2,929 wild-caught adult *An. gambiae s.l* and 1,431 wild adults *An. funestus* were exposed to deltamethrin (0.05%) using WHO Bioassay test. *Anopheles* mosquitoes emerging from pupation and wild-caught *Anopheles* mosquitoes sampled via indoor resting collection were given 5% sugar solution as awaited for the bioassay. The wild-caught adult female mosquitoes were allowed to stay up to 3 days before they were used for bioassay. Prior to exposing them to the insecticide, six sheets of clean white paper (12 x 15 cm) impregnated with 0.05% deltamethrin; rolled into cylinder shape were inserted into four holding tubes (one per tube) and fastened into position with a steel spring-wire clip. Hundred and twenty (120) to hundred and fifty (150) mosquitoes were used for a single test. They were aspirated from paper cups into four holding tubes through the filling hole in the slide to give four replicate samples of 20-25 mosquitoes per tube. The slide unit was closed once mosquitoes have been transferred and the holding tubes were set in an upright position for an hour. Each of the four red-dotted exposure tubes was lined with a sheet of deltamethrin (0.05%) impregnated papers whereas the two yellow-dotted control tubes

were lined with silicone oil impregnated papers; each was fastened into position with copper spring-wire clip. The control mosquitoes (Kisumu strain) were treated in the same way as the exposed mosquitoes; they were treated in parallel and under the same conditions. The purpose of the control was to provide an estimate of natural mortality during the bioassay and to account for all variables that may induce mortality other than the insecticide being tested. The empty exposure tubes were attached to the vacant position on the slides and with the slide unit opened so that the mosquitoes are blown gently into the exposure tubes and once they were in the tubes, the slide unit is closed and the holding tubes were detached and set aside. Exposure tubes containing mosquitoes were set in a vertical position with mesh the mesh-screen and uppermost for a period of 1 hour. In this 1 hour period, the number of knockdown mosquitoes was recorded every 10 minutes. At the end of the 1 hour exposure period, the mosquitoes were transferred back into the detached holding tubes. A pad of a cotton wool soaked in 10% sugar water was placed on the mesh-screen end of the holding tubes. Afterwards, the mosquitoes were maintained at 25± 2°C and 80 ± 10% relative humidity and mortality were scored at 24-hours post exposure. At the end of the 24 hour post-exposure, the dead mosquitoes were counted and recorded. Adult mosquito was considered to be alive if it was able to fly regardless of the number of legs remaining. Any knocked-down mosquito having lost wings or legs was considered moribund and was counted as dead. Once mortality was scored, surviving mosquitoes were knockdown by freezing. All mosquitoes were placed in individual Eppendorf tubes and frozen at -20^{0}C for molecular analysis. Only female *Anopheles* mosquitoes were used for bioassay and molecular analysis.

3.6 DNA Extraction using Ethanol Precipitation Method

Prior to DNA extraction, the grinding buffer was prepared using the following reagents and their concentratons.

Homogenization Buffer consisted of 0.10m NaCl (0.59g), 0.20m sucrose (6.84g), 0.01m EDTA (0.37g), 0.03m Trizma base (0.36g) and 100ml sterile water (pH 8.0).

Lysis buffer consisted of 0.25m EDTA (9.28g), 2.5% (w/v) SDS (1.88g), 0.5m Trizma base (6.03g) and 100ml sterile water (pH 9.2)

The grinding buffer was prepared by mixing 4 parts homogenization buffer and 1 part lysis buffer

Genomic DNA was extraction from three hundred and sixty (368) wild *Anopheline* mosquitoes and two hundred and seventy three (273) larvae raised mosquitoes. The extracted DNA samples were subjected to coventional PCR analysis for species identification and susequently genotyped for kdr east (L1014S) and kdr west (L1014F) mutations.

Genomic DNA samples from malaria vectors were extracted for each population using Ethanol Precipitation Method. This is a standard recommended method developed by Collins *et al*., (1987) for extracting genomic DNA from mosquitoes. With this method, DNA could be extracted from body parts (legs, antenna or wings). Mosquitoes that were exposed to deltamethrin via WHO Bioassay procedure and frozen at -20^{0}C were ground immediately after removing them from the freezer to avoid degradation of the DNA by nucleases. The grinding of the body parts (legs and wings) of each mosquito was done using a sterile pestle by adding 100uL grind buffer to the Eppendorf tubes until no mosquito parts were identifiable. The tubes were immediately placed in +65^{0}C water bath for 30 minutes to degrade any nucleases released after grinding the mosquito parts so that they do not degrade the DNA. While the tubes were still warm, 14uL of 8M potassium acetate (KAc) were added

to each tube to achieve a final concentration of 1M and mixed thoroughly by vortexing or tapping the tubes. Afterwards, the tubes were incubated on ice for at least 30 minutes. The salt solution precipitated out the mosquito parts and other insolubles including proteins denatured by sodium dodecyl sulfate (SDS). The tubes were then centrifuged at a maximum speed of 13,200 rpm at room temperature for 15 minutes. New sterile tubes were labeled and the supernatants were transferred into the new tubes. Care was taken not to transfer any of the precipitates. A volume of 200uL of ice-cold 100% ethanol was added to the supernatants and mixed well by inverting the tubes. They were then incubated at room temperature for 5 minutes to precipitate the DNA. At this point, some of the samples were stored at -20^{0}c and -80^{0}C for long term storage. Later, the tubes were centrifuged at a maximum speed of 13,200 rpm at room temperature for 20 minutes to pellet the DNA. The ethanol was poured off immediately and care was taken not to disturb the pellets. In some instances where the pellets have dislodged, the tubes were spun again for 5 minutes before pouring off all the ethanol. A volume of 200uL of cold 70% ethanol was added and spun for 5 minutes at top speed to wash the pellets. The final stage of the washing was done by adding 200uL of cold 100% ethanol and spun for 5 minutes at top speed. The ethanol was poured off carefully at each stage of the washing of the DNA pellet. After washing, the pellets of DNA were dried on the bench for at least 1 hour. In some cases, the pellets of DNA were left to dry on the bench overnight. Finally, a solution of Tris-EDTA buffer with 1uL/mL of RNase/DNase-free (this was useful to remove any RNA that co-precipitated with the DNA) was prepared. The DNA was re-suspended in 100uL of this solution and pellets were dissolved by gently tapping the tubes. The DNA was allowed to fully re-suspended and stored at -20^{0}C for PCR analysis.

3.7 Identification of *An. gambiae s. s.* and *An. arabiensis* using Polymerase Chain Reaction

Conventional polymerase chain reaction (PCR) was used to distinguish *An. gambiae s.s.* and *An. arabiensis* following a method described by Scott *et al.*, (1993). Following this method, 1.5 µl of the extracted DNA samples was loaded into the wells of the PCR plate and amplification of the DNA was performed using Biorad T100™ Thermal Cycler (Bio-rad laboratories, inc., www.bio-rad.com, Singapore). Table 3.1 shows the PCR master mix of the various reagents used in the DNA amplification.

Table 3.1: PCR master mix reagents for *An. gambiae s.l* discrimination

Reagent	Volume for 1 sample(µl)	Volume for 100 samples(µl)
Water	4.98	498
5× PCR buffer	3	300
$MgCl_2$	1.8	180
dNTP mix	1.14	114
Primer GA	1	100
Primer AR	1	100
Primer UN	1	100
Taq DNA Polymerase	0.08	8
Total	**14**	**1400**

GA: An. gambiae ss, AR: A. arabiensis, UN: universal, NTP: nucleotide primers

The nucleotide primers (NTP), the universal primers, primers for *An. gambiae s.s.* and *An. arabiensis*, the Taq enzyme, 5×buffer and the water were kept at -20^{0}C freezer and $MgCl_2$ was kept at 4^{o}C prior to PCR reactions. The specific primers which defined the mosquitoes DNA sequence to be amplified includes 5`-AAG TGT CCT TCT CCA TCC TA-3` for *A. arabiensis*, 5`-CTG GTT TGG TCG GCA CGT TT-3` for *An. gambiae s.s* and a universal primer, 5`-GTG TGC CCC TTC CTC GAT GT-3` which played a role in extension of the DNA after denaturation of nucleotide primers (dCTP), dCTP, dATP and dTTP). Water was added first and the enzyme, DNA Taq polymerase was added last to the PCR plate. The total volume of the master mix per well used for the amplification was 14µl (Table

3.1) for 96-well plate. Controls and blanks were also added at 14 µl. After loading the plate, it was sealed with plate sealer and loaded directly onto the Thermal Cycler (gene machine) (Bio-rad laboratories, inc., www.bio-rad.com, Singapore). The lid was closed and the machine was switched on. In some cases, the plates were stored at 4^0C for future use when not loading immediately into the PCR machine. The cyclic conditions included, 94^0C (1minutes) (denaturation) for 1 cycle followed 30 cycles of 94^0C for 1 minute, 54^0C (1minute) for annealing and 74^0C (1 minutes) for extension. This is repeated 30 times and the whole process of amplification took 1hour and 30 minutes. The gene machine was programmed to rest at 4^0C after the last cycle so that the amplified DNA could be processed further.

3.8 Identification of *An. funestus* using Polymerase Chain Reaction

PCR discrimination of *An. funestus* was performed based on species-specific single nucleotide polymorphisms in the second internal transcribed region following a method developed by Koekemoer *et al.*, (2002) which was later modified by incorporating internal mismatches into the primers to enhance specificity and simplicity (Wilkins *et al.*, 2006). DNA amplification was done using Biorad T100™ Thermal Cycler (Bio-rad laboratories, inc., www.bio-rad.com, Singapore). For 96-well plate, 15µl of master mix was added per well and the 96-well plate was sealed and loaded onto the Thermal cycler (Bio-rad laboratories, inc., www.bio-rad.com, Singapore). Then, the Thermal cycler was switched on at the power source to amplify the DNA. The PCR conditions included melting at 94^0C for 4 minutes for 1 cycle followed by 30 cycles of 94^0C for 30 seconds, 58^0C for 30 seconds and 72^0C for 45 second followed by 1 cycle of 72^0C for 7 minutes. The reagents and their respective concentrations were added in the following order as presented in the Table 3.2.

Table 3.2 PCR master mix reagents for *An. funestus* discrimination

Reagents	1 Sample	100 sample
Sterile H_2O	4.99.0 µl	499µl
Taq 5×PCR Buffer with $MgCl_2$	3.0 µl	300 µl
dNTPs (2 mM mix)	1.14 µl	114.0 µl
$MgCl_2$ (25mM)	1.80 µl	180 µl
UV (F, 5 pmol/µl) [5`-CCGATGCACACATTCTTGAGTGCCTA-3`]	1.0 µl	100 µl
FUN (R, 5 pmol/µl) [5`-CTCGGGCATCGATGGGTTAATCATG-3']	1.0 µl	100 µl
LEES (R, 5 pmol/ µl [5`-GACGGCATCATGGCGAGCAGC-3`)	1.0 µl	100 µl
Taq DNA polymerase (5U/ µl)-MR4 uses GoTaq, Promega	0.08 µl	8 µl
Total	**14.73** µl	**1401 µl**

F: forward primer R: reverse primer

3.9 Agarose Gel Electrophoresis and Visualisation of Amplified DNA

After DNA amplification, the plates were removed and the plate sealer was carefully removed to avoid spills. The agarose (2%) was prepared by weighing 3g of agarose powder and mixed with 150ml TBE (Tri Boric Acid EDTA) buffer and heated for 3minutes until molten. Ethidium bromide (4µl) was added into the agarose gel then the molten gel was poured into the casting tray containing combs and allowed to solidify for 30 minutes. The gel was transferred to the electrophoresis tank containing TBE buffer then loaded the wells with 16µl of the amplified DNA using multi-channel pipette. The Power Pac (Bio-Rad, USA) of the electrophoresis tank was switched on and the DNA was allowed to run at 100 volts for 15 minutes. Then the gel was transferred to the UVP GelDoc It^2 imager machine (UPV, Analytik Jena Company, UK) for DNA visualization. The machine captured high resolution images and enhances quantitative analysis of fluorescent gels. The GelCam 315 camera attached to UVP GelDoc It^2 imager machine helped to take pictures of the gel and VisionWorks Software installed in the computer was used for images enhancement and analysis. The DNA bands were scored on the electrophoresis loading map.

3.10 *Plasmodium falciparum* Sporozoite Rate Detection using ELISA

Plasmodium falciparum circumsporozoite protein was detected in the head and thoraces of the *Anopheles* using antigen-capture ELISA method (Durnez *et al.*, 2011; Wirtz *et al.*, 1987). Enzyme Linked Immunosorbent Assay (ELISA) is a sensitive immunoassay that uses an enzyme linked to antibody or antigen as a marker for the detection of a specific protein (sporozoite). *Plasmodium falciparum* sporozoite ELISA was performed using reagent kit (MRA-890, MR4, ATCC, Manassas, VA). Preparation of ELISA reagents solutions is outlined in Appendix 3. Three thousand three hundred and sixty (4,360) wild-caught adult mosquitoes were subjected to enzyme-linked immunosorbent assay (ELISA) to test for *P. falciparum* sporozoite positivity rate. Of this total, 2,929 were *An. gambiae s.l* and 1,431 were *An. funestus.*

Using this method, 50µl of capture monoclonal antibodies (mouse Ab *Pf*2A 10-CDC) specific for *P. falciparum* sporozoite was added to each well of the ELISA plate. The plate was covered with aluminum foil to prevent evaporation and incubated for 30 minutes. Afterwards, the well contents was removed by holding the trays firmly by their sides, inverted over the sink and banged gently to remove the solution. This was followed by blotting the plates on clean absorbent paper. Thereafter, the wells were filled with 200µl blocking buffer (containing IGEPAL CA-630) and incubated for 1 hour. When the time elapsed, the contents were drained by banging plates sharply to remove the solution. 50µl mosquito homogenate (test samples) were then added per well. Positive and negative controls were also added (50µl per well). The plates were incubated 2 hours at room temperature. Near the end of the incubation period, the ABTS-substrate solution; mixture of solution A (ABTS) and solution B (hydrogen peroxide) 1:1 was prepared. Also, the Mab-peroxidase conjugate was mixed with the blocking buffer: 0.05ug/50µl blocking buffer. In a 1.5ml microfuge tube, the enzyme activity was confirmed by mixing 5µl of the conjugate with 100µl ABTS. A rapid colour change observed

indicated that the peroxidase enzyme and the substrate were functional. After the incubation period, homogenates were removed and the plates were washed 2× with PBS-Tween-20 by filling and emptying the wells. After washing, 50µl of the conjugate was added to each well and incubated for 1 hour. Later the conjugate was removed and the wells were washed with 3× with PBS-Tween-20. Finally, 100µl of ABTS was added per well and incubated for 30 to 60 minutes for colour change to be observed. Colour change was read visually and the results were scored on the data form (Appendix 2).

3.11 *KDR* Genotyping

Real-time polymerase chain reaction (RT-PCR) using Applied Biosystem Real-time machine was used to detect kdr genotypes (L1014S and L1014F) at amino acid position 1014 of the voltage-gated sodium channel, following a method developed by Bass *et al.*, (2007) and Mathias *et al.*, (2011). Frozen genomic DNA samples were thawed at room temperature. A 96-well plate was placed on ice-cold pack. Using a pipette, 10ul pipette tips was used to dispense 1.5 µl of each sample genomic DNA to appropriate wells. Samples were genotyped using probes for the wild type (5'-CTTACGACTAAATTTC-3') and L1014S (5`-ACGACTGAATTTC-3`) alleles. The same samples were also genotyped for the L1014F allele (5'-ACGACAAAATTTC-3'). RT-PCR reactions were runned on Applied Biosystem Real-time machine using a 96-well format. Each reaction involved 5.0 µl of 2×Taqman RT-PCR master mix (Applied Biosystems), 0.2 µl , primer forward for 1014S (5`-CGA TCT TGG TCC ATG TTA ATT TGC A-3`), 0.20 µl primer reverse for 1014S (5`-CAT TTT TCT TGG CCA CTG TAG TGA T-3`), 0.2 µl for the wild type (5'-CTT ACG ACT AAA TTT C-3') and 0.15µl L1014S probe (5`-ACG ACT GAA TTT C-3`) at respective concentrations of 10 µM, 1.50 µl DNA template, and 2.75 µl sterile water in a final volume of 10 µl. Each 96-well plate contained positive controls for all three genotypes in triplicate along with a no-template. PCR conditions for L1014S were initial DNA melting and activation of hot-start taq polymerase step at 95°C for 10

minutes for 45 cycles, 95° for 30 seconds, 60°C for 30 seconds (annealing) and 72°C for 30 seconds (extension). Kdr west (L1014F allele) involves, 0.2µl for the wild type probe (5'-CTTACGACTAAATTTC-3'), 0.2µl for forward primer (5`-CAT TTT TCT TGG CCA CTG TAG TGA T-3`), 0.2µl for reverse primer (5`-CGA TCT TGG TCC ATG TTA ATT TGC A-3`). The rest of the reagents and volumes used were the same as those mentioned above when genotyping for L1014S. The final volume was 10µl. PCR conditions for L1014F include 95°C for 10 minutes for 1 cycle, 95°C for 10 seconds for 40 cycles and 60°C for 45 seconds. Reaction curves for each set of reactions were visualized using TaqMan® Genotyper software version 1.3.1. Absence of alleles was confirmed by the text reports generated by the TaqMan® Genotyper software version 1.3.1.

3.12 Data Analysis

Data analysis was done using SAS Version 9.1. Results from phenotypic resistance to deltamethrin were analysed following WHO recommended criteria for testing for resistance (WHO, 2013). The mortality rates of the tested samples were calculated by summing the number of dead mosquitoes and expressing this as a percentage of the total number of exposed mosquitoes:

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

Following the WHO guidelines for testing insecticide resistance in malaria vectors, mortality range of 98-100% indicates susceptibility to the insecticide tested, less than 98% mortality suggest existence of resistance in the population which warrant further investigation and a population with less than 90% mortality were considered as resistant.

Independent t-test was used to determine whether there are significant differences in the level of deltamethrin phenotypic resistance in *An. gambiae s.l* field larvae raised mosquitoes and wild-caught adult mosquitoes. Also, Independent t-test was used to compare the mean deltamethrin phenotypic resistance between wild adult *An. gambiae s.l* and *An. funestus*. One way Analysis of Variance (ANOVA) was used to establish whether there was a significant difference in mean *P. falciparum* sporozoite of *An. gambiae s.l* across the three sites and independent t-test was used to compare the mean sporozoite in *An. gambiae s.l* and *An. funestus*.

Two-way classification chi-square test of association was used to determine whether there was an association between *P. falciparum* sporozoite rates and *kdr* genotype frequency in *An. gambiae s. l.* In the same way, Two-way classification chi-square test of association was used to associate *kdr* allele

frequency in field larvae raised mosquitoes and wild-caught adult mosquitoes sampled from households.

3.13 Ethical Considerations

Ethical approval was obtained from Maseno University Ethics Review Committee. The study conducted under the Scientific and Ethical Review Unit of the Kenya Medical Research Institute (SSC 2267).

Informed consent was also obtained from owners of households in Ahero, Bondo and Busia where mosquitoes were collected. Details of informed consent are outlined in Appendix 6 and ethical approval letter is in Appendix 4.

CHAPTER FOUR

RESULTS

4.1 Deltamethrin Phenotypic Resistance of Wild Population and Larvae Raised Population of *An. gambiae s.l*

This study pointed out that larvae raised *An. gambiae s.l* from the three sites were all resistant to deltamethrin with Ahero having highest mortality rate of 88% followed by Busia, 62% and Bondo, 52%. Hence the population from Bondo recorded the highest resistance to deltamethrin.

Using WHO standard method, the mortality rates of *An. gambiae s.l* exposed to deltamethrin were 50%, 39% and 52% for Ahero, Bondo and Busia respectively. The mortality rates for *An. funestus* sampled in Bondo and Busia were 29.70% and 33.05% respectively. These mortality rates of both larvae raised and wild-caught adult *An. gambiae s.l* sampled from the three sites were considerably lower than the recommended WHO >90% threshold for resistance (Table 4.1). Independent t-test showed that there was a significant differences in deltamethrin phenotypic resistance between larvae raised mosquitoes and wild caught mosquitoes sample from the study sites ($P<0.001$) at 95% confidence interval. Also, *An. funestus* was more phenotypically resistant to deltamethrin than *An. gambiae s.l* ($P<0.001$). However, was no significant difference in phenotypic resistance in *An. funestus* from Bondo and Busia for ($P=0.303$). Table 4.1 details the comparison of mortality rate in larvae raised mosquitoes and wild caught mosquitoes.

Table 4.1: Comparison of the mortality rate of *An. gambiae s.l* wild mosquitoes and larvae raised mosquitoes exposed to 0.05% deltamethrin for 24 hour post-exposure

Site	Wild-caught Adult Mosquitoes		Field larvae raised mosquitoes		*P*-value
	No. per site	Mortality	No. per site	Mortality	
Ahero	1,407	698 (50%)	398	353 (89%)	<0.001
Bondo	569	223(39%)	430	224 (52%)	<0.001
Busia	953	499 (52%)	1,358	848 (62%)	<0.001
Total	2,929	1420 (48%)	2,186	1425(65%)	<0.001

4.2 Percentage Density of *Anopheles* Mosquitoes from the Study Sites

Of two thousand nine hundred and twenty nine (2,929) wild caught *An. gambiae s.l* sampled, 48.04%, 19.45% and 32.54% were from Ahero, Bondo and Busia respectively. Two thousand one hundered and eighty six (2,186) larvae raised *An. gambiae s.l* were identified. Out of this number, 18.21%, 19.67% and 62.12% were from Ahero, Bondo and Busia respectively. A total of one thousand four hundred and thirty one (1,431) *An. funestus* was sampled from Busia and Ahero. One thousand one hundred and ninety two (1,192) was was sampled from Bondo and two hundred and thirty nine (239) was sampled from Busia. Table 4.2 shows the percentage density of *Anopheles* mosquitoes from the study sites.

Table 4.2: Percentage density of larval raised and wild adults *Anopheles* species in the study sites

Wild Caught *Anopheles* Mosquitoes		
Site	***An. gambiae s.l***	***An. funestus***
Ahero	1,407 (48.04%)	-
Bondo	569 (19.45%)	1,192 (83.30%)
Busia	953 (32.54%)	239 (16.70%)
Total	**2,929**	**1,431**
Larval Raised *An. gambiae s.l*		
Ahero	398 (18.21%)	-
Bondo	430 (19.67%)	-
Busia	1,358 (62.12%)	-
Total	**2,186**	-

4.3 Sporozoite Rate of *P. falciparum* in Wild *Anopheles* Mosquitoes

Sporozoite rate for *An. gambiae s.l* sampled from the three sites were 1.56%, 0.2% and 1.78% for Ahero, Bondo and Busia respectively whereas the positivity rate for *An. funestus* from Bondo was slightly higher (4.45%) compared to Busia (3.77%). *An. funestus* however, had the higher *P. falciparum* sporozoite rate compared to the *An. gambiae s.l* counterparts in all the study sites and there was a significant difference between them ($P<0.001$). The study identified *An. funestus* to be the most abundant vector population in Bondo region but no *An. funestus* was sampled from Ahero. One way Analysis of Variance (ANOVA) showed that the mean difference in sporozoite rate *of An. gambiae s.l* across the three sites were statistically significant at 95% confidence interval ($P=0.022$) (Table 4.4). However, independent-t test failed to show any significant difference in sporozoite infectivity rate for *An. funestus* sampled from Bondo and Busia ($P=0.637$) (Table 4.4). Table 4.3 Shows *Plasmodium falciparum* sporozoite negative and positive of the species from the study sites.

Table 4.3: *P. falciparum* sporozoite rate of wild *Anopheles* mosquitoes

Total Number tested	*An. gambiae s.l*					
N=4,360	Ahero N=1407		Bondo N=569		Busia N=953	
	Pf sporozoite negative	*Pf* sporozoite positive	*Pf* sporozoite negative	*Pf* sporozoite positive	*Pf* sporozoite negative	*Pf* sporozoite positive
2,929	1,385	22 (1.56%)	568	1 (0.2%)	936	17 (1.78%)
	An. funestus					
	Ahero		Bondo N=1,192		Busia N=239	
	-	-	*Pf* sporozoite negative	*Pf* sporozoite positive	*Pf* sporozoite negative	*Pf* sporozoite positive
1,431	-	-	1,140	52 (4.45%)	230	9 (3.77%)

**Pf: Plasmodium falciparum*

Table 4.4: Mean *P. falciparum* sporozoite difference in wild *Anopheles* across the study sites

	An. gambiae s.l			*An. funestus*		
Site	*Pf* sporozoite mean (N=2929)	*F-value*	*P*-value	*Pf* sporozoite mean (N=1431)	Statistic value	*P*-value
Ahero	0.01563611			-		-
Bondo	0.00175747	3.82	0.022	0.04446309	0.22	0.637
Busia	0.01783841			0.0376569		

Ahero*Bondo (*P*=0.042), Ahero*Busia (*P*=0.8932), Bondo *Busia (*P*=0.0242)

*ANOVA was used to compare the mean difference of *Pf* sporozoite in *An. gambiae s.l* in Ahero, Bondo and Busia

*Independent t-test was used to compare mean difference of *Pf* sporozoite in *An. funestus* between Bondo and Busia

4.4 Knockdown Resistance Genotype Frequency and Distribution in Wild adult *Anopheline* Mosquitoes

Coventional PCR revealed that most of the samples from the study sites were *An. arabiensis*. The number of *An. arabiensis* identified was 86 (88.66%) , 91 (67.91%) and 96 (70.07%) for Ahero, Busia and Bondo respectively. *An. gambiae s.s.* on the other hand, recorded low numbers across the study sites. Results from conventional PCR analysis indicated that 2 (2.06%), 39 (29.10%) and 24 (17.52%) of *An. gambiae s.s.* were recorded in Ahero, Busia and Bondo respectively. However, 9 (9.28%), 4 (2.99%) and 17 (12.41%) of the samples from Ahero, Busia and Bondo respectively did not amplify. The reason for not amplifying could be that they were other species of the *An.gambiae s.l* complex. The frequency of *kdr* east (L1014S) mutation observed in *An. arabiensis* mosquitoes was 26.74%, 35.71% and 21.88% from Ahero, Busia and Bondo respectively. *Kdr* west (L1014F) was 7.56% in Ahero, 28.02% in Busia and 30.21% in Bondo. The homozygote form of *kdr* west was not detected in Ahero but 13 (7.56%) of heterozygote form was observed in only *An. arabiensis* mosquitoes. In general *kdr* east frequency was high across the three sites compare to *kdr* west. However, in Busia, *An. arabiensis* population recorded the highest number of homozygote form of *kdr* west. Bondo recorded only 1(one) homozygote form of *kdr* west in the *An. arabiensis* mosquitoes. The heterozygote form of *kdr* west on the other hand, is present in all the *An. arabiensis* mosquitoes with highest number (56, 30.21%) being observed in Bondo.

The frequency of *kdr* east and west in *An.gambiae s.s* across the study in was relatively low compared to the frequencies in *An. arabiensis*. Only one homozygote *kdr* east allele was recorded in Ahero but *kdr* west allele was not obseverd. In Busia, the frequency of *kdr* east and west in *An. gambiae s.s* was 38.46% and 19.23% respectively. The frequency of *kdr* east and west in *An. gambiae s.s* from Bondo

was the same (12.5%). The proportion and distribution of *kdr* east and west mutations in the study sites was showed in Table 4.5

Table 4.5: The frequency and distribution of the *kdr* east (1014S) and west (1014F) alleles of wild *Anopheline* mosquitoes

Site	Species	Proportion	East *Kdr* (1014S)						West *Kdr* (1014F)				
		N=368	SS	LS	LL	NA	Freqency, %		FF	LF	LL	NA	Frequeny, %
Ahero	A	86 (88.66%)	3	40	39	4	26.74		0	13	57	16	7.56
	G	2 (2.06%)	1	0	1	0	50		0	0	2	0	0
	NA	9 (9.28%)	0	4	3	2	22.22		0	1	6	2	5.56
Busia	A	91 (67.91%)	12	41	24	14	35.71		11	29	30	21	28.02
	G	39 (29.10%)	7	16	9	7	38.46		3	9	10	17	19.23
	NA	4 (2.99%)	0	2	2	0	50		1	1	2	0	3.75
Bondo	A	96 (70.07%)	7	28	46	15	21.88		1	56	29	10	30.21
	G	24 (17.52%)	0	6	14	4	12.5		0	6	9	9	12.5
	NA	17 (12.41%)	1	2	12	2	11.76		0	10	5	2	29.41

A: *An. arabiensis*, G: *An. gambiae s.s.*, NA: Not amplified, SS: homozygote allele for L1014S mutation, LS: heterozygote allele for L1014S mutation, FF: homozygote allele for L1014F mutation, LF: heterozygote allele for L1014F mutation, LL: wild genotype (not resistant allele) L1014 codon

*The frequency was calculated using the formula: 2(SS) +LS)/2N*100 for *kdr* east and 2(FF) +LF)/2N *100 for *kdr* west

4.5 Knockdown Resistance Association with *P. falciparum* Sporozoite Rate in *An. gambiae s.l*

An. gambiae s.s and *An. arabiensis* mosquitoes tested for *P. falciparum* sporozoite and proved positive were subjected to *kdr* (L1014S and L1014F) analysis (Table 4.3). Samples that were tested negative for *P. falciparum* sporozoite were also genotyped for *kdr* (L1014S and L1014F) mutations along side the ELISA positive ones.

Two way classifications Chi-square (X^2) revealed that there was significant association between vgsc-L1014S mutation with sporozoite rate in *An. gambiae s.s* (X^2_1=5.48, P=0.032) though the number of sporozoite positive alleles were very low. In contrast, there was no association between L1014S with sporozoite rate in *An. arabiensis* population. Moreover, the presence of L1014F mutation did not correlate with sporozoite rate in either *An. gambiae s.s* or *An. arabiensis* populations investigated (Table 4.6).

Table 4.6: *Kdr* Mutations (L1014S and L1014F) associations with *P. falciparum* sporozoite rate in *An. gambiae s.s* and *An. arabiensis*

***Kdr* east (L1014S) association with *P. falciparum* sporozoite rate**				
Species	***Pf* sporozoite**	***Kdr* frequency**	**X^2 test value**	***P*-value**
An. gambiae s.s	N=8 5 (62.5%)	N=22 4 (18.18%)	5.48	0.032
An. arabiensis	N =22 2 (9.09%)	N=109 2 (1.83%)	3.26	0.131
***Kdr* west (L1014F) association with *P. falciparum* sporozoite rate**				
Species	***Pf* sporozoite**	***Kdr* frequency**	**X^2 test value**	***P*-value**
An. gambiae s.s	N=3 0 (0%)	N=15 2 (13.33%)	0.45	1
An. arabiensis	N=12 0 (0%)	N=98 2 (2.04%)	0.249	1

Pf: *Plasmodium falciparum*
*The results were analysed using two way classifications Chi-square (X^2)

4.6 Frequency of *Kdr* Genotype in Lavae Raised *Anopheline* Mosquitoes

The PCR analysis results indicated that 223 (81.68%) were *An. arabiensis*, 6 (2.20%) were *An. gambiae s.s* and 44 (16.12%) did not amplified. The distribution of *An. arabiensis* in the study areas was 76 (81.72%) for Ahero, 70 (75.27%) for Busia and 77 (88.51%) for Bondo. No *An. gambiae s.s.* was was recoreded in Ahero. However, only 2 (2.15%) and 4 (4.60%) of *An. gambiae s.s.* identified in Busia and Bondo respectively. The frequency and distribution of *kdr* allele varied across the study sites. Bondo recorded the highest number of homozygote and heterozygote forms of *kdr* west (16),

followed by Busia (6) and Ahero (1). The proportion of each of the species per study site and the frequency and distribution of the *kdr* alleles for 1014S and 1014F were indicated in Table 4.7

Table 4.7: The frequency and distribution of *kdr* east (1014S) and west (1014F) alleles of field collected larvae raised *Anopheline* mosquitoes

Site	Species	Proportion	East ***Kdr*** (1014S)						West ***Kdr*** (1014F)				
		N=273	SS	LS	LL	NA	Frequency, %		FF	LF	LL	NA	Frequency, %
Ahero	A	76 (81.72%)	2	33	38	3	24.34		1	13	48	14	9.87
	G	0 (0%)	0	0	0	0	0		0	0	0	0	0
	NA	17(18.28%)	1	10	6	0	35.29		1	4	10	2	17.65
Busia	A	70 (75.27%)	0	31	38	1	22.14		9	15	40	6	23.57
	G	2 (2.15%)	0	2	0	0	0.5		0	0	2	0	0
	NA	21 (22.58%)	3	11	6	1	40.48		1	5	14	1	23.81
Bondo	A	77 (88.51%)	3	7	64	3	8.44		16	31	17	13	40.90
	G	4 (4.60%)	0	3	1	0	37.5		1	0	2	1	25
	NA	6 (6.90%)	0	2	2	2	16.67		0	4	2	0	33.33

A: *An. arabiensis*, G: *An. gambiae s.s.*, NA: Not amplified, SS: homozygote allele for L1014S mutation, LS: heterozygote allele for L1014S mutation, FF: homozygote allele for L1014F mutation, LF: heterozygote allele for L1014F mutation, LL: wild genotype (not resistant allele) L1014 codon

*The frequency was calculated using the formula: 2(SS) +LS)/2N*100 for *kdr* east and 2(FF) +LF)/2N *100 for *kdr* west

4.7 Comparison of the Frequency of *Kdr* Genotypes (Vgsc-L1014S and Vgsc-L1014F) in Larvae Raised Mosquitoes and Wild Mosquiotoes

Two way classifications Chi-square (X^2) showed that there was no significant association between kdr east (vgsc-L1014S) in larvae raised mosquitoes and wild-caught female mosquitoes (X_1^2=2.630, P=0.108) at 95% confidence interval (Table 4.8). Similarly, the result also showed that there was no significant association between kdr west (vgsc-L1014F) in larvae raised mosquitoes and wild-caught female mosquitoes (X^2_1=0.141, P=0.742).

Table 4.8: Comparison of *kdr* genotypes frequencies in larvae raised and wild caught adult mosquitoes

Kdr Genotypes	Wild caught mosquitoes N (%)	Larvae Raised mosquitoes N (%)	X^2 value	*P*-value
L1014S	170 (46.2)	109 (39.78)	2.630	0.108
L1014F	141 (38.32)	101 (36.86)	0.141	0.742

*The result was analysed using two way classification Chi-square (X^2)

CHAPTER FIVE

DISCUSSION

The low mortality rates (<90%) following WHO Bioassay guidlines observed in all the malaria vectors used in this study showed that there was a dramatic increased in phenotypic resistance to deltamethrin in all the study sites. Earlier, high mortality rate to deltamethrin using WHO Bioassay method was documented in *An. arabiensis* population in western Kenya (Mathias *et al.*, 2011; Ochomo *et al.*, 2012) but recent study revealed low mortality to deltamethrin (Wanjala *et al.*, 2015) confirming the findings of the current study. There was significant difference in mortality rates between larvae raised *An. gambiae s.l* and wild caught *An. gambiae s.l* mosquitoes resting indoors in all the study sites. Wild-caught *An. gambiae s.l* mosquitoes were far more resistant compare to larvae raised mosquitoes. Even though earlier studies in western Kenya reported high resistance to pyrethroids, most of these studies used *An. gambiae s.l* larvae collected from the fields to determine insecticide resistance (Ochomo *et al.*, 2012; Ochomo *et al.*, 2014; Wanjala *et al.*, 2015). The higher resistance recorded in wild-caught adult *An. gambiae s.l* mosquitoes compare to larvae raised ones could be ascribed to other mutations or mechanisms of resistance which might not be in the larvae raised mosquitoes. Asparagine-to-tyrosine substitution mutation on the voltage-gated sodium channel at position 1575 (N1575Y) has been identified to be responsible for pyrethroid resistance in *An. gambiae s.l* (Djègbè *et al.*, 2014; Jones *et al.*, 2012). Furthermore, the significant difference recorded in the level of deltamethrin resistance between wild mosquitoes and larvae raised mosquitoes could be attributed to pyrethriods used in the control of livestock disease vectors (Diabate *et al.*, 2002; Overgaard, 2006); since *An. arabiensis* also depends on cattle for blood meals. A study conducted in some farming communities in Benin had shown that pyrethroids use for agricultural activities resulted in high level of resistance to deltamethrin (Nazaire *et al.*, 2014). The high level of resistance recorded in this study

could be as a result of development of other unknown molecular markers of resistance as the mosquitoes come into contact with insecticide. This warrant further investigations through genome sequencing to detect resistance markers.

Anopheles funestus was found to be more resistant compared to *An. gambiae s.l* counterparts but there were no significant difference between resistance in *An. funestus* species found in Bondo and Busia populations. Globally, Insecticide resistant data on *An. funestus* were documented to be limited (Ranson and Lissenden, 2016) though there are studies which reported high level of pyrethroids resistant in this malaria vector (Hargreaves *et al.*, 2000; Kawada *et al.*, 2012; McCann *et al.*, 2014). This study pointed out that *An. funestus* resistance to deltamethrin had increased significantly as compared to previous studies. McCann *et al.*, (2014) documented 60% mortality rate to deltamethrin in Asembo, Bondo region in 2010 and 2011 wheras this study revealed 29.7% and 33.05% mortality rate for Bondo and Busia regions respectively.

Since Bondo and Busia regions were among areas under ITNs coverage, insecticide resistance could play a major role in the abundance of *An. funestus* vector population in the region. Use of insecticides for agricultural activities such as for controlling livestock vector desease and crop pest, could also play a role in offering selection pressure for resistance in western Kenya. The mechanisms for extremely high level of deltamethrin phenotypic resistance in *An. funestus* mosquitoes was not elucidated in this study but other studies documented that over-expression of CYP6P9a and CYP6P9b oxidation enzymes which result in rapid detoxification of pyrethroids were responsible for pyrethroids resistance in this vector (Brooke *et al.*, 2001; Djouaka *et al.*, 2011). Metabolic resistance mechanisms have been implicated for insecticide resistance in *An. funestus* (Hemingway, 2014; Mulamba *et al.*, 2014). Furthermore, vgsc-1014S and vgsc-1014F are not associated with target site pyrethroids resistance in

An. funestus but mutations in other exons have been suggested to play a role in target site resistance (Djouaka *et al.*, 2011).

The current study revealed that *An. arabiensis* is the major vector of all the *An. gambiae s.l* complex in Ahero, Bondo and Busia (>70%). Earlier studies had shown that *An. arabiensis* was the main vector in Ahero due to rice irrigation farming which provide breeding grounds for that vector (Ochomo *et al.*, 2012; Ochomo *et al.*, 2014). In Bondo region, *An. arabiensis* was previously discovered to be a minor vector (Beier *et al.*, 1990; Taylor *et al.*, 1990) but subsequent studies proved that the population of *An. arabiensis* became the most abundant vector with a decrease in *An. gambiae s.s.* population; which was in agreement with this study (Bayoh *et al.*, 2010; Ochomo *et al.*, 2014). Though *An. gambiae s.s.* is known to be a major vector in Busia/Teso region with the presence of *An. arabiensis* as a minor vector (Ochomo *et al.*, 2012), this study found out that *An. arabiensis* is the most abundant vector. This therefore necesitates further mornitoring. The feeding behaviour of *An. arabiensis* (more exophilic and endophilic) and resistance to pyrethriods could be the reason for the resurgence of this vector in western Kenya. High proportion of *An. funestus* was sampled from Bondo district via indoor resting collection and small proportion was observed from Busia region. Previously, *An. funestus* population in Bondo Region had witnessed a drastic reduction following the distribution of ITNs in the 1990s (Gimnig *et al.*, 2003), hence sampling of this vector was low (Lindblade *et al.*, 2006); but later there was resurgence of this vector in Asembo (Bondo sub-County), western Kenya (McCann *et al.*, 2014). This study comfirmed that *An. funestus* has reemerged and is now the major vector and the main player of malaria transmission in the region and could dominate other areas if other vector control efforts are not employed. The remergence of *An. funestus* could be attributed to insecticide resistance since this vector showed high resistance to deltamethrin in this study.

Sporozoite infectivity rate in malaria vectors is a major components of entomological inoculation rate (EIR) (President's Malaria Initiative, 2016). In general, the sporozoite infectivity rate recorded in this study varies across all the study sites. *Plasmodium falciparum* sporozoite infectivity rate in *An. gambiae s.l* varied across Bondo, Ahero and Busia. High sporozoite rate observed in Busia and Ahero in *An. gambiae s.l* mosquitoes sampled via indoor resting collection suggested that these vectors are the major players of malaria transmission in those regions. The relative low rate of sporozoite in *An. gambiae s.l* in Bondo could be as a result of ITNs intervention and a significant resurgence of *An. funestus* species. *Anopheles funestus* had the higher *P. falciparum* rates in Bondo and Busia but there was no significant difference in sporozoite rate between the two regions. But in general, there was a significant difference in sporozoite rate between *An. gambiae s.l* and *An. funestus* suggesting that *An. funestus* is likely to transmit malaria more than *An. gambiae s.l*. This also implies that the parasite can adapt and be sustained more in *An. funestus* mosquitoes compare to *An. gambiae s.l* counterpart.

This study confirmed a previous study in Asembo, Bondo District in 2010 and 2011 which also reported high sporozoite rate (4.5%) in *An. funestus* (McCann *et al.*, 2014). High proportion of *An. funestus* and high level of resistance to deltamethrin in Bondo Region indicated the impact of this vector in malaria transmission in the region. *Anopheles funestus* resistance to pyrethroids was linked to increase in malaria transmission in South Africa (Hargreaves *et al.*, 2000). Moreso, Chanda *et al.*, (2011) noted that *P. falciparum* prevalence in ITNs and IRS implementation zones could be ascribed to increasing level of pyrethriods resistance which could lead to malaria control failures. Undoubtedly, the prevalence of *P. falciparum* malaria cases in Bondo and Busia regions are due to high vectorial capacity of *An. funestus* population in those regions though other vectors could also play a role. The reemergence, relatively high resistance to deltamethrin, anthropophagic nature and high *P. falciparum* sporozoite rate of *An. funestus* female mosquitoes in Bondo and Busia regions contribute significantly

to malaria transmission in those regions in western Kenya. This should be of great concern to National Malaria Controls Programs and other agencies aiming at malaria elimination in western Kenya.

Knockdown resistance (L1014S and L1014F) genotype frequencies observed in this study were high and could be associated with *An. arabiensis* vector population in all the study sites though some proportions were observed in *An. gambiae s.s.* population. An earlier study associated L1014S mutation with *An. gambiae s.s.* and L1014F was associated with *An. arabiensis* (Kabula *et al.*, 2014). *Kdr* east (L1014S) was first discovered in Kenya in 2000 (Ranson *et al.*, 2000) and *kdr* west (L1014F) was discovered in 2012 in western Kenya (Ochomo *et al.*, 2015). Since the discovery of kdr mutations, they vary from area to area and from one species to another. Previously, no kdr allele was observed in *An. arabiensis* vector but the frequency of L1014S in *An. gambiae s.s.* was reported to be high (Mathias *et al.*, 2011; Ochomo *et al.*, 2012); though Kawada *et al.*, (2011) documented 1014S in *An. arabiensis* mosquitoes in high proportions. Later in 2011 to 2013, Ochomo *et al.*, (2015) recorded low frequencies of L1014S in *An. Arabiansis* population in Bondo and Ahero but no L1014S was observed in *An. gambiae s.s* in Ahero and Bondo.

The frequency of L1014F recorded in Bondo and Ahero in 2012 and 2013 in *An. Arabiansis* population was in very low proportions compared to the frequency observed in the current study. Also, no vgsc-1014S and vgsc-1014F were detected from 2011 to 2013 in Bondo and Ahero in *An. gambiae s.s.* mosquitoes (Ochomo *et al.*, 2015). Moreover, Wangala *et al.*, (2015) reported no detection of 1014F in *An. gambiae s.s.* and *An. Arabiansis* in Ahero but L1014S was low in *An. arabiensis* population. The increased in *kdr* allele frequency detected in this study could be associated with low migration of *Anopheles* mosquitoes and selection overcome gene flow (Protopopoff *et al.*, 2008). An earlier study noted that extensive use of LLINs had resulted in relatively high level of gene flow between *An. arabiensis* mosquitoes in about 300km apart (Wondji *et al.*, 2005). However, increased in

kdr frequency could not have any negative effect on malaria control interventions implemented in western Kenya. Some studies noted that high kdr frquency did not have negative effects on the bio-efficacy of pyrethriod treated bed nets (Ouattara *et al.*, 2014) or reduce the bioefficacy of pyrethriod IRS (Hemingway *et al.*, 2013) though one study associated the failure of IRS pyrethroids with high frequency of *kdr* L1014F in *An. gambiae s.s* (Sharp *et al.*, 2007).

Earlier studies to elucidate the possibility of kdr (L1014S and L1014F) impact on malaria transmission in some areas in Africa had reported conflicting results. Even though the current study detected increased *kdr* mutations (L1014S and L1014F) especially in *An. arabiensis*; there was no association between L1014S mutations and *P. falciparum* sporozoite infectivity rate in *An. arabiensis* mosquitoes. Similarly, it was also observed that L1014F had no association with sporozoite rate in *An. gambiae s.s* and *An. arabiensis*. In contrast, L1014S had significant association with sporozoite rate in *An. gambiae s.s* population though the number of sporozoite positive alleles were very low to make any meaningful interpretation. A previous study in Benin (Ossegrave *et al.*, 2012) had shown that L1014F had no influence on *P. falciparum* sporozoite rate in *An. gambiae s.s* which confirmed the current study. Moreover, Hemingway *et al.*, (2013) noted that homozygote form of kdr pyrethroids-resistant could transmit less malaria compared to the susceptible one. However, a study had also shown that *An. gambiae s.s* having L1014F mutation had high sporozoite rates which could impact malaria transmission (Alout *et al.*, 2013). Furthermore, in Senegal, Trape *et al.*, (2011) reported *kdr* L1014F had effect on increased malaria transmission after implementation of malaria control programmes. The underlying factors by which L1014F mutation influenced malaria transmission in some areas contrary to other areas is still not understood though in the current study there was no detection of homozygote form of L1014F in both species investigated. The significant association of L1014S with sporozoite rate in *An. gambiae s.s* population detected in the current study confirmed a similar study in Tanzania

which reported significant association of L1014S mutation with sporozoite rate in *An. gambiae s.s* mosquitoes (Kabula *et al*., 2016). Thus, *An. gambiae s.s* having kdr east (L1014S) are more likely to sustain the *P. falciparum* parasite and could contribute to malaria transmission in areas recording high level of resistance to pyrethroids.

High frequency of *kdr* genotypes were detected in both larvae raised adult mosquitoes and wild mosquitoes collected indoors in households having ITNS; meanwhile there was no significant association between *kdr* east (L1014S) in larvae raised mosquitoes and wild-caught mosquitoes and also no significant association between *kdr* west (L1014F) and *kdr* east (L1014S) in larvae raised mosquitoes and wild-caught mosquitoes. But from the forgoing discussion, there was significant difference in deltamethrin phenotypic resistance between larvae raised mosquitoes and wild-caught adult mosquitoes. This did not however reflect in *kdr* frequencies in larvae raised mosquitoes and wild-caught adult mosquitoes suggesting that the wild adult mosquitoes' population developed other mechanisms of insecticide resistance possibly not in larvae raised populations as they came into contact with ITNs and IRS pyrethriods.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 SUMMARY

High level of deltamethrin phenotypic resistance was observed in larvae raised versus wild caught adult *An. gambiae s.l* populations implying that wild adults *Anopheles* developed other resistance mechanisms when they came in contact with ITNs during host seeking. Higher sporozoite rate, relative abundance and higher resistance observed in *An. funestus* compared to *An. gambiae s.l* suggest that the former is playing a major role in malaria transmission in Bondo and Busia sub-Counties. Only L1014S mutation had significant association with *P. falciparum* sporozoite rate in *An. gambiae s.s* though the number of sporozoite positive alleles was low; suggesting that that marker may play a role in malaria transmission. The insignificant association between *kdr* L1014S and L1014F in larval raised mosquitoes and wild caught mosquitoes implies that wild caught adult mosquitoes developed other mechanisms of insecticide resistance apart from *kdr* mutations as a result of their interaction with ITNs during host seeking for blood meal.

6.2 CONCLUSIONS

1. This study demonstrated significant increase in deltametrin phenotypic resistance in both larval raised and wild caught *Anopheles* sampled from Ahero, Busia and Bondo.
2. There was a higher sporozoite rates and relative abundance of *An. funestus* in Bondo and Busia compare to *An. gambiae s.l* therefore *An. funestus* is more competent in malaria transmission than *An. gambiae s.l* populations

3. The L1014S mutation may influence malaria transmission in pyrethroid resistant *An. gambiae s.s.*

4. There were no association between L1014S and L1014F mutations in larval raised versus wild caught *Anopheles.*

6.3. RECOMMENDATIONS

6.3.1 Recommendations from the study

1. Larvae alone should not be used to test for insecticide resistance in *Anopheles* mosquitoes since wild-caught adult mosquitoes proved more resistant than larvae raised counterparts. Wild mosquitoes should also be used alongside. Furthermore, other vector control methods or new insecticides apart from pyrethroids should be explored for use in malaria vector control programmes since mosquitoes are highly resistant to current pyrethroids.
2. Effort should be geared towards *An. funestus* control especially in Bondo and Busia Regions since they are the main players of malaria transmission in those areas
3. Insecticide resistant molecular markers should be continously monitored in malaria control programme
4. Other mechanisms such as metabolic resistance or Ace-1 mutations might have played a role in resistance in wild caught adults mosquitoes.

6.3.2. Recommendations for Further Studies

1. The high level of phenotypic resistance observed in this study suggest that there could be other unknown insecticide resistance molecular markers, therefore further studies should be carried out to elucidate these markers in both *An. gambiae s.l* and *An. funestus* populations.
2. Further studies should explore possible use of new classes of insecticide for vector control

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