AN ASSESSMENT OF THE HETEROGENEITY, AGE MODULATION AND PROTECTIVE EFFECT OF INSECTICIDE RESISTANCE AND EVALUATION OF INSECTICIDE TREATED NET CONDITION AMONG CHILDREN UNDER FIVE YEARS OF AGE INFECTED WITH MALARIA IN WESTERN KENYA.

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOMEDICAL SCIENCE AND TECHNOLOGY (ENTOMOLOGY AND VECTOR SCIENCE)

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ABSTRACT

Vector control remains central to the fight against malaria with insecticide-treated bednets (ITNs) and indoor residual spraying (IRS) being a central component of the World Health Organization (WHO) global strategy. The recent upsurge of insecticide resistance in various parts of the world has led to fears that control efforts may be compromised. The frequency of insecticide resistance in western Kenya has not been mapped in a large scale and the effect it is likely to have on ability of vectors to survive exposure to ITNs is unknown. With malaria control programs embarking universal coverage every 3 years, it is important to determine whether ITNs are able to protect person using them once they have holes. The effect massive vector control have on vector age structure needs to be evaluated if successful transmission reduction is to be achieved, hence the need to evaluate age grading tools. *Anopheles* mosquitoes were sampled from clusters (sub-locations) then susceptibility to permethrin or deltamethrin determined. A subset of *An. arabiensis* samples from Bondo and Nyando underwent gene-expression profiling using whole genome microarray. Cross-sectional surveys of ITNs were conducted in houses in Gem and Bungoma in May 2013 where ITN condition, number and species of mosquitoes resting inside, and insecticidal activity against susceptible and resistant mosquitoes were quantified. Children under 5 years of age were recruited into a case control study at the local health facility and followed back to their homes and their ITNs taken for evaluation. A questionnaire detailing household characteristics and net use was administered. Ages of wild mosquitoes collected at different times were estimated using the Near Infra-Red Spectrometry (NIRS). Mortality to deltamethrin ranged from 45-100%, and to permethrin from 30-100% and varied between *An. arabiensis* and *An. gambiae* s.s. although this was not statistically significant. There was no correlation between mortality to permethrin and deltamethrin in *An. arabiensis* (Z=2.9505, P=0.2483). Microarray results revealed that CYP6M2 (the mutant allele) was consistently expressed at higher levels in Bondo. A total of 552 *An. gambiae* s.l., 5 *An. funestus* s.l. and 137 *Culex spp.* were found resting inside nets in Bungoma. The ITNs from Bungoma retained strong activity against a susceptible laboratory (>90% mortality) but not against first filial generation (*F1*) offspring of field-collected *An. gambiae* s.s. (<60% mortality). There was no difference in the number and size of holes in ITNs used by cases versus controls thus there was no association of the presence of malaria infection with sleeping under a holed ITN. Roof type and number of people that slept inside the households was associated with malaria infection (P=0.013; P=0.006). Higher resistance was observed in older mosquitoes compared to the younger ones. Insecticide resistance is heterogeneous within small geographical areas. The CYP6M2, identified in this study is associated with resistance to multiple insecticide classes. In areas with pyrethroid resistant vectors, mosquitoes are able to enter ITNs and survive. In an area of high insecticide resistance, ITNs may not offer protection against malaria once they have holes. Age was observed to select for resistance in mosquitoes such that there was an increase in insecticide resistance with increasing mosquito age. Results from this study will inform resistance monitoring programs on the need for multiple sampling within a district. The finding of mosquitoes inside ITNs indicates the need for integrated vector management for example IRS with a non-pyrethroid. There is a need for prompt replacement of ITNs especially those used by children once they start acquiring holes. The observation of older mosquitoes having higher insecticide resistance means these mosquitoes may survive insecticide exposure in ITNs and thus the need for insecticide resistance mitigation strategies.

CHAPTER ONE: INTRODUCTION

1.1 Background

Malaria continues to be the most important parasitic disease, killing more than 627,000 people globally in 2012 (WHO, 2013b). Sub-Saharan Africa continually suffers the greatest burden; harboring about 90% of the cases (WHO, 2010b). The use of insecticide treated nets (ITNs) has been shown to contribute to a reduction in entomologic (Mbogo et al., 1996; Lindblade et al., 2006) and epidemiologic indices (Vulule et al., 1994; Gimnig et al., 2003b; O'Meara et al., 2010) and, most importantly a reduction in morbidity and mortality in children under the age of five years (Nevill et al., 1996; Phillips-Howard et al., 2003; Lengeler, 2004). ITNs have been observed to reduce the morbidity and mortality among Gambian children by over 60% and greatly reduce the vector density and consequently malaria transmission of indoor resting mosquitoes in at coastal Kenya (Alonso et al., 1991; Mbogo et al., 1996) as well as other parts of Africa (Lindsay et al., 1991). Thus, ITN use has been a major contributor to the declines in malaria endemicity in Africa. In addition to ITNs, the recent WHO position statement on indoor residual spray (IRS) has brought an important change in the landscape of malaria control in Africa. The use of IRS has increased almost six-fold since 2001 (WHO, 2009b) and has stimulated a renewed interest in malaria prevention with emphasis on vector control. ITNs are now considered efficient tools for vector control when used in large scale (Curtis et al., 1990).

In the recent past, there has been an upsurge of insecticide resistance in different parts of the world (Wood and Bishop, 1981; WHO, 1986; Kristan et al., 2003; Marcombe et al., 2009; Ramphul et al., 2009; Ranson et al., 2009; Yewhalaw et al., 2010; Ranson et al., 2011) resulting in fears that insecticide based vector control may be compromised especially after large scale interventions. In western Kenya, a reduction in susceptibility to pyrethroid

insecticides was reported after one year of a large-scale permethrin impregnated bednet programme (Vulule et al., 1994; Vulule et al., 1999) and has since been reported in multiple sites (Kawada et al., 2011a; Kawada et al., 2011b; Mathias et al., 2011; Ochomo et al., 2013). The National Malaria Control Program advocates the use of ITNs in malaria endemic areas and IRS in endemic and epidemic prone areas. The insecticides of choice in both strategies have traditionally been synthetic pyrethroids. As insecticide resistance particularly to this important class of insecticides increases, programs could get to levels where the insecticides no longer work. While studies have attempted to describe frequencies of insecticide resistance to pyrethroids, the available information has widely generalized resistance status based on just a few sample points, for example one sample point representative of a district. Secondly, these studies have only attempted to associate such resistance with known markers like knock down resistance (*kdr),* even though the genes driving resistance are bound to vary from place to place. Despite these, the variations in insecticide resistance and genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando remains unknown. As such, the current study compared the variations in insecticide resistance and identified genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando.

In western Kenya, malaria transmission in the lowland areas around Lake Victoria has historically been very high, with entomological inoculation rates (EIR) estimated to be as high as 300 infectious bites per person per year (Beier et al., 1990; Beier et al., 1994; O'Meara et al., 2010). *An. gambiae s.l.* and *An. funestus* population density declined markedly in a randomized evaluation trial of permethrin-treated bed nets in treatment compared to control villages in western Kenya (Gimnig et al., 2003b; Lindblade et al., 2006). Since the completion of the ITN trial, the distribution of nets in malaria endemic areas has risen over the years with household ownership in the Kenya lake endemic zones up to 70% of

households, and ITN ownerships up to 60% (DOMC, 2010). Between 2003 and 2007, demographic surveillance revealed a 42% reduction in all-cause mortality among children less than 5 years of age coinciding with a period of ITN scale up (Adazu et al., 2008; Hamel et al., 2011b). Increased availability of ITNs due to large scale distribution campaigns and improved awareness for proper use have contributed to the efficacy of ITNs in reducing malaria transmission (Noor et al., 2009; Chukwuocha et al., 2010; PMI, 2011). All ITNs act as a physical barrier, preventing access to humans by vector mosquitoes and thus providing personal protection against malaria to the individuals using the nets. Pyrethroids used to treat the ITNs, have an exito-repellent effect thus adding a chemical barrier to the physical one (Curtis et al., 1990; Miller et al., 1991; WHO, 2005a). Insecticides incorporated in the ITNs kill the malaria vectors that come into contact with it and when used by a majority of the target population, may provide protection for the community including those who do not themselves sleep under an ITN (Binka et al., 1998; Takken, 2002; Hawley et al., 2003a). A more recent innovation is the long-lasting insecticidal net (LLIN), in which insecticide is either incorporated into the fiber during extrusion, or coated on the fiber or the finished net with a binding agent. Unlike conventional ITNs, which lose effective insecticide after one or two washes and last only 6-12 months, LLINs retain effective doses of insecticide up to 20 washes and have are marketed to have lifespan of 3 to 4 years (WHO, 2005a). The actual duration the ITN physical integrity is maintained is dependent on the individuals using them. In area with emerging resistance to insecticides used in the ITNs, compromise in the physical integrity of the ITNs could mean mosquitoes are able to pass through the ITN and bite persons sleeping under the ITN. The actual duration an ITN is effective in killing mosquitoes may be dependent on the resistance status of the local vectors. Whether an ITN remains effective once it has holes in an area of high pyrethroid resistance remains unknown. Therefore, this study investigated the presence of mosquitoes resting inside ITNs and further

assessed the bioefficacy and condition of the ITNs in which mosquitoes were found to be resting.

In contrast to the largely encouraging reports of ITN efficacy from Kenya and Rwanda (O'Meara *et al.,* 2010), data from a highland and a lowland area in western Uganda showed steadily increasing numbers of malaria cases and deaths in district hospitals from 1991 to 2000, with a two-fold to four-fold overall increase in the number of children admitted to hospital with the disease despite use of ITNs (Ndyomugyenyi and Magnussen, 2004). A slight decline in the proportion of positive blood films was seen in a single facility in an area of moderate transmission in Uganda after one round of indoor residual spraying (IRS) in 2007. About 14 months after IRS, the proportion of blood films that tested positive began to increase, suggesting that trends are easily reversed if control measures are not sustained for whatever reason (Bukirwa et al., 2009). In a study conducted in Macha in Choma district of Zambia, mosquito surveillance collections were performed, and findings demonstrated that the area has 100% ITN coverage yet 25-28% of *An. arabiensis* collected in CDC light traps were engorged, with human blood indices of 94-96% (Fornadel et al., 2010). This indicates that despite high ITN use, *An. arabiensis* mosquitoes were still obtaining human blood meals. It is possible that this could be due to either decreased efficacy of ITNs in use in Macha, or pyrethroid resistance in the vector (Norris and Norris, 2011). If holes are very large, as was the case for some nets in a study conducted in Macha, Zambia, a mosquito can easily enter with very little searching, limiting the amount of insecticide contact, and again negating the ITN's protective effect (Norris and Norris, 2011). Other recent studies have also reported the presence of mosquitoes inside ITNs (Gnanguenon *et al.,* 2013). In addition to killing mosquitoes using insecticides, ITNs also function as a barrier to prevent bites. Holes in the net can undermine this function, particularly when residual insecticide concentrations in the

net are too low to efficiently kill mosquitoes. A study in Kenya found that 40% of ITNs currently being used for malaria prevention were of poor quality due to the number of holes (Githinji et al., 2010). Unlike ITNs, that have to be retreated every 6 months, the insecticide content in LLINs lasts throughout the useful lifetime of the net (WHO, 2005a; WHO, 2011a). With this in mind, it is assumed that since the LLINs have a long lasting residual insecticidal effect, they remain efficacious even when they acquire because of the insecticide content. With increasing insecticide resistance, it is unclear whether LLINs would remain protective once they acquire holes. The current study sought to investigate whether sleeping under an ITN with holes could expose a person sleeping under it to a greater risk of infection with malaria compared to an intact one especially in an area of high insecticide resistance.

The two most common malaria vector control methods, IRS and ITNs are based on the pyrethroid class of insecticides due to their rapid efficacy and low toxicity to humans. In Africa, the first case of insecticide resistance, involving *Anopheles gambiae* s.s. was observed in 1967 in Bobo Dioulasso (Burkina Faso) and attributed to the use of DDT against cotton pests (Curtis et al., 1990; Chandre et al., 1999). Pyrethroid resistance in *An. gambiae* s.s. has been documented from multiple parts of Africa (Elissa et al., 1993; Vulule et al., 1994; Vulule et al., 1996; Martinez-Torres et al., 1998; Darriet et al., 1999; Santolamazza et al., 2008; Ranson et al., 2009). Tolerance to insecticides has been recorded to be higher in younger compared to older insects, mosquitoes included (Bouvier et al., 2002; Rajatileka et al., 2011). Mosquito age is an important entomological parameter for use in evaluation of the efficacy of vector control interventions as it is used in determining the entomological inoculation rate which is an index of the number of infectious bites a person is likely to receive.. In the light of insecticide resistance, it is important to understand whether mosquito ages modulate their resistance to insecticides thus enabling them survive longer. The current

study evaluated the variation in insecticide resistance between young and old mosquitoes from Ahero, Bungoma, Teso and Bondo.

1.2 Problem Statement

Pyrethroid resistance in local *Anopheles* vectors could largely undermine vector control programs. There is a need for extensive monitoring of insecticide resistance in areas with vector control. In addition, apart from *kdr*, there are no other markers of insecticide resistance used as indicators of resistance. Whether pyrethroid resistance protects mosquitoes against lethal effects of insecticides, enabling them to enter holed ITNs remains unknown. As nets acquire holes prior to replacement, it is important to understand whether there is an increased risk in sleeping under a holed net compared to an intact one especially in children under 5 years of age who spend most of their time under these nets. Insecticide resistance has been shown to vary with age of mosquitoes. Determination of average vector ages could help predict the success of control programs, however, the only method currently available for age grading is ovarian dissection which is slow, labor intensive and appropriate for large sample numbers, thus creating a need to evaluate new age grading tools. The overall objective of the current study was to determine the effect of insecticide resistance and insecticide treated nets (ITNs) condition on malaria infection rates among children under five years of age and on entomological parameters of malaria vectors in western Kenya.

1.3 Research Objectives

1.3.1 General Objective

To assess the heterogeneity, age modulation and protective effect of insecticide resistance in malaria vectors and evaluate the Insecticide treated net condition among children under five years infected with malaria in western Kenya.

1.3.2 Specific Objective

1. To compare variations in insecticide resistance and identify genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando.

2. To assess the association of insecticide resistance with the ability of *Anopheles* mosquitoes to rest in long lasting insecticide treated nets in Bungoma and Gem.

3. To assess the association between sleeping under a holed ITN and the presence of malaria infection in children under five years in Bungoma.

4. To assess age-modulated insecticide resistance in natural *Anopheles* populations of Bungoma, Teso, Asembo and Ahero in western Kenya.

1.4 Study Questions

1. What are the comparisons in variations in insecticide resistance and the genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando?

2. What is the association between insecticide resistance and the ability of *Anopheles* mosquitoes to rest in long lasting insecticide treated nets in Bungoma and Gem.

3. What is the association between sleeping under a holed ITN and presence of malaria infection in children under five years in Bungoma?

4. What is the association between age and insecticide resistance in natural *Anopheles* populations of Bungoma, Teso, Asembo and Ahero in western Kenya?

1.5 Justification of the study

Vector control has contributed to the declines in malaria prevalence in areas that were traditionally burdened with the disease (WHO, 2009b; WHO, 2013b). However, the rate of decline has slowed and even reversed (Hamel et al., 2011a; Zhou et al., 2011) perhaps due to a myriad of factors among them, speculations of insecticide resistance compromising vector control. This study mapped out the distribution of insecticide resistance to the 2 most common insecticides used in ITNs in western Kenya and further investigated the mechanisms involved, understanding of mechanisms would facilitate the development of better control tools. LLINs are marketed as having life spans of up to 3 years, this study examined ITNs for the presence of resting mosquitoes to determine their ability to repel and kill resistant mosquitoes and the contribution of holes to the presence of the mosquitoes. The study investigated the risk of malaria in children sleeping under a holed versus intact ITN. This study would help determine whether there is an increased risk of sleeping under torn ITNs so that replacement can be done as soon as nets acquire holes. Furthermore, knowledge of the mosquito ages would enable understanding of the impact age has on the ability of mosquitoes to tolerate insecticides and may provide information on the effectiveness of ITNs.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria

Malaria is one of the primary causes of human morbidity and mortality with about 40% of the world's population lives in areas where malaria is endemic (WHO, 2008). Malaria epidemics and seasonal outbreaks have devastated large populations posing a serious barrier to economic progress in many developing countries (WHO, 2008). There are an estimated 300-500 million cases of clinical disease resulting in 627,000 deaths in 2012 (WHO, 2013b). In addition, malaria affects as many as 30,000 visitors to the tropics annually, and it is estimated that 59% of the world's clinical malaria cases occur in Africa, 38% in Asia and 3% in the Americas (WHO, 2005b). The disease is also responsible for 60% of foetal losses and over 10% of maternal deaths (WHO, 2005b). Malaria mostly affects the poor tropical and sub-tropical countries and traps them in poverty (WHO, 2007). About 10% of the world's population suffers a clinical attack of malaria each year, however, most survive after an illness lasting 10 to 20 days, but during the clinical illness, they are usually unable to attend school or work, diminishing educational attainment and productivity (WHO, 2011b). Between 2008 and 2010, nearly 300 million insecticide treated bed nets (ITNs) were distributed and over 78 million people were protected by indoor residual spraying (IRS) of insecticides in malaria-endemic areas (WHO, 2011b). These achievements, along with improvements in malaria case management through use of drugs, have resulted in fewer cases of malaria and fewer deaths due to malaria in many regions of sub-Saharan Africa (O'Meara *et al.*, 2010).

2.2 Integrated malaria control

Malaria control is currently centred around the distribution of ITNs to pregnant women and children under 5 years of age through maternal and child health clinics and universal

coverage in multiple countries, Kenya included (WHO, 2009b; WHO, 2010b). Along with IRS, these two vector control measures have been widely advocated as part of the governments policy along with prompt diagnosis and treatment (DOMC, 2010; WHO, 2010b).

Malaria is caused by members of the genus *Plasmodium*, an apicomplexan which exhibits a heteroxenous life cycle involving a vertebrate intermediate host and an arthropod definitive host which acts as a vector. Vertebrate hosts include: reptiles, birds, rodents, monkeys and humans. The *Plasmodium* species are generally host-specific in that each species will only infect a limited range of hosts and they differ in their clinical manifestations. So far, five distinct species infect humans: *P. falciparum*, *P. vivax*, *P. ovale, P. malariae and P. knowlesi* (Chin et al., 1965; Cox-Singh et al., 2008). *P. falciparum* is the deadliest of the five malaria parasites, being responsible for almost all of the observed malaria-related morbidity and mortality (WHO, 2007).

2.3 Malaria transmission in Kenya

In Kenya, areas of stable malaria have altitudes ranging from 0 to 1300m and are found around Lake Victoria in western Kenya as well as in the coastal region (DOMC, 2009). Approximately 70% of the country's surface area is prone to malaria epidemics with regular epidemics occurring in the western highlands (DOMC, 2009). The semi-arid regions in the north-east and eastern parts of the country are involved in epidemics only during intense flooding and about 170 million working days are lost due to malaria illness per year (PMI, 2008).

2.4 The malaria vector

Human malaria parasites are transmitted almost exclusively by female mosquitoes of the genus *Anopheles* Giles, which feed on vertebrate blood, with the exception of very few cases of transplacental and blood transfusion–associated transmission (Ouedraogo *et al.,* 2012). Malaria was once present on all continents except Antarctica with vectors like *An. freeborni* and *An. quadrimaculatus* in North America; *An. pseudopunctipennis* and *An. darling* in South America (Root, 1926; Davis, 1927); *An. culicifacies* and *An. minimus* in Asia and finally *An. arabiensis*, *An. gambiae* s.l. and *An. funestus s.l.* in the African tropics, including Kenya (Gilles and DeMeillon, 1968).

Kenya and the Afro-tropical region in general have two main vectors: *An. gambiae* complex and the *An. funestus* complex (Garnham, 1938; Surtees, 1970). Members of the *An. funestus* group were initially observed in the rice field of Nyanza Province (Chandler et al., 1975) then in other places but were greatly reduced through house spraying and the introduction of ITNs (Hawley et al., 2003a; Lindblade et al., 2006). However, a recent study reports the emergence of the *An. funestus* as vectors of malaria in western Kenya (McCann *et al.,* 2014) with densities in the range of 30-50% indoors (unpublished data). Members of the *An. gambiae* complex thus remain the principal vectors of malaria in western Kenya.

2.4.1 The *An. gambiae* complex

Anopheles gambiae sensu lato (s.l.) is a species complex composed of seven biological species, including two widely distributed and important vectors of human malaria in sub Saharan Africa, *An. gambiae* s.s. and *An. arabiensis*, whose geographical ranges broadly overlap (White, 1974; Coluzzi et al., 1979; White, 1985; Gillies and Coetzee, 1987). Two species (*An. melas* and *An. merus*) are restricted to brackish water environments of coastal Africa, and are of regional importance as vectors; the other 3 species (*Anopheles bwambae*,

and *An. quadriannulatus* species A and species B) are species of localized distribution (Coetzee et al., 2000). For purposes of basic and operational research and as part of malaria control programmes, it is necessary to identify field caught *An. gambiae* s.s. and *An. arabiensis*, but they are not distinguishable morphologically. Cytological methods based upon chromosome inversion patterns were the original method to distinguish the species (Cornel et al., 1996). Currently a ribosomal DNA PCR is used to distinguish the species (Scott et al., 1993).

Members of the *An. gambiae* complex are morphologically indistinguishable but exhibit distinct genetic and eco-ethological differences reflected in their ability to transmit malaria (dellaTorre *et al.,* 2002). The value of studies on the biology and behaviour of malaria vectors lies mainly in the relationship of these properties to the epidemiology and control of the disease (Goriup and Kaay, 1984). *An. quadriannulatus* is less widespread in its distribution and has been found in three widely separated areas: (White, 1974), Ethiopia and extensively in southern Africa (White, 1974). In Zanzibar and Southern Africa, *An. quadriannulatus* is markedly exophilic while it tends to be endophilic at high altitudes in Ethiopia (White, 1974; Coluzzi et al., 1979). This species feeds principally on animals other than man (Mahon et al., 1976). The Ethiopian population of *An. qudriannulatus* is a different species and designated it as *An. qudriannulatus* B (Hunt et al., 1998). *An. merus* is confined to the East coast of Africa, adjacent inland areas, coastal islands and at inland localities in association with salt pans (Muirhead-Thomson, 1951; Gilles and DeMeillon, 1968; White, 1974; Sharp, 1983; Gillies and Coetzee, 1987; Le_Sueur and Sharp, 1988; Sharp and Le_Sueur, 1990; Service, 1993). This species is regularly zoophagic (White, 1974) and has been shown to be involved in low rate malaria transmission (Muirhead-Thomson, 1951) and efficient filariasis transmission in Tanzania (Bushrod, 1981) and Kenya (Mosha and Petrarca, 1983). *Anopheles merus* plays an unexpectedly important role in malaria transmission in

coastal Tanzania (Temu et al., 1998). *Anopheles melas* is a malaria vector in West Africa (White, 1974; Gillies and Coetzee, 1987; Service, 1993); this species is the sole member of the complex known to feed readily on goats and sheep (White, 1974). It is considered that they probably do not discriminate between man, cow, pig and goat (Muirhead-Thomson, 1948). *Anopheles bwambae* is known only from the Semliki forest area of the Uganda/Zaire border, where breeding is apparently confined to mineral water swamps, vegetated principally with *Cyperus laevigatus* sledge, and formed by geothermal activity in the Rift valley (White, 1974). This species is a local vector of malaria and filariasis in the Bwamba County of Uganda (White, 1985). Thus only *An. gambiae* s.s. and *An. arabiensis* are members of this complex that transmit malaria in western Kenya. *An. gambiae* s.s. predominates in humid situations, whereas *An. arabiensis* is relatively successful in arid zones. *An. arabiensis* bites predominantly outdoors while *An. gambiae* s.s. predominantly bites indoors (Service et al., 1978). *An. arabiensis* is more zoophilic and endophilic (Coluzzi et al., 2002), while *An. gambiae* s.s., is highly anthropophagic and rests predominantly in houses making it a better vector of malaria (Githeko et al., 1994).

2.4.2 The *An. funestus* complex

Anopheles funestus Giles has been shown to be an important malaria vector, in some cases playing a more important role than *An. gambiae* Gilles and *An. arabiensis* Patton (Fontenille *et al.*, 1997). The *Anopheles funestus* Giles is comprised of at least nine members: *An. funestus, Anopheles vaneedeni* Gillies & Coetzee, *Anopheles parensis* Gillies, *Anopheles aruni* Sobti, *Anopheles confusus* Evans & Lesson, *Anopheles rivulorum* Lesson, *Anopheles fuscivenosus* Leeson, *Anopheles lessoni* Evans, and *Anopheles brucei* Service (Gillies and Coetzee, 1987; Koekemoer et al., 1999; Hargreaves et al., 2000; Brooke et al., 2001; Kamau et al., 2002). Only two species within this group are implicated in malaria trasmission: *An. funestus* and *An. rivulorum.* The latter has been implicated as a minor vector in Tanzania

(Wilkes et al., 1996). Within this group, *An. funestus* is the most abundant and widespread in eastern and southern Africa, and is highly endophilic, endophagic and anthropophilic. The other species are typically more limited in density and distribution, and mainly bite animals outdoors (Bruce-Chwatt, 1954; Hackett *et al.,* 2000). However, they avidly bite humans outdoors in the absence of other hosts (Gilles and DeMeillon, 1968). *Anopheles rivulorum* is the second most abundant and widespread species in the *funestus* group (Hackett et al. 2000).

2.5 Malaria Vector Control

Various methods exist for the control of mosquitoes including but not limited to use of repellents, larvicides and biological control, insecticide vaporizers, space sprays, IRS and Insecticide-treated nets (ITNs). Most of these control techniques are expensive to implement and limited in distribution (Barat et al., 2004; WHO, 2006). Most countries, including Kenya, that bear the burden of malaria rely on ITNs and IRS for vector control (WHO, 2009b). ITNs are an important tool to protect individuals against the morbidity and mortality caused by malaria (Alonso et al., 1991; Phillips-Howard et al., 2003; Lengeler, 2004). ITNs are also effective in preventing transmission of other vector-borne diseases (Dapeng et al., 1994; Reyburn et al., 2000; Fumiya et al., 2001). A more recent innovation is the long-lasting insecticide-treated net (LLIN), in which insecticide is either incorporated into the fiber during extrusion, or coated on the fiber or the finished net with a binding agent. Unlike conventional ITNs, which lose effective insecticide after one or two washes and last only 6-12 months, LLINs retain effective doses of insecticide up to 20 washes and have an expected lifespan of 3 to 4 years (WHO, 2005a). These are the only types currently recommended for distribution in malaria endemic regions and per the WHO recommendation, these nets are considered efficacious if they can kill 80% of susceptible mosquitoes (WHO, 2011a) which may be inaccurate, given the rise in insecticide resistance in local vector populations.

2.6 Insecticide Resistance

Insecticide resistance is the selection of a heritable trait in an insect population that results in an insect control product no longer performing as intended. Exposure to sub-lethal rate applications may allow these individuals to survive and pass on the resistance genes (IRAC, 2011). Sub-lethal doses may arise in IRS due to poor choice of product, under-dosing during application or due to poor application technique (IRAC, 2011). ITNs may also deliver sub-lethal doses due to poor product choice, inappropriate storage, use or washing, and resistance traits and genes have been seen to increase, coinciding with implementation of ITN programs (Elissa et al., 1993; Vulule et al., 1994; Vulule et al., 1996; Martinez-Torres et al., 1998; Darriet et al., 1999; Santolamazza et al., 2008; Ranson et al., 2009; Mathias et al., 2011). Both ITNs and IRS use pyrethroids because of their cost efficacy and mild toxicity to human beings (Naumann, 1990; Smolen et al., 1999; Zaim et al., 2000). Selection pressure from exposure to the insecticides used for vector control is expected to result in resistance to insecticides potentially leading to program failure, as has been observed in several African countries. Countries that have faced insecticide resistance to pyrethroids have resorted to the use of carbamates such as bendiocarb, as in the case in Benin (Akogbéto et al., 2010) and Uganda (USAID, 2010) and organochlorines such as DDT in Sudan for IRS (Abate and Hadis, 2011) which are more expensive options compared to the pyrethroids. Resistance to the pyrethroid class of insecticides is currently the biggest threat to programs implementing vector control using ITNs or IRS (Ranson et al., 2011). The variation of insecticide resistance in districts implementing vector control in western Kenya remains unknown. As such, the current study compared the variations in insecticide resistance and identified genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando.

2.7 Mechanisms of insecticide resistance

There are several ways insects can become resistant to insecticides and insects can exhibit more than one of these mechanisms at the same time. Behavioural resistance where resistant insects may detect or recognize a danger and avoid the toxin. This mechanism of resistance has been reported for several classes of insecticides, including organochlorines, organophosphates, carbamates and pyrethroids. Insects may simply stop feeding if they come across certain insecticides, or leave the area where spraying occurred (Chareonviriyaphap *et al.*, 1997; Roberts *et al.*, 2000). Altered target-site resistance where, the site where the toxin usually binds in the insect becomes modified to reduce the insecticide's effects. This is the second most common mechanism of resistance examples being knock down resistance (*kdr*) (Diabate et al., 2002; Stump et al., 2004) and Acetylcholinesterase (*Ace-1*) (Essandoh et al., 2013). Metabolic resistance where resistant insects may detoxify or destroy the toxin faster than susceptible insects, or quickly rid their bodies of the toxic molecules. Metabolic resistance is the most common mechanism and often presents the greatest challenge. Insects use their internal enzyme systems to break down insecticides. Resistant strains may possess higher levels or more efficient forms of these enzymes. In addition to being more efficient, these enzyme systems also may have a broad spectrum of activity and therefore can degrade many different insecticides (Hemingway, 2000).

2.7.1 Behavioral resistance

Changes in mosquito behaviour as a result of ITNs, including reduced indoor biting, increased outdoor biting, shifts in host preference and time of biting have been noted (Takken, 2002). It is possible that some of these apparent behavioural changes may be because of changes in the sibling species profile in an area (Takken, 2002). Behavioural resistance results from actions that have evolved in response to selective pressures presented by a toxicant (Pates and Curtis, 2005). This type of resistance occurs in the form of actions

that increase a population's ability to avoid the lethal effects of a pesticide. This response can be stimulus-dependent or -independent. For example, a stimulus-dependent response would involve the mosquitoes sensing the insecticide and then avoiding it. Such resistance occurs with insecticides that are in baits, or insecticide treated surfaces (Pates and Curtis, 2005). A stimulus-independent response occurs when mosquitoes start occupying an area, or micro-habitat, that is not treated with pesticide. Effective indoor residual spraying against malaria vectors depends on whether mosquitoes rest indoors (that is; exhibit endophilic behavior). This varies among species and is affected by insecticidal irritancy (Pates and Curtis, 2005). Exophilic behaviour has evolved in certain populations exposed to prolonged spraying programs (Pates and Curtis, 2005). Optimum effectiveness of insecticide-treated nets presumably depends on vectors biting at hours when most people are in bed. Time of biting varies among different malaria vector species, but so far there is inconclusive evidence for these evolving so as to avoid bednets. Use of an untreated net diverts extra biting to someone in the same room who is without a net (Stone et al., 2012). Many insecticides such as DDT and permethrin also influence behavioural changes in the insect—for example, by reducing the rate of mosquito entry into houses, and/or the rate of early exit from houses is increased and a shift in biting times is induced (Lines et al., 1984; Miller et al., 1991; Mbogo et al., 1996; Mathenge et al., 2001).

2.7.2 Target site resistance

There are three major target sites for most insecticides: the GABA receptor is the target of cyclodiene insecticides, the voltage-dependent sodium channel is the target site for DDT and pyrethroids, and acetylcholinesterase (AChE1) is quasi-irreversibly inhibited by organophosphorous and carbamate compounds, which are substrate analogues (Brogdon, 1987).

2.7.2.1 Knock down resistance (*kdr*)

Pyrethroids act on the insect nervous system by altering the normal function of the *para*-type sodium channel, resulting in prolonged channel opening that causes increased nerve impulse transmission, leading to paralysis and death (Soderlund and Bloomquist, 1989; Narahashi, 1992). Resistance to pyrethroids is caused by point mutations in the *para*-type sodium channel gene, which result in reduced neuronal sensitivity. This resistance mechanism was first identified in the house fly *Musca domestica* and was termed knock down resistance or *kdr* (Busvine, 1951). *kdr* is a well-characterized mechanism of resistance to pyrethroid insecticides in many insect species and is caused by point mutations of the pyrethroid target site the *para*-type sodium channel (Bass *et al.*, 2007). The *kdr* mutation involves a replacement of leucine at position 1014 in transmembrane segment 6 of domain II of the sodium channel (Williamson et al., 1996). Two forms exist: a leucine–phenylalanine substitution (1014F) of the voltage-gated sodium channel which is associated with resistance to permethrin and DDT in many insect species, including *An. gambiae* from West Africa, referred to as the West African *kdr*. The other is a Leucine-Serine substitution (1014S) at the same position, referred to as the East African *kdr* (Ranson et al., 1997; Martinez-Torres et al., 1998). The *kdr* has also recently been observed in *An. arabiensis* (Diabate *et al.*, 2004).

The East African *kdr* mutation in *An. gambiae* s.s. has been observed widely in western Kenya and has been associated with the presence of DDT resistance and pyrethroid tolerance observed in the region. The pharmacological effect of DDT and pyrethroids is to cause persistent activation of the sodium channels by delaying the normal voltage dependent mechanism of inactivation (Soderlund and Bloomquist, 1989), hence *kdr* fully or partially blocks the persistent activation of *para*-type sodium channel which would normally lead to

prolonged channel opening that causes increased nerve impulse transmission, leading to paralysis and death.

2.7.2.2 Modified Acetylcholinesterase

Acetylcholinesterase (AChE) is critical for hydrolysis of acetylcholine at cholinergic nerve synapses and is a target for organophosphate and carbamate insecticides (Rocheleau et al., 1995). Modified acetylcholinesterase (AchE), a major mechanism for organophosphate and carbamate resistance, can be identified using both biochemical (Hemingway and Karunaratne, 1998) and molecular assays (Weill *et al.,* 2003). In Africa, propoxur resistance was first detected in a population of *An. gambiae* Giles from Co^te d'Ivoire (Elissa et al., 1994). Insensitive AChE1 was next confirmed as the resistance mechanism (N'Guessan et al., 2003). Two forms of target site resistance have been demonstrated in mosquitoes, that is, knock down resistance (*kdr*) and Insensitive Acetylcholinesterase (Ace1r).

2.7.3 Metabolic resistance

The enzymes responsible for detoxification of xenobiotics in living organisms are transcribed by members of large multigene families of esterases, oxidases, and glutathione S-transferases (GST). The most common resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize a wide range of insecticides (Brogdon and McAllister, 1998). Esterase, GST and the target site mechanism (*kdr*) occur in areas where bed-nets are used (Elissa et al., 1993; Vulule et al., 1994; Vulule et al., 1999). Since bed-nets are currently the most potentially sustainable vector control measure against anophelines in developing countries, it is imperative that the dynamics of detoxification enzyme activation and selection are understood. While much has been studied in knock down resistance genotyping, less is known of detoxification genes expressed in resistant *An. arabiensis.* Three enzyme families are involved in metabolic-based insecticide resistance mechanisms: the

cytochrome P450 monooxygenases (P450s), the glutathione-S-transferases (GSTs), and the carboxylesterases (COEs) (Hemingway and Karunaratne, 1998; Hemingway, 2000; Hemingway and Ranson, 2000; Vontas et al., 2001). Given the genetic redundancy present in superfamilies of genes involved in insecticide metabolism, it is possible that alternative routes of detoxification may have been selected for in different mosquito populations (David *et al.*, 2005). There is need to develop accurate PCR-based methods for detection of metabolic resistance just as are available for target site resistance (Bass *et al.*, 2007). Before equivalent assays can be developed for metabolic resistance mechanisms, candidate genes must be identified, and their role in insecticide metabolism must be verified. Microarray techniques have taken the analyses of insecticide resistance mechanisms to genome-wide expression profiling (David et al., 2005; Vontas et al., 2007). A microarray is a multiplex 2 dimensional array on a silicon chip onto which large amounts of biological material are assayed using high throughput screening miniaturized, multiplexed and parallel processing and detection methods. *An. gambiae* s.s. has been investigated in a few studies using the microarray technique (Muller et al., 2007; Djouaka et al., 2008; Müller et al., 2008; Mitchell et al., 2012).

Previous studies in Kenya have looked extensively at *kdr* as a marker for resistance (Kawada et al., 2011a; Kawada et al., 2011b; Mathias et al., 2011; Ochomo et al., 2013), infact one study (Stump *et al.*, 2004) had indicated *kdr* as a suffient marker for estimation of resistance levels. While these studies were able to observe presence of *kdr* in vector populations, a direct correlation between *kdr* and resistance has not been observed indicating that *kdr* may not be a proper marker of resistance. As such, the current study explored and identified genes over expressed in resistant versus susceptible mosquitoes.

2.8 Efficacy of insecticide treated nets

Ownership and use of ITNs within households, as measured by the number of children less than 5 years of age reported to have used an ITN the previous night, increased by three to ten times between 2000 and 2008 in many African countries (Baume and Marin, 2008; Macintyre et al., 2011). The distribution of ITNs in malaria endemic areas of Kenya has increased net ownership up to 70% for any net and up to 60% household ownership for ITNs (DOMC, 2010). All mosquito nets act as a physical barrier, preventing access to vector mosquitoes and thus providing personal protection against malaria to the individual(s) using the nets (Curtis et al., 1990). Pyrethroids used to treat the ITNs, have an exito-repellent effect thus adding a chemical barrier to the physical one (Curtis *et al.,* 1990; Miller *et al.,* 1991). Insecticides incorporated in the ITNs kills the malaria vectors that come into contact with it and when used by a majority of the target population, may provide protection for the community including those who do not themselves sleep under an ITN (Binka et al., 1998; Takken, 2002; Hawley et al., 2003b). With the emergence and sudden increase in the frequency of insecticide resistance, it is likely that the ability of insecticides incorporated ITNs to kill mosquitoes may deteriorate. Whether insecticide resistance protects mosquitoes against the lethal effects of insecticides incorporated in the net fabric remains unknown. As such, the current study investigated the association between insecticide resistance and the ability of mosquitoes to rest inside nets.

In western Kenya, malaria transmission in the lowland areas around Lake Victoria has historically been very high, with entomological inoculation rate (EIR) estimated to be as high as 300 infectious bites per person per year (Beier et al., 1990; Beier et al., 1994; O'Meara et al., 2010). *An. gambiae* s. l. and *An. funestus* densities declined markedly in a randomized evaluation trial of permethrin- treated bed nets in treatment compared to control villages in western Kenya (Gimnig et al., 2003a; Lindblade et al., 2006). Following the large scale trial,

ITNs have been scaled up through multiple distribution channels. Between 2003 and 2007, demographic surveillance indicated a 42% reduction in all-cause mortality among children less than 5 years of age coinciding with a scale up of ITNs as well as improvement in diagnostics and introduction of Artemisinin-based combination therapy ACTs (Adazu *et al.*, 2008). The same period saw a decline in the proportion of *An. gambiae* s.s., the principal vector of malaria, relative to *An. arabiensis* (Bayoh *et al.*, 2010).

Resurgence in parasite prevalence and malaria vectors have been observed in several sites in western Kenya despite a high ownership of ITNs (Zhou *et al.*, 2011). This resurgence could be due to one or a combination of the following factors: reduced efficacy of ITNs, insecticide resistant mosquitoes, improper use of ITNs, or stock outs of anti-malarial drugs (Zhou *et al.*, 2011). ITNs, in addition to killing mosquitoes via insecticide action, also function as a barrier to prevent bites. Holes in the net can undermine this function, particularly when residual insecticide concentrations in the net are too low to efficiently kill mosquitoes or when mosquitoes are resistant to the pyrethroid insecticides used to treat the nets (Norris and Norris, 2011). For example, in recent field studies in Bungoma district, an area of high pyrethroid resistance, blood-fed *Anopheles* vectors resting inside ITNs were observed, suggesting malaria transmission is maintained due to a combination of pyrethroid resistance and deteriorating ITNs (Ochomo et al., 2014). Given this scenario, the increased risk of malaria infection when sleeping under a holed ITN compared to an intact one remains unknown. As such, the current study investigated the malaria risk in children under 5 years of age that could be associated with sleeping under an ITN with holes versus one without holes.

2.9 Insecticide resistance and mosquito age

The initial meal choice of a large majority of female *An. gambiae* s.s. is human blood rather than plant sugar (Stone *et al.*, 2012). This means at any one time, both young freshly emerged adults and older multi-parous mosquitoes will seek blood meals and therefore come

into contact with insecticide treated interventions such as LLINs, IRS and wall linings. It has been observed that younger mosquitoes are less susceptible to insecticides as compared to older mosquitoes (Rowland and Hemingway, 1987; Lines and Nassor, 1994; Rajatileka et al., 2011) and is a probable reason why WHO recommends the use of 1-3 day old mosquitoes in insecticide resistance diagnostics and monitoring (WHO, 1981; WHO, 1998; WHO, 2013a). Whether this difference in mosquito ages influences susceptibility to insecticides in actual field conditions remains undetermined. As such, this study assessed the age modulated insecticide resistance status of *Anopheles* populations of Ahero, Asembo, Teso and Bungoma in western Kenya.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study sites

This multifaceted study was conducted in five sites in western Kenya, Bungoma district in Bungoma County, Rarieda, Gem and Bondo districts in Siaya County, Teso North and Teso South districts in Busia County, Rachuonyo district in Homa Bay County and Nyando district in Kisumu County depending on the objective of study (Figure 1).

3.1.1 Bondo district is located in Siaya County. The district has a population of 309,190 (KNBS, 2010) and has recently been split into two, Bondo and Rarieda districts. For this study, Bondo refers to the greater district with both Bondo and Rarieda districts. The district borders Siaya to the North, Gem district to the East and Lake Victoria to the South. The altitude of the district rises from 1,140m in the eastern parts to 1,400m above sea level in the west. River Nzoia and Yala traverse the district and enter Lake Victoria through the Yala Swamp. The district experiences bimodal rainfall. The relief and the altitude influence its distribution and amount. On the highlands, the rainfall ranges between 800mm and 2000mm. The lower areas receive rainfall ranging from 800 and 1600mm. The long rains occur between April and June while the short rains occur between August and November. The mean minimum and maximum temperatures are 15°C and 30°C, respectively. Humidity is relatively high with mean evaporation being between 1800mm to 2000mm per annum. Malaria is a major problem in this district with a prevalence of about 40% in 2009 (Hamel et al., 2011a). Bondo district has had long term use of bednets with net distribution having started in the early 2000s in some parts of the district. The early intervention with the bednets led to a drastic decline in *An. funestus* s.l. and a shift from a largely *An. gambiae* s.s. population to a largely *An. arabiensis* population (Bayoh *et al.*, 2010), currently *An. funestus* s.l. have re-emerged in the district (McCann *et al.*, 2014).

3.1.2 Gem district is also located in Siaya County, bordering Siaya district to the North, Bondo district to the west, Vihiga district to the east and Kisumu district to the south. Residents of Gem are primarily subsistence farmers growing food crops. The district has an altitude averaging 1070m above sea level and average rainfall about 1200mm. The district has a malaria prevalence rate of about 25% and an ITN coverage of 80% (Okiro et al., 2010). KEMRI/CDC is currently carrying out a study to evaluate the longevity of 7 different types of LLINs in the district.

3.1.3 Bungoma district is located in Bungoma county and has a total population of 1,2020,646 (KNBS, 2010). The district borders Kakamega district to the south, Teso district to the south west, Uganda to the North West. The economy of Bungoma County is mainly agricultural, centering on cash crops such as sugar cane and tobacco as well as horticulture and cereal farming. The district averages an altitude of 1,385m above sea level with annual rainfall averaging 1800mm. The district is endemic for malaria with prevalence of between 35 and 40% among children under 5 years of age (Okiro *et al.,* 2010). The first universal coverage with LLINs was done in 2011 but has not received IRS.

3.1.4 Rachuonyo District is located in Homabay County and has a total population of 400,802 (KNBS, 2010). The district borders Lake Victoria to the North, Nyando district to the North East, Kisii district to the South and Homabay district to the West. The altitude of the district is around 1300m above sea level with an annual rainfall around 1700mm. The temperature ranges between 15 and 30° C. This district is also endemic for malaria with a prevalence of 26% (Unpublished data) and has received bednets through the national programme for over a decade now and IRS from 2007 with coverage of >80% for LLINs and >90% for IRS. Little has been published on the malaria vectors within this district but *An. arabiensis* and *An. funestus* are the most abundant (Bayoh *et al.,* unpublished).

3.1.5 Nyando district is in Kisumu county and has a population of 389,351 (KNBS, 2010). The district is located on the northeast shore Lake Victoria, the Gulf of Winam. It is bordered to the North by Nandi district, to the east by Kericho district, to the South by Rachuonyo and Kisumu district to the West. The areas along the Nyando plateau have an altitude as low as 1100m above sea level and are prone to flooding. Other areas are as high as 1540m above sea level. Rice farming is a major economic activity and provides conducive breeding habitats for *An. arabiensis* and *An. funestus* mosquitoes which drive transmission. This district has had long term intervention with bednets, the last mass campaign being in 2011 and more recently IRS in the same year (PMI, 2011; WHO, 2013b). Malaria prevalence in children under 5 years in this district is about 40% while bednet coverage is more than 85% (unpublished data).

3.1.6 Teso district is located in Busia county and has a population of 252,884 (KNBS, 2010). The district has been split into Teso North and Teso South districts and borders Busia district to the South West, Bungoma district to the East, and Tororo district in Uganda to the North. The average altitude of the district is 1208m above sea level. Trade is a major economic activity alongside subsistence farming. Malaria is a endemic in this district with a prevalence of 32% (unpublished data). Bednets have been distributed in this district with the aim of universal coverage (2 people per bednet) and as such are the main intervention against malaria vectors (PMI, 2011). *An. gambiae* s.s is the major vector of malaria but *An. arabiensis* and *An. funestus* s.l. are also present but in lower proportions.

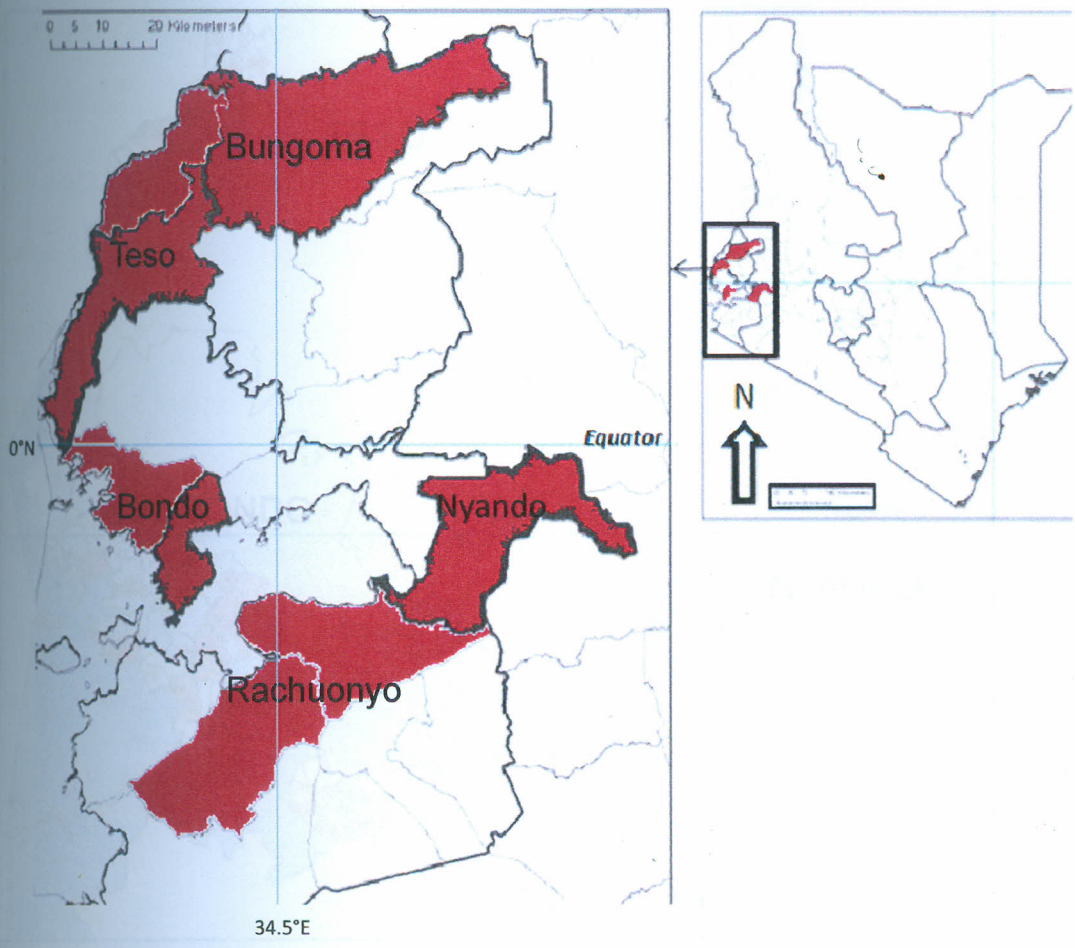


Figure 1: Map of the study sites showing the study districts on the left and the map of Kenya on the right. Source-KEMRI/CDC, Kisumu.

3.2 To compare variations in insecticide resistance and identify genes over expressed in pyrethroid resistant versus susceptible Anopheles mosquitoes from Western Kenya

A baseline insecticide resistance survey was conducted in 4 malaria endemic districts (Bondo, Rachuonyo, Nyando and Teso) in western Kenya. There were 2 distinct vector control interventions implemented in the districts: Rachuonyo and Nyando: IRS combined with LLINs; Bondo and Teso: LLINs only (Figure 2). In each district, 20 sub-locations (clusters) were randomly selected for baseline resistance determination. In Kenya, sub-locations are the smallest administrative units and are composed of multiple households. Each sub-location can have as many as 10-30 villages each having about 100 households.

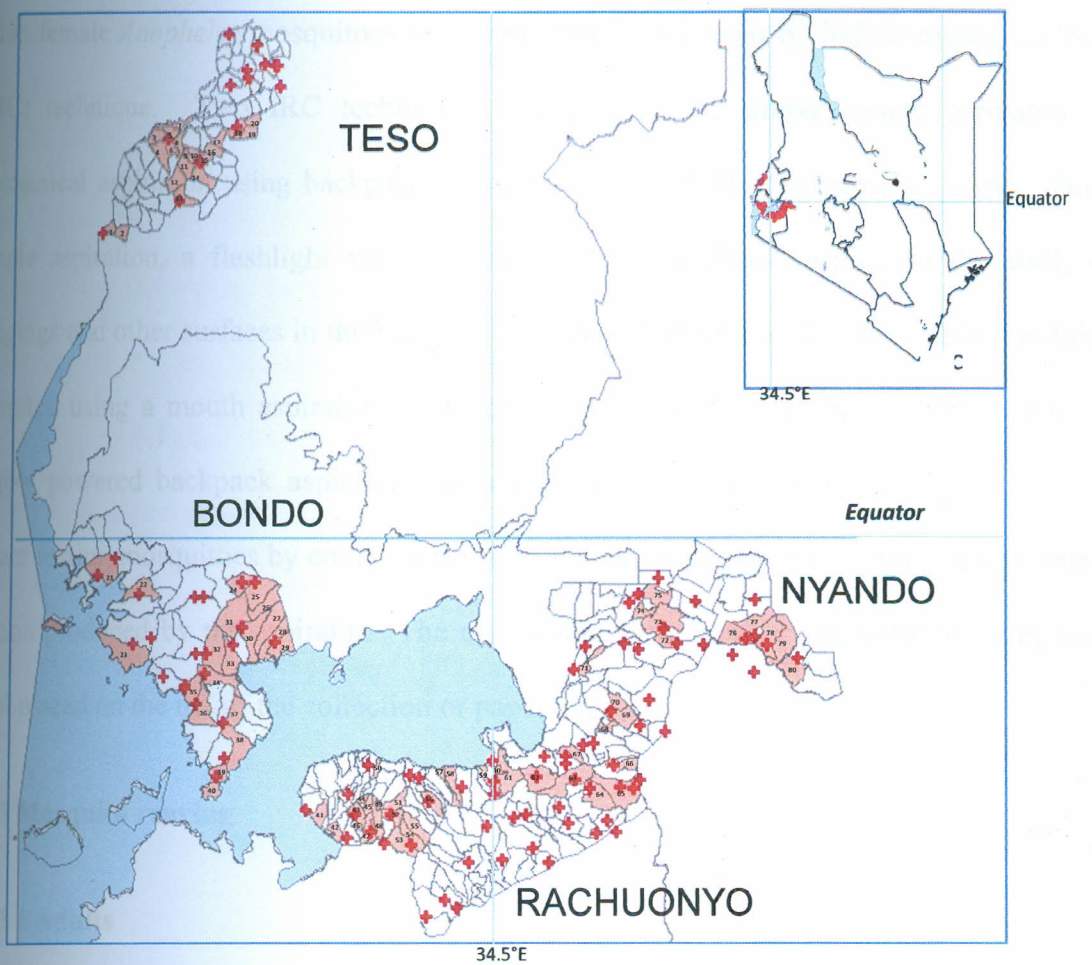


Figure 2: Map of Kenya showing the study Districts (right) and the specific study Districts with study clusters highlight in orange. The red crosses represent health facilities.

3.2.1 Larval sampling

Dippers were used to collect *Anopheles* larvae from a variety of larval habitats around health facilities including ponds, potholes, tire tracks, rice fields, drainage channels and mud paths. Individual larvae were picked from the dippers using wide-mouthed plastic pipettes and placed in plastic tins to be transported back to the laboratory at KEMRI-CGHR, Kisumu for rearing.

3.2.2 Indoor resting collections

Adult female *Anopheles* mosquitoes were collected from houses by indoor resting collection (IRC) technique. Two IRC techniques were employed; simple mouth aspiration and mechanical aspiration using backpack aspirators (John W. Hock Company, USA). During simple aspiration, a flashlight was used to locate mosquitoes resting on the wall, wall hangings and other surfaces in the house. Once spotted, the mosquito was gently but quickly aspirated using a mouth aspirator and placed in a paper cup. In mechanical aspiration, a battery powered backpack aspirator was used. Backpack aspirators were used to collect indoor resting mosquitoes by combing walls, roofs and other potential resting places with the suction produced by the aspirator. The mosquitoes were given sugar solution using cotton wool placed on the net of the collection or paper cup.

3.2.3 Mosquito rearing

3.2.3.1 Adults

Once samples were obtained from the field, the females were sorted based on species (*An. gambiae* s.l. and *An. funestus* s.l.) and on abdominal status. Single females (gravid, half-gravid or freshly fed) were placed in labelled oviposition cups containing laying pads made of moist cotton wool covered with filter paper. Once females had laid eggs, they were removed from the laying cup and desiccated in preparation for species identification by PCR so that the resulting *F1* generation could be put together in cages of the same species. Only larvae of the *An. gambiae* s.s. species were raised further. To ensure maximum hatch rate, the laying pads with eggs were transferred to a hatching bowl. Hatched larvae from each cup were transferred to a larval tray with same sample label and fed on a mixture of brewer's yeast and fish food. On pupation, all pupae from the same larval tray were transferred to the same pupal cup. Pupal cups contained water and were placed inside individual paper cups for

emergence. Both pupal cups and paper cups were labelled with the sample label. Freshly emerged adults were fed on 5% sugar solution until ready for bioassays at 3 days.

3.2.3.2 Larvae

Once larvae were brought back from the field, they were poured into larger trays and debris removed from the water. Larval samples from each collection site were pooled together and sorted according to instar stage. Similar instar stages were transferred to the same larval tray. All the trays were labelled with collection date and site. Separation of instar stages continued every 2 days. Upon pupation, pupae were collected and placed in pupal cups inside a labelled cage for emergence. Each day emerged adults were removed and placed in a new cage with sample ID and date of emergence. These were provided with 5% sugar solution as they awaited assay.

3.2.4 WHO tube Bioassays

This is the WHO approved test for diagnosing resistance to insecticides in mosquito populations and involves the use of specially designed plastic tubes that are lined with insecticide impregnated papers (Science University of Malaysia, Malaysia). For this study, papers impregnated with 0.75% permethrin or 0.05% deltamethrin were used. Tubes were lined with treated papers in such a way that the part with the name of insecticide used and dosage indicated is visible. Adult mosquitoes were aspirated into the tubes and then exposed to the insecticide for one hour. Mosquitoes used in this assay were three day old sugar fed samples that have not had a blood meal according to WHO recommendations (WHO, 2013a) this is informed by the fact that this is the age at which female mosquitoes are likely to seek their first bloodmeal. A maximum of 25 mosquitoes were then aspirated into each tube and the timer set at one hour. The sample size required for WHO tube Bioassays is 100 per insecticide according to the guidelines though this is dependent on site specific mosquito

densities(WHO, 2013a). After the exposure mosquitoes were transferred from exposure tube to tubes with untreated paper. Mosquitoes were provided with 5% sugar water on a cotton pad and held at 25-27°C and 70-80% humidity and mortality scored after 24 hours.

3.2.5 DNA extraction

The DNA extraction protocol used was derived from the protocol as previously developed (Collins *et al.*, 1987) with a few modifications and is described below. Before starting the actual DNA extraction, a +65 °C waterbath was prepared. Reagent preparation is described in Appendix 1. The frozen samples were placed in individual sterile centrifuge tubes and crushed in 100µl of grinding buffer. Once ground, the samples were incubated at 65°C water bath for 30 minutes. This step denatures nucleases that would further degrade DNA and is the optimum temperature at which lysis buffer degrades cell membranes. A volume of 14 µl potassium acetate was then added and the samples vortexed to mix. The samples were then incubated in ice for 30 minutes during which tubes were labelled for transfer of the supernatant. Once incubation time was over, the samples were spun for 10 minutes at 13,200 rpm. Supernatant was then removed and placed in sterile vials. Cold absolute ethanol (volume of 200µl) was added and samples placed at -20°C for 20 minutes. A final spin was done for 20 minutes at 13,200 rpm to pellet the DNA and then vials washed first in 200 µl of 70% ethanol and then in the same volume of absolute ethanol. The tubes were then inverted to dry overnight. The samples were reconstituted in 100µl of TE buffer. This is important to remove any RNA that co-precipitated with DNA. DNA was stored at -20 °C until use.

3.2.6 Species Identification using PCR

Species were identified using the method proposed by Scott (Scott et al., 1993). The Master Mix was made up of: 5X GoTaq PCR buffer (500 mM KCl; 100 mM Tris-HCl (pH 8.3); 15 mM $MgCl_2$, primers: forward Universal primer (5`-GCT GCG AGT TGT AGA GAT GCG-3`), reverse *An. gambiae* primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3`) and the reverse *An. arabiensis* primer (5`-GCT TAC TGG TTT GGT CGG CAT GT-3'). Primers were obtained from DNA chemistry section, Biotechnology Core Facility Branch, Division of Scientific Resources, Centre for Disease Control and Prevention, Atlanta, USA. The reaction mixture was prepared in a single tube, which can was then aliquoted into individual tubes. The template DNA template was then added. The reaction mixture calculations described in Appendix 2. The cycling conditions were as follows; Initial denaturation: an initial heating for 10 minutes at 95°C to completely denature complex genomic DNA so that the primers can anneal to the template as the reaction mix is cooled; subsequent cyclic denaturation was done at 95°C for 30 seconds, annealing at 64°C for 30 seconds and extension at 72°C for 45 seconds. This was done for 35 cycles followed by a final extension at 72°C for 5 minutes to promote completion of partial extension products and annealing of single-stranded complementary products, after which amplified DNA was stored at 4°C until use. The amplified DNA was then loaded onto a 2% agarose gel stained with 3% ethidium bromide and electrophoresis run for 30 minutes. DNA bands were finally visualised on a UV illuminator.

3.2.7 *KDR* genotyping

Forward and reverse primers and three minor groove binding (MGB) probes (Applied Biosystems, TX, USA) were used for the Taqman 5' allelic discrimination assay. The exact sequences were: Primers: *kdr*-Forward (5'-CATTTTTCTTGGCCACTGTAGTGAT-3'), and *kdr*-Reverse (5'-CGATCTTGGTCCATGTTAATTTGCA-3') which are standard

oligonucleotides with no modification. The probe WT (5'-CTTACGACTAAATTTC-3') was labelled with HEX at the 5' end for the detection of the wild type allele, the probes *kdr*W (5'- ACGACAAAATTTC-3') and *kdr*E (5'-ACGACTGAATTTC-3') were labelled with 6-FAM for detection of the *kdr*-w and *kdr*-e alleles, respectively.

PCR reactions (10µl) contained 1.5 µl of genomic DNA, 5.0µl of 2x Taqman mix (ABI), 0.2µl of 10µM reverse and forward primers and 0.2µl of 10µM of wild-type probe and 0.175µl of 10µM East or West probe. Samples were run on a Stratagene Mx 3005P QPCR machine with MxPro QPCR software using the following temperature cycling conditions: 10 minutes at 95°C followed by 45 cycles of 95°C for 25 seconds and 64°C for 1 minute. The increase in HEX and FAM fluorescence were monitored in real time by acquiring each cycle on the green (535 nm excitation and 555 nm emission) and blue channel (492 nm excitation and 516 nm emission) of the Stratagene, respectively.

3.2.8 Microarray analyses of insecticide resistance in An. arabiensis from Bondo and Nyando districts

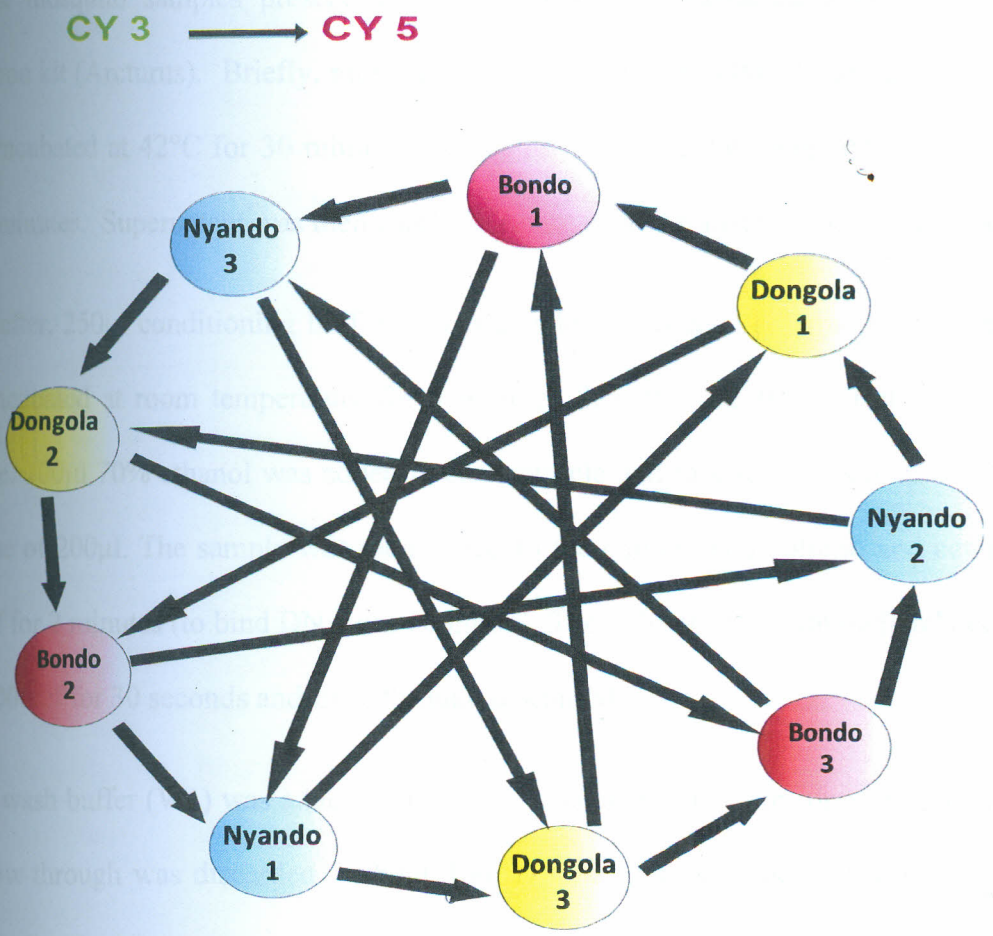
3.2.8.1 Microarray sample selection and analyses

Due to lack of a parapatric susceptible *An. gambiae* s.s. population, *An. arabiensis* was selected for the microarray analyses. Based on the bioassay data, where Bondo had the highest proportion of resistant *An. arabiensis* and Nyando the lowest, *An. arabiensis* from Bondo were selected as the resistant group while those from Nyando formed the parapatric susceptible population with the Dongola lab strain originating from Sudan being used as the lab susceptible control. A 3-day old *An. arabiensis* adults raised from larval collections in study clusters of Bondo and Nyando were exposed to 0.05% Deltamethrin using the WHO tube bioassay method, surviving mosquitoes from Bondo and knocked down individuals from Nyando were kept in RNAse free microfuge tubes and preserved in RNA later (Ambion, TX,

USA) and frozen at -80°C. Assays could only be done to one insecticide and Deltamethrin was chosen because it has been used for Both ITNs and IRS in western Kenya while Permethrin has only been used for ITNs. Individual mosquitoes' legs were pulled out and DNA was extracted using the Alcohol precipitation method described in section 3.2.5 followed by species identification PCR as described in section 3.2.6.

3.2.8.2Microarray Design

This study utilised the Agilent 8 × 15 K *An. gambiae* microarray design (Mitchell et al., 2012). The array contains 14,071 probes for the 12,604 *An. gambiae* genes identified in the Ensembl P3.5 annotation (September 2009). For genes with alternative splice variants, separate probes target individual variants. An additional three unique probes per gene were designed for the 281 insecticide-resistance candidate gene sequences from the *An. gambiae* detox array (David et al., 2005): thus, detoxification candidates were covered by four separate probes. Total RNA was extracted from pools of 10 mosquitoes which were selected above. The interwoven loop design was used to perform the comparisons (Figure 3).



Each pool, indicated by a circle, represents RNA extracted from 10 female *An. arabiensis* mosquitoes that were 3–5 day old. Arrows indicate individual microarrays (18 in total), with direction representing microarray Cy dye labelling

Figure 3: Interwoven microarray experimental loop design for a comparison between deltamethrin-resistant field-collected *An. arabiensis* from Bondo, a parapatric susceptible field-collected *An. arabiensis* from Nyando and a laboratory colony of *An. arabiensis* originating from Dongola in Sudan that was fully susceptible to deltamethrin.

3.2.8.3 RNA extraction

Whole mosquito samples preserved in RNALater were extracted using PicoPure RNA isolation kit (Arcturus). Briefly, mosquitoes were ground in 100µl of extraction buffer (XB) then incubated at 42°C for 30 minutes then centrifuged at room temperature at 32,000rpm for 2 minutes. Supernatant was then carefully removed and transferred to a new tube.

Thereafter, 250µl conditioning buffer (CB) was added into each column onto the membrane and incubated at room temperature for 5 minutes thereafter centrifuged at 16,000rcf for 1 minute. 100µl 70% ethanol was added to each sample and mixed by pipetting to give a total volume of 200µl. The sample was then added to the conditioned column and centrifuged at 100rcf for 2 minutes (to bind DNA onto column). The columns were immediately centrifuged at 16,000rcf for 30 seconds and flow-through discarded.

100µl wash buffer (W1) was added and column spun at 16,000 rpm for 1 minute after which the flow-through was discarded. About 40µl DNase was then added directly onto column and incubated at room temperature for 15 minutes. Once the incubation period had elapsed, 40µl of buffer W1 was added onto column and spun at 16,000 rpm for 15 seconds and flow-through discarded. 100µl wash buffer 2 (W2) was then added and spun at 16,000 rpm for 1 minute and flow-through discarded. Finally, 100µl wash buffer 2 (W2) was added and column spun at 32,000 rpm for 2 minute and flow-through discarded then spun again at 32,000 rpm for 1 minute. The column was then placed into a new 0.5ml tube and 30µl elution buffer (EB) added onto the membrane and incubated at room temperature for 1 minute at 32,000 rpm to distribute EB onto column.

The total RNA was then kept on ice and 1µl RNA stored for NanoDrop (Table 1) and 2µl stored separately for the Bioanalyzer (Table 2). RNA concentration was estimated using NanoDrop at setting 40 (RNA) with EB as a blank and concentration of RNA (ng/µl) recorded at 260/280 ratio and concentration of protein impurities at 230/280 ratio (Table 1).

Tubes were then labelled clearly and stored at -80°C. Before labelling of RNA for micriarrays, the yield of RNA (260/280 ratio) was used to calculate the volume required for use in microarray experiments, where all concentrations were diluted or concentrated by centrifugation to 100 ng/µl.

Table 1: Nanodrop readings of RNA extracted from Bondo and Nyando samples for microarrays

Group	Population	Lab Code	260:280	230:280
Resistant	Bondo	B11	116.9	2.19
		B12	68.6	2.18
		B14	123.2	2.18
Parapatric Susceptible	Nyando	B21	56	2.09
		B22	105.7	2.11
		B23	61.1	2.09

3.2.8.4 Procedure for Bioanalyser evaluation of RNA samples

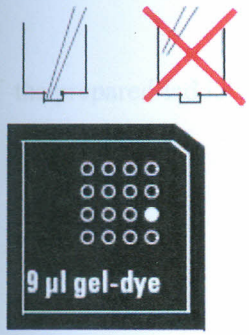
About 25-500ng of the RNA sample was analyzed for quality before proceeding with microarray labelling as the degradation of RNA is not detectable via NanoDrop readings using the Agilent 2100 bioanalyzer.

The dye concentrate (blue lid) and filtered gel aliquot (65µl) were removed from the fridge and equilibrated in the dark to RT for 30 minutes. Once equilibrated the dye mix (blue lid) was vortexed for 10 seconds and spun down. About 1µl of dye was added to the 65µl gel aliquot, mixed thoroughly by vortexing and centrifuged for 10 minutes at 14,000rpm. The tube was covered in foil to protect from light and stored at RT until required. Gel dye mix was used within one day.

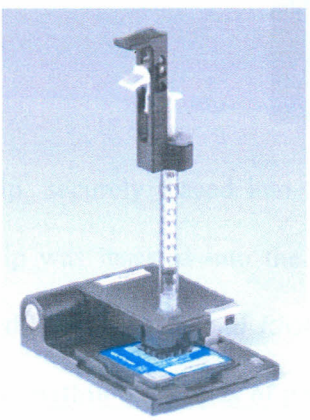
Meanwhile RNA aliquots and a ladder aliquot were collected from the -80°C freezer and placed on ice. Samples were diluted as necessary, with water to achieve concentration within the range (50-500ng) then denature in a thermocycler for 2 minutes at 70°C and placed back on ice.

Bioanalyzer electrodes were decontaminated by pipetting 350µl of RNAse Zap into the labelled wash chip, 350µl of RNAse free water was then added to the other chip. The lid was opened and the RNAse Zap filled chip placed into the bioanalyzer, then timed for one minute. This was then removed and the water filled chip inserted into the machine, timed for ten seconds, lid opened and chip removed leaving lid open for ten seconds to allow electrodes to dry.

The computer and bioanalyzer were switched on and the 2100 expert software opened. A new chip was placed into the chip priming station and 9µl of the gel-dye mix pipetted into the well-marked ⓖ ensuring the pipette tip is inserted straight to the bottom of the well as below left.



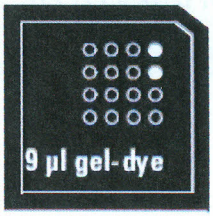
The timerwas then set for 30 seconds then the station and the plunger pressed until it is held by the clip as shown below.



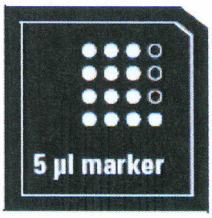
After 30 seconds the clip was releaed and the plunger will moved towards the 1ml position.

After 5 seconds the plunger was slowly pulled back to the 1 ml postion and priming opened.

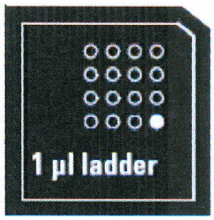
Exactly 9 µl of the gel dye mix was pipetted into wells marked G as below.



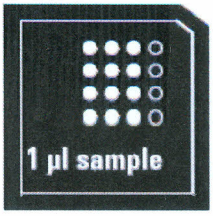
Then 5µl of the nano-marker was pipetted (green lid) into all 12 sample wells and into the ladder well marked .



Then 1µl of the prepared ladder was into well marked .



Finally 1µl of each sample was Pipetted into wells 1-12.



The timer was set for 1 minute and the chip securely placed into the IKA vortexer then Vortexed at 2,400 rpm for 60 seconds. Chip was inserted into the bioanalyzer and *Start* clicked ensuring the correct assay is selected – *Eukaryote Total RNA nano* is sufficient for examining total RNA. **The chip had to be run within 5 minutes of preparation.**

While the chip is running sample names and information was added to the data file by clicking on the *Data* tab and typing next to the appropriate wells. Once the run is finished the chip was removed and the electrode decontamination step repeated.

Below is a typical trace for **good quality mosquito total RNA**: the 26S is cleaved resulting in triple peaks around the 18S region, there is also a relatively flat line between 200 and 1500nt indicating little degradation of the sample.

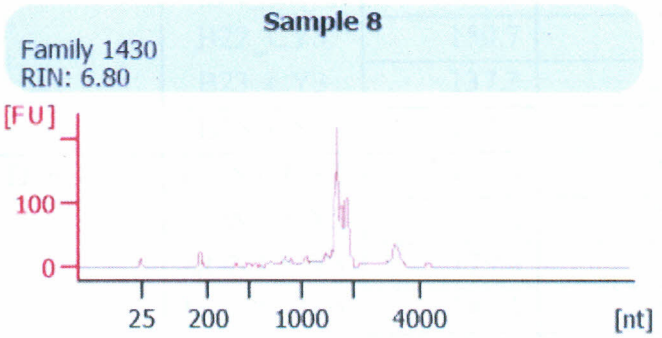


Table 2: Bioanalyser results of CY3 and CY5 bye labelled RNA for microarrays

Group	Population	Lab Code	260:280	230:280
Resistant	Bondo	B11_CY3	118.5	2.29
		B11_CY5	65.7	2.35
		B12_CY3	62.4	2.25
		B12_CY5	113.2	2.32
		B14_CY3	143.2	2.34
		B14_CY5	109.3	2.29
Parapatric Susceptible	Nyando	B21_CY3	179.9	2.29
		B21_CY5	165.7	2.32
		B22_CY3	295.7	2.31
		B22_CY5	150.7	2.34
		B23_CY3	137.3	2.32
		B23_CY5	140.3	2.32
Lab Susceptible	Dongola	DON1_CY3	190.8	2.31
		DON1_CY5	252.2	2.32
		DON2_CY3	179.4	2.3
		DON2_CY5	145.1	2.32
		DON3_CY3	144.4	2.31
		DON3_CY5	319.1	2.32

3.2.8.4 Microarray Hybridization and Analysis

The RNA pools selected for microarray analysis were labelled and purified separately with Cy3 and Cy5 dyes using the Low Input Quick Amp Labelling Kit (Agilent Technologies, USA) (Appendix 2) Only 1.5 µl of 100ng/µl RNA was used for each experiment. Labelled RNA quantity and quality were assessed using a NanoDrop spectrophotometer and a Bioanalyzer (Table 2). All samples passed Agilent recommendations for yield greater than 825 ng and specific activity greater than 6.0 pmol of cynanine (Cy) per microgram of cRNA. Array hybridization, washing, scanning, and feature extraction were performed according to the manufacturer's recommendations (Agilent Techlnologies, USA) (Appendix 3). All arrays passed the Agilent quality control thresholds (QC score ≥ 10). Microarray normalization was performed in the statistical program R using Limma 3.2.3 (Smyth, 2005), as described previously (Muller *et al.,* 2008). Analysis of normalized signal intensities was performed using the MAANOVA package in R (Wu et al., 2003). In brief, because there were three treatment groups, an ANOVA F-test approach was applied. The significance level for the FDR-corrected data (Benjamini and Hochberg, 1995) was set at log10(Q value) > 4 ($q < 0.0001$). Within this subset of differentially expressed probes, those that were significantly over expressed in the deltamethrin-resistant group were identified by examining expression patterns in all pair-wise comparisons. Deltamethrin was chosen due to its use in both ITN and IRS programs.

3.3 Assessing the effect of holes on ITN involvement in reduction of malaria transmission in two sites in western Kenya with emerging insecticide resistance

3.3.1 Bed net cross-sectional survey

Community-based, cross-sectional surveys were carried out in the two study sites. Sampling was done in all the houses in the selected villages in Bungoma and Gem. The surveys were conducted in all houses in the selected villages since mosquito densities are variable even within the same village and random sampling would have either under or over estimated mosquito numbers. In total, 303 houses were sampled in Bungoma and 179 in Gem. The surveys consisted of interviews with household heads and an inspection of all nets in the house. Interviews were conducted using a structured questionnaire administered on personal data assistants (PDAs, Dell Axim X50, Dell Inc., Dallas, TX, USA) and the collected information on the household characteristics included wall type, roof type, whether eaves were open or closed, insecticide use and application within the household, frequency of net use, the number of people who used the nets the previous night and the brands and ages of the nets. The interviewers examined the nets for the presence of mosquitoes resting inside them using torches, mosquitoes outside the nets were not sampled in this study (Appendix 4). Any mosquitoes observed were collected using a mouth aspirator, transferred into a paper cup and labeled with the house ID and net type. The samples were placed in a cool box and maintained on a 10% sugar solution for transport to the insectary. The interviewers then examined each net and recorded the presence, number and size of holes. Hole sizes were categorized using methods recommended by the WHO Pesticide Evaluation Scheme: the thumb was used to estimate hole sizes ≤2 cm in diameter (small), holes larger than the thumb but smaller than the fist were estimated to be between 2 and 10 cm (medium), while those larger than the fist were estimated to be ≥10 cm (large). Holes that fell within the largest hole

size category (>25 cm) recommended by WHOPES (WHO, 2011a) were recorded as large holes (≥10 cm).

3.3.1.1 Mosquito rearing

Mosquitoes were reared according to the protocol described in (Mathias *et al.*, 2011) where, female mosquitoes were sorted based on species (*An. gambiae* s.l. and *An. funestus* s.l.) and on abdominal status. Fed and half-gravid samples collected from the nets were maintained on 10% sugar solution at the KEMRI/CDC insectary until they became gravid. Gravid mosquitoes were placed in oviposition cups containing laying pads made of moist cotton wool covered with filter paper. Mosquitoes were pooled into 34 oviposition cups with one to five females per cup. To ensure maximum hatch rate, once the females laid eggs, the laying pads with eggs were transferred to a hatching bowl. Hatched larvae from each cup were transferred to a larval tray. Larvae were fed daily on a mixture of brewer's yeast and fish food, and their water changed every two days. Pupae from the same larval tray were transferred to the same eclosion cup and placed inside individual paper cups for emergence. Freshly emerged adults were fed on 5% sugar solution for three days after which they were ready for bioassays. In addition, mosquitoes were collected as larvae from each site and reared until three days old adults as described earlier in this section.

3.3.1.2 Susceptibility testing

To assess susceptibility to insecticides, field collected mosquitoes (three days old) were exposed to permethrin (0.75%) and deltamethrin (0.05%) for 1 hour using WHO tube tests (WHO, 2013a) as described in section 3.2.4 above. Three sets of samples were exposed: *F1* offspring of the *Anopheles* samples collected inside mosquito nets from Bungoma and adult samples from larval collections in Bungoma and Gem.

3.3.1.3 Molecular assays

Conventional polymerase chain reaction (PCR) was used to distinguish between the two sibling species of the *An. gambiae* species complex native to western Kenya, *An. gambiae* s.s. and *An. arabiensis* (Scott *et al.,* 1993) as described in section 3.2.6. Further, the mosquito samples were tested for the presence of the 1014S *kdr* genotype using the methods previously described (Bass et al 2007) as described in section 3.2.7. Detailed procedure is described in section 3.2.7 above. Sporozoite ELISA was conducted on the heads and thoraces of all the collected female mosquitoes briefly: the capture monoclonal antibody (Mab) was added to the wells of a microtiter plate and incubated at room temperature for 1 hour. Once bound to the plate, the well contents were aspirated and the remaining binding sites blocked with blocking buffer. Mosquitoes to be tested were ground in blocking buffer containing IGEPAL CA-630. Positive and negative controls were also added to specific plate wells at this time. If CS antigen is present it would form an antigen-antibody complex with the capture Mab. After a 2-hour incubation at room temperature, the mosquito homogenate was aspirated and the wells are washed. Peroxidase-linked Mab is then added to the wells, completing the formation of the "sandwich". After 1 hour, the well contents were aspirated, the plate is washed again and the clear peroxidase substrate solution added. As the peroxidase enzyme reacts with the substrate, a dark green product was formed, the intensity of the color being proportional to the amount of CS antigen present in the test sample. Results were read visually or at 405-414 nm using an ELISA plate reader 30 minutes after the substrate had been added (Wirtz *et al.,* 1987).

3.3.1.4 Bioefficacy of Long Lasting Insecticide treated Nets (LLINs) against pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes from Bungoma

From the nets collected in Bungoma in section 3.3.1 above, 68 of the nets with mosquitoes resting inside and 31 nets without were collected from the field and the owners provided with

new LLINs in exchange. The WHO cone bioassays using ten mosquitoes on five pieces cut from these nets, one piece per side and one from the top were performed using the susceptible *An. gambiae* s.s. Kisumu strain (WHO 2012). In addition, the progeny of females collected from inside nets were exposed to new, unused, unwashed PermaNet. 2.0 (Vestergaard Frandsen SA, Aarhus, Denmark) and Olyset (Sumitomo Chemicals, Osaka, Japan) as these were the primary net brands observed in the field. For all bioassays, mosquitoes were exposed in plastic cones for 3 min and then transferred to holding cages with access to 10% sucrose solution. Knockdown was recorded 60 min after exposure and mortality was recorded 24 hours after exposure.

3.3.2 Case control study

This study evaluated the effect of holes on ITN involvement in reduction of malaria transmission. The study participants were recruited from patients visiting the Kimaeti health centre in Bungoma. The rapid diagnostic kits (RDTs) were distributed to the study clinics prior to the study and health centre personnel trained on proper use and interpretation of the results. RDTs are approved by WHO for use in malaria diagnosis and are currently implemented by the Ministry of health as government policy because of the lack of microscopy in major rural setups (WHO, 2010a; PMI, 2011). In addition, WHO only approves RDTS with >80% sensitivity and specificity for use therefore the cases diagnosed in this study were at least 80% accurate (WHO, 2010a). The case included children aged less than 5 years with RDT confirmed malaria. The control arm was composed of children aged less than 5 years visiting the health centre presenting with ailments other than malaria. All children below the age of 5 visiting the facility were eligible for enrolment into the study. The sample size for participants in the case control study was determined using the formula for the method described in (Kelsey, 1996). Calculating sample size per arm for the population at 95% confidence interval, 80% power, with a 1.0 ratio of controls to cases at an

odds ratio of finding a damaged net amongst the cases versus controls of 2.0 assuming 20% of the controls have damaged nets, giving a total sample size of 173 participants per arm.

Sample size for participation was estimated at 173 persons per arm. Sample size estimates were done using the openepi calculator: (http://openepi.com/OE2.3/Menu/OpenEpiMenu.htm).

The parents or guardians of the children aged less than 5 years were consented by the study counsellor (Appendix 5). Once consent was obtained, the participants were followed to the home where a questionnaire was administered to obtain information on the net used by the study participant. Factors observed included the type of net and the age of the net along with other potential confounding factors the type of wall and roof of the house, presence or absence of open eaves, the number of other nets in the house and the number of other people living in the house net used, condition, age and vector presence (Appendix 6). All nets were checked for physical condition. Holes were counted and measured to determine their size. Each study participant's net was then collected along with the net from the matched case or control and carried back to the KEMRI/CDC laboratory for bioassays and chemical analysis and replaced with a new Permanet 2.0 LLIN. Finally a pyrethrum spray collection (PSC) was conducted in the household to estimate vector density.

3.3.2.1 Study design

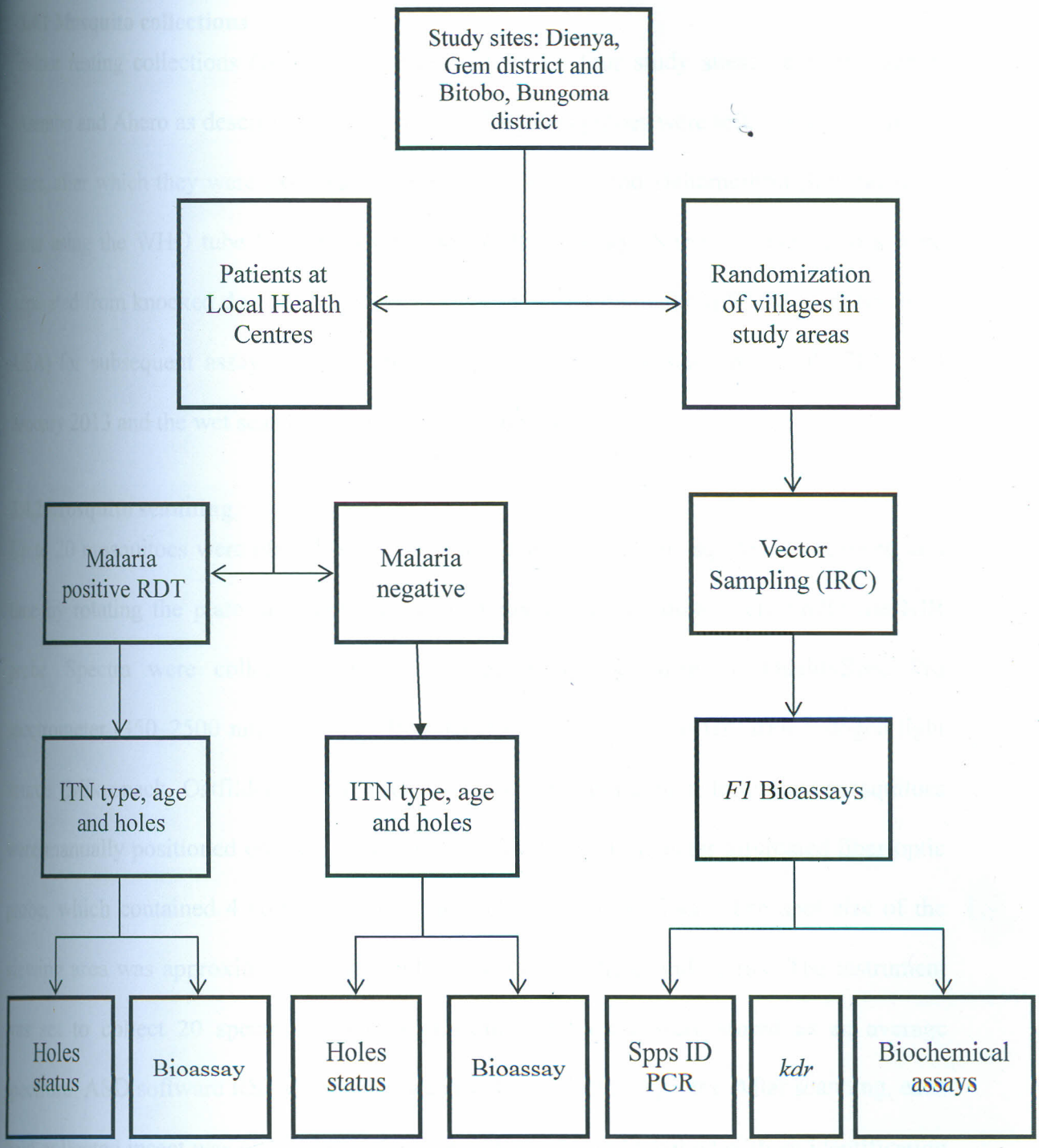


Figure 4: An illustration of the design of the vector survey that was conducted in Bungoma and Gem and case control study conducted in Bungoma.

3.4 Age modulated resistance in natural Anopheles populations in western Kenya

3.4.1 Mosquito collections

Indoor resting collections (IRC) were conducted in the four study sites: Teso, Bungoma, Asembo and Ahero as described in section 3.2.3. The mosquitoes were left to rest for 2 hours later, after which they were exposed to Permethrin (0.75%) and Deltamethrin (0.05%) for 1 hour using the WHO tube bioassay technique (WHO, 2013a). Surviving mosquitoes were separated from knocked down mosquitoes and preserved individually in RNALater (Ambion, USA) for subsequent assays. Collections were done in the dry seasons of July 2012 and January 2013 and the wet seasons of May and October 2012.

3.4.2 Mosquito scanning

Up to 20 mosquitoes were placed on a spectralon plate, and one mosquito was scanned at a time by rotating the plate until the head and thorax of the mosquito were under the NIR probe. Spectra were collected from individual mosquitoes using a QualitySpec Pro spectrometer (350–2500 nm; ASD Inc, Boulder, CO) (Figure 4). An HL-2000 halogen light source (Mikropack, Ostfildern, Germany) was used for illumination. Individual mosquitoes were manually positioned on their backs 2mm below a 3 mm-diameter bifurcated fiber-optic probe, which contained 4 collection fibers and 33 illumination fibers. The spot size of the viewing area was approximately 3mm and focused on the head and thorax. The instrument was set to collect 20 spectra from each mosquito and these were stored as an average spectrum. ASD software RS3 version 3.1 was used to collect all spectra. After scanning, each field-collected insect was placed in a 1.5ml eppendorf tube and labelled with an identification that corresponded to the spectrum number.

3.4.3 Data conversion

The "Asd to Spc" software was used to convert the Asd files to Spc files and the Grams IQ Predict software was used to estimate the ages of the mosquito samples using a

predetermined age grading calibration. Predictions from the age-grading calibration are the approximate age of each mosquito.

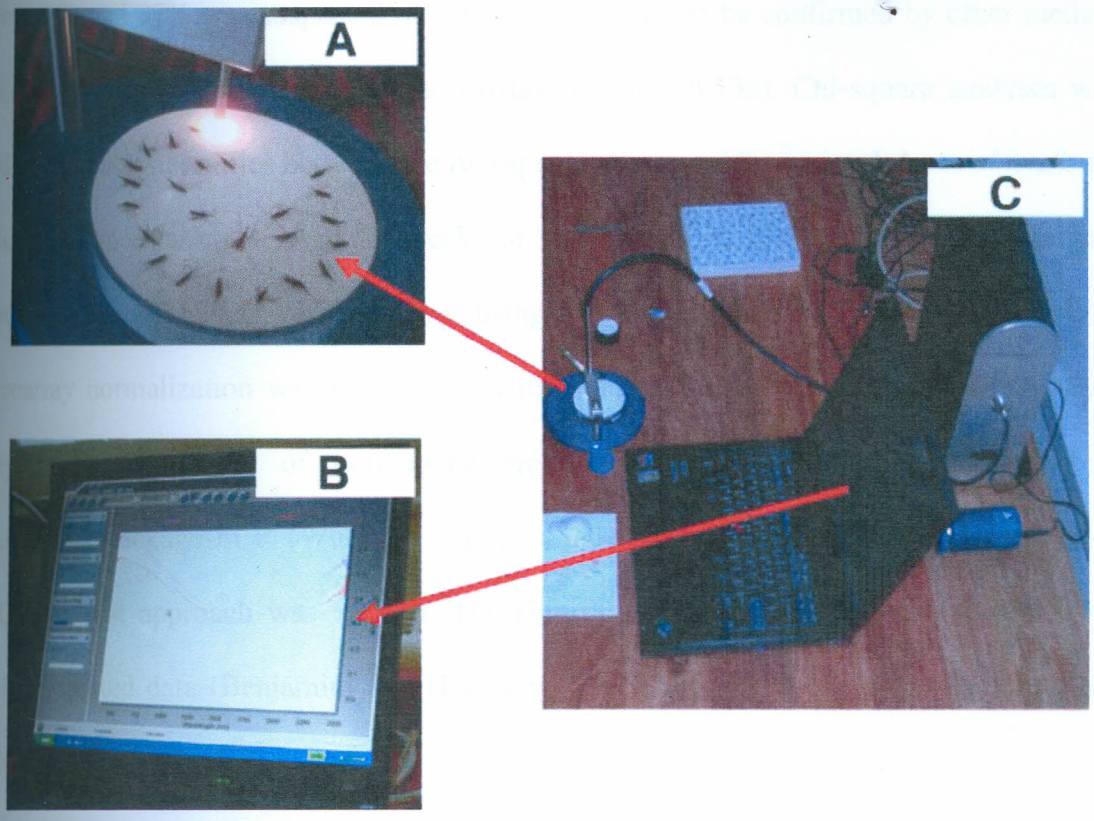


Plate 1: Scanning mosquitoes using a near-infrared spectrometer. A , Plate with mosquitoes positioned for scanning. B , Near-infrared (NIR) spectra of mosquitoes. C , Complete NIR system including the spectrometer (ASD Inc., Boulder, CO) (Mayagaya *et al.*, 2009).

3.5 Data analysis

Data analysis for objective 1: Population susceptibility to insecticide status was inferred following WHO guidelines where mortality ≥98% was regarded as susceptible, mortality between 90 and 97% was suspected of resistance, and are to be confirmed by other methods while mortality <90% was regarded as resistant (WHO, 2013a). Chi-square analyses were used to compare mortalities between the two species *An. gambiae* s.s. and *An. arabiensis* and Wilcoxon signed-rank test used to check for consistency in the comparison. Similarly, frequencies of L1014S *kdr* were compared using Chi-square analysis.

Microarray normalization was performed in the statistical program R using Limma 3.2.3 (Smyth, 2005), analysis of normalized signal intensities was performed using the MAANOVA package in R (Wu *et al.*, 2003). Since there were three treatment groups, an ANOVA F-test approach was applied. The significance level for the false discovery rate (FDR)-corrected data (Benjamini and Hochberg, 1995) was set at log10(Q value) > 4 ($q < 0.0001$).

Data analysis for objective 2: The average hole area was estimated according to the methods recommended by the WHO Pesticide Evaluation Scheme (WHO, 2012b) and the data presented in square centimetres summing up the total hole area of the net derived from calculating the hole area using the formula $A = \pi r^2$. The hole area was categorized as ≤50 cm^2, 50–500 cm^2 and >500 cm^2. Nets with no holes were used as the reference category in the regression analysis. A Poisson regression model, corrected for over-dispersion, was used to estimate the effect of net type, net age and the physical condition of the net, on the number of mosquitoes resting inside and to compare the hole sizes and bioassay data of nets from cases and controls.

Data analysis for objective 3: Chi-square analysis was done to compare mean knockdown and mean mortality of susceptible mosquitoes when exposed to nets collected from cases and

controls. The average hole area was estimated according to the methods recommended by the WHO Pesticide Evaluation Scheme (WHO, 2012b) and the data presented in square centimetres summing up the total hole area of the net derived from calculating the hole area using the formula $A = \pi r^2$. Chi-square analysis was also used to test for significant differences in the mean hole sizes of the nets used by the cases and controls as well as the average number of mosquitoes collected in these houses using PSC.

Data analysis for objective 4: Bioassay results were interpreted following the WHO tube assay guidelines (WHO, 2013a) and Chi-square analyses used to test for significant differences in mortality between the two species *An. gambiae* and *An. funestus*. Data was separated according to species and time of collection. Tukey Kramer HSD test was used in conjunction with ANOVA to find mean ages that were significantly different from each other. Simple logistic regression for the binary outcomes, live and dead upon exposure to insecticides, based on the three predictors, insecticide, species and age category. Age was categorized into two <= 8days indicative of mosquitoes unable to transmit *Plasmodium* parasites and >8days indicative of mosquitoes with ability to transmit parasites.

3.6 Ethical clearance

Participants were recruited once they read/or been read to, understood and signed the consent forms (See Appendix 6). Their questions and concerns were addressed at any time during the study. For specific objectives 2, 3 and 4, verbal consent was obtained from participants prior to entry into houses for mosquito collection or bednet survey. Ethical clearance was obtained from the Ethical Review Committee of the Kenya Medical Research Institute, SSC #2267 and the Institutional Review Boards of the US Centers for Disease Control and Prevention IRB #6395 (See Appendix 7).

CHAPTER FOUR: RESULTS

4.1 To compare variations in insecticide resistance and identify genes over expressed in pyrethroid resistant versus susceptible *Anopheles* mosquitoes from Bondo, Rachuonyo, Teso and Nyando.

4.1.1 Phenotypic assays

Mortality to insecticides varied widely between clusters even within one district, mortality to deltamethin ranged from 45 to 100%, while mortality to permethrin ranged from 30 to 100% (Figures 5 and 6). Nyando district had the most clusters with low (<90%) insecticide resistance while Bondo and Teso had clusters with the highest levels of insecticide resistance to the two insecticides.

4.1.2 Species distribution

Apart from Teso, the vector species most abundant in all the districts was *An. arabiensis*. This species comprised at least 94% of all vector population in Bondo, Rachuonyo and Nyando. In Teso, 77% of all samples were *An. gambiae* s.s. Results are depicted in Table 3.

Table 3: The distribution of *An. gambiae* s.l. sibling species in the four study districts Bondo, Rachuonyo, Teso and Nyando.

District	Total	Arab	Gam	Upper CI	Lower CI	Prop Arab
Bondo	3372	3159	213	94.47	92.796	93.683
Rachuonyo	1487	1451	36	98.274	96.628	97.579
Teso	1332	306	1026	25.347	20.757	22.973
Nyando	1147	1101	46	97.018	94.643	95.99

Arab=> *An. arabiensis*, Gam=> *An. gambiae* s.s., Upper CI=> Upper confidence interval of percentage of *An. arabiensis*, lower CI => Lower confidence interval of percentage of *An. arabiensis*, Prop Arab=> Proportion of *An. arabiensis* in the population.

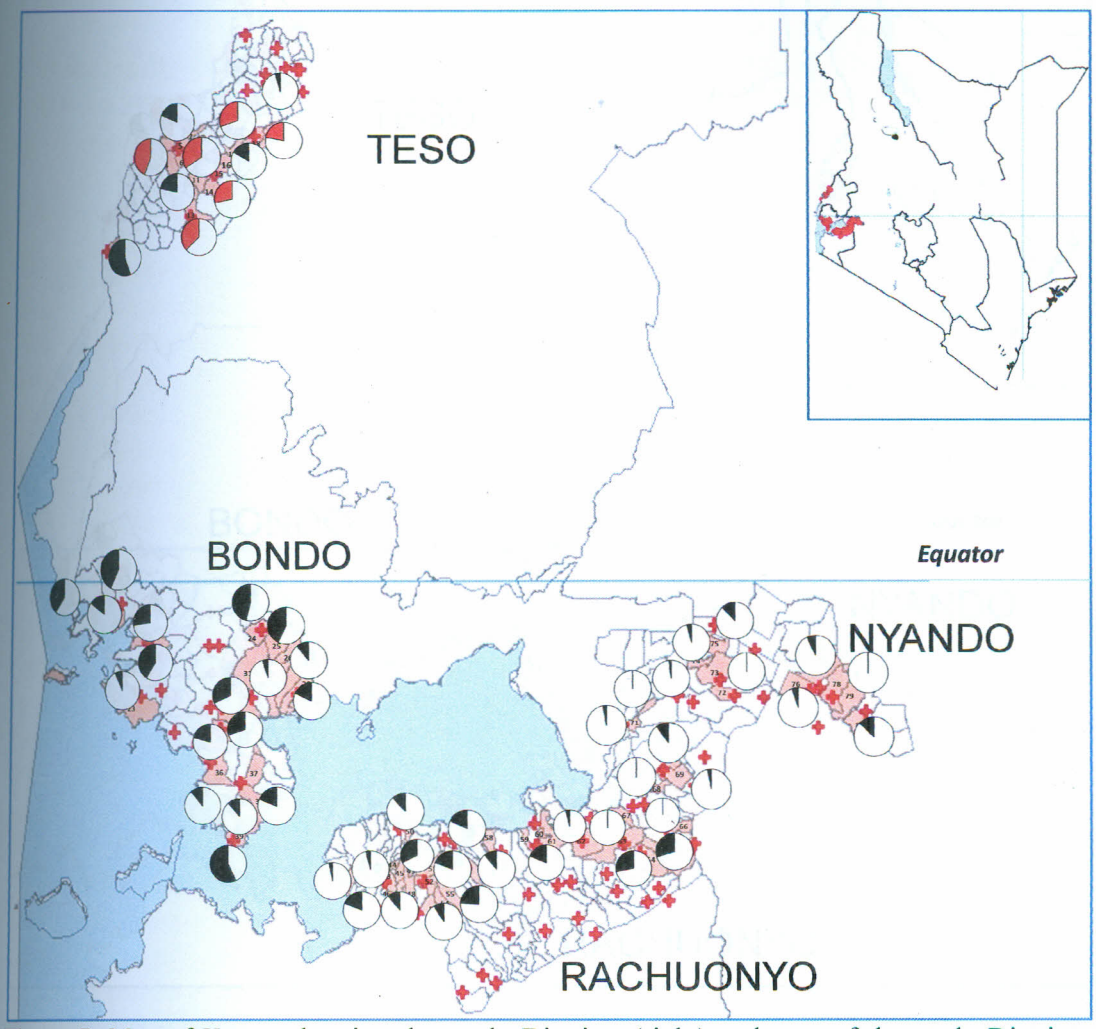


Figure 5: Map of Kenya showing the study Districts (right) and map of the study Districts with clusters highlight in orange. The red crosses represent health facilities. The pie charts indicate susceptibility status of mosquito populations to deltamethrin in the study clusters. The black charts indicating the resistance status of *An. arabiensis* while the red charts indicate resistance status of *An. gambiae* s.s.

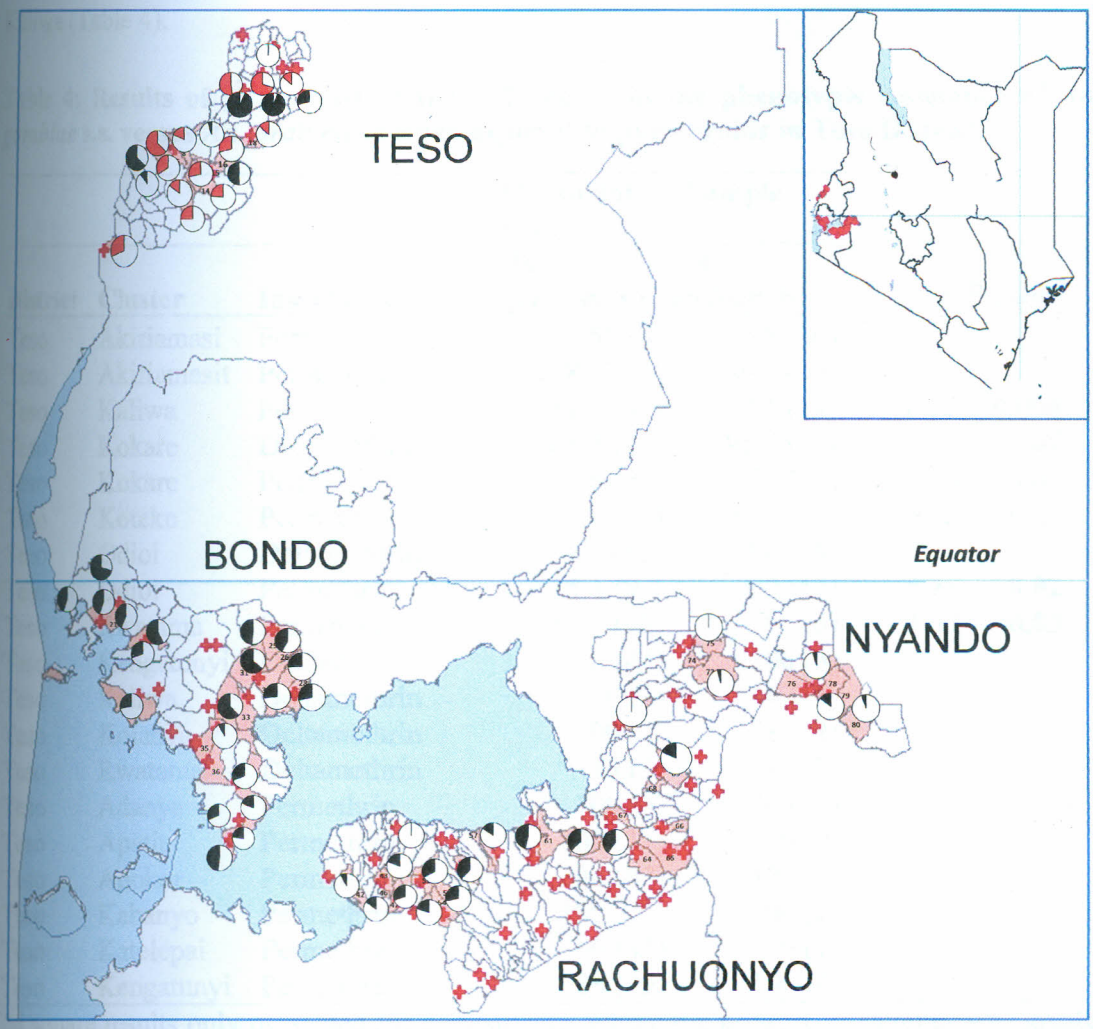


Figure 6. The pie charts indicate susceptibility status of mosquito populations to permethrin in the study clusters. The red charts indicate the resistance status of *An. Arabiensis*, while the black charts indicate resistance status of *An. gambiae* s.s.

4.1.3 Susceptibility status by species

Susceptibility data for Bondo, Rachuonyo and Nyando could not be split down to the two species since *An. gambiae* s.s population was less than 6% in all the clusters in these sites. The pooled mortality for all *An. gambiae s.s.* from all 4 districts is reported in Table 4. Overall, Teso had a higher proportion of *An. gambiae s.s.* 1 even though the species distribution was not the same in all clusters. In two clusters in South Teso: Akiriamasi and Akiriamasit, more *An. arabiensis* were observed compared to *An gambiae* s.s. with higher resistance observed in *An. gambiae* s.s. (χ^2= 7.89, P=0.005; χ^2=0.1, P=0.75, respectively) with the rest of the clusters having more *An. gambiae* s.s. A few instances of resistance to

permethrin were observed in *An. arabiensis* compared to *An. gambiae* s.s. in Odioi and Kaliwa (Table 4).

Table 4: Results of χ^2 analysis of the differences in the phenotypic resistance of *An. gambiae* s.s. versus *An. arabiensis* when exposed to pyrethroids in Teso District.

			% Mortality (Sample size)			
District	Cluster	Insecticide	*An. gambiae* s.s.	*An. arabiensis*	χ^2	*P* value
Teso	Akiriamasi	Permethrin	50 (8)	95 (20)		
Teso	Akiriamasit	Permethrin	85.7 (7)	89.7 (39)		
Teso	Kaliwa	Permethrin	51.4 (37)	7.1 (14)	8.32	**0.004**
Teso	Kokare	Deltamethrin	85.4 (48)	95.4 (65)	3.4	**0.07**
Teso	Kokare	Permethrin	34.6 (26)	71 (31)	7.53	**0.006**
Teso	Koteko	Permethrin	82.4 (51)	90.5 (21)	0.76	0.21
Teso	Odioi	Deltamethrin	47.4 (19)	83.3 (6)		
Teso	Odioi	Permethrin	35.1 (97)	5.9 (17)	5.78	**0.02**
Teso	Rwatama	Permethrin	83.5 (79)	50 (16)	4.62	**0.03**
Teso	Kengatunyi	Deltamethrin	28 (43)	100 (5)		
Teso	Kaliwa	Deltamethrin	50 (2)	44 (18)		
Teso	Kolanya	Deltamethrin	100 (2)	78 (72)		
Teso	Rwatama	Deltamethrin	33 (3)	81 (77)		
Teso	Adanya	Permethrin	100 (6)	36 (14)		
Teso	Apatit	Permethrin	100 (17)	0 (0)		
Teso	Apokor	Permethrin	96 (47)	100 (1)		
Teso	Kabanyo	Permethrin	100 (17)	100 (2)		
Teso	Katelepai	Permethrin	50 (42)	0 (1)		
Teso	Kengatunyi	Permethrin	86 (79)	100 (4)		

Chi square results only presented for comparisons where the number of samples was greater than 10 in each species.

4.1.4 Gene expression associated with resistance in Bondo versus Nyando and Dongola

When exposed to deltamethrin using the WHO tube bioassay technique, *An. arabiensis* samples from Bondo had 74% mortality (N=1208) while those from Nyando had a mean mortality of 92% (N=554). *An. arabiensis* samples from Bondo had an L1014S *kdr* mutation frequency of 2.1% while those from Nyando had a frequency of 13.3%. The frequency was significantly higher in Nyando than Bondo ($\chi^2 = 13.32$ $P<0.0001$).

The expression levels of genes putatively involved in insecticide resistance were compared by co-hybridising labelled RNA from the deltamethrin resistant Bondo population with both parapatric susceptible Nyando population and laboratory susceptible Dongola strain. Out of the more than 14, 000 gene probes on the microarray, the top 100 most highly overexpressed genes in the comparison of the resistant versus susceptible populations were compiled and checked for involvement in metabolic insecticide resistance in GenBank (http://www.ncbi.nlm.nih.gov/genbank). The over expression of different genes in the resistant population was compared in each of the 4 different replicates and multiple testing *P*-values adjusted to control for false discovery rate (FDR) to 10^{-4} and presented in the volcano plots with each dot indicating the level of gene expression, the scales were different depending on the populations compared. The dots represent the level of expression of the genes in the two populations being compared, with outliers to the top right representing those genes over expressed in the more resistant population compared to the latter (Figure 7). The 3 position of cytochrome P450 and 4 GST gene clusters identified, including CYP6M2 are shown in circles on each plot. Analysis of the arrays revealed that among the top 100 genes significantly over expressed ($P<0.0001$) in Bondo versus Nyando and Dongola samples were 2 non-specific cytochrome P450 genes, a gene for GSTE4, 2 genes for CYP6M2, and 3 for GSTD 1_5. The full list of the first 100 upregulated genes is given in Appendix 8.

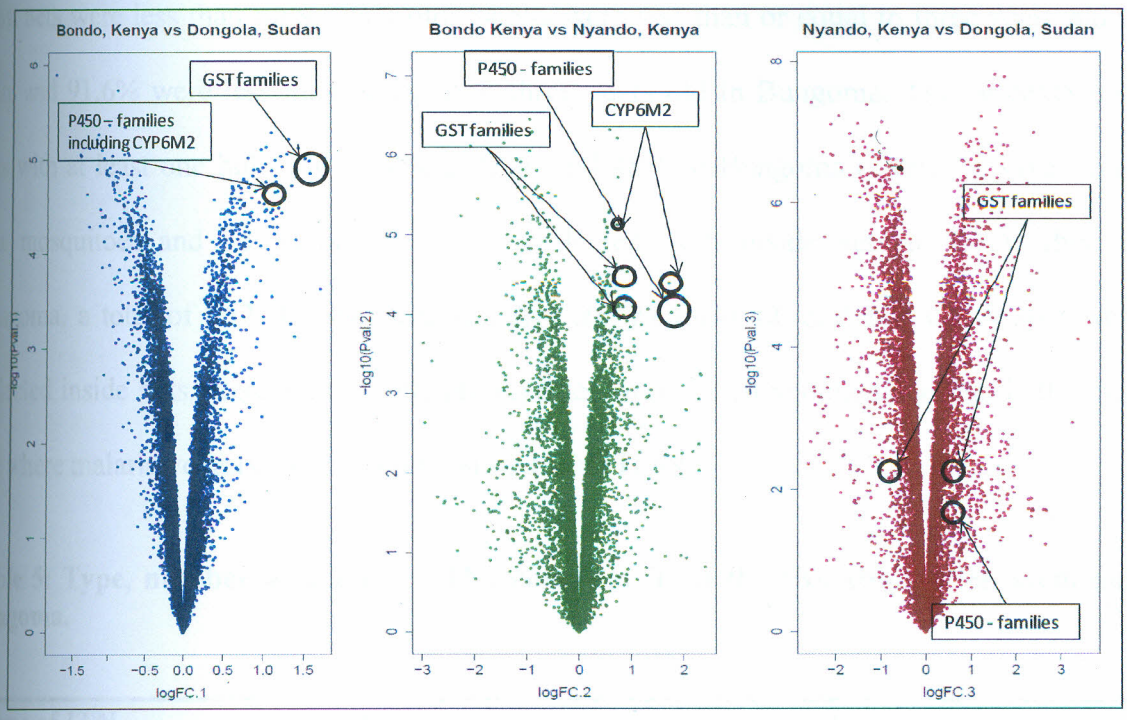


Figure 7: Volcano plots showing differences in expression levels between deltamethrin resistant *An. arabiensis* from Bondo and laboratory susceptible Dongola (blue), resistant *An. arabiensis* from Bondo and parapatric susceptible population from Nyando (green) and parapatric susceptible population from Nyando and laboratory susceptible Dongola (red). Also shown is the position of resistance gene cluster in each comparison plot.

4.2 To assess the association of insecticide resistance with the ability of *Anopheles* mosquitoes to rest in long lasting insecticide treated nets in Bungoma and Gem

4.2.1 Bed net cross-sectional survey

The bed net survey was conducted in all 303 houses in Bitobo village in Bungoma District and all the 179 households in Dienya village in Gem District, with a total of 590 nets being sampled. The numbers of the different types of nets and their ages are shown in Table 5. In Gem, just over half (117/216, 54.0%) of the nets were PermaNets while 69/216 (32.1%) were Olyset nets. In Bungoma, most of the nets were Olyset nets (279/374, 74.7%) while only 58/374 (15.6%) were PermaNets. The remainder of the nets sampled in both sites (57/590, 6.7%) was SupaNets [commercially available, conventionally treated nets bundled with

deltamethrin treatment kits (KO-Tab®)] or nets that could not be identified. In both sites, most nets were less than three years old; 84.5% were less than or equal to three years old in Gem and 91.6% were less than or equal to three years old in Bungoma. The percentage of nets with at least one hole was 32.4% in Gem and 48% in Bungoma (Table 5). Five *Culex spp.* mosquitoes and no anophelines were collected from inside nets in Gem while in Bungoma, a total of 552 *An. gambiae* s.l., five *An. funestus* s.l. and 137 *Culex spp.* were collected inside nets. Hereafter, results are reported only for Bungoma since this is the only site where malaria vectors were caught resting inside ITNs.

Table 5: Type, number and age of ITNs collected from the two study sites, Gem and Bungoma.

Type of ITN	Gem			Bungoma		
	≤ 3 years	> 3 years	Unknown	≤ 3 years	>3 years	Unknown
Olyset	59 (85.5)	10 (14.5)	0 (0.0)	254 (91.7)	14 (5.1)	9 (3.3)
Permanet 2.0	96 (82.1)	20 (17.1)	1 (0.9)	48 (78.7)	8 (13.1)	5 (8.2)
Supanet*	1 (33.3)	2 (66.7)	0 (0.0)	27 (75.0)	8 (22.2)	1 (2.8)
Other	24 (88.9)	3 (11.1)	0 (0.0)	0	0	0
Total	**180 (83.3)**	**35 (16.2)**	**1 (0.5)**	**329 (88.0)**	**30 (8.0)**	**15 (4.0)**

Numbers in parentheses indicate the percent of each net type that are less or equal to 3 or than or greater than three years of age as reported by the owner in each site. * A conventional net available at local shops.

Overall, 36% of nets in Bungoma had at least one *Anopheles* mosquito resting inside the net. Among the different net types, 38% of the Olyset nets, 33% of the Permanet 2.0 nets and 29% of all Supanet nets had at least one *An. gambiae* s.l. resting inside. The proportion of nets less than or equal to three years old and those older than three years that had at least one mosquito resting inside were 36.9% and 36.6%, respectively. The proportion of intact nets

(without holes) and damaged nets (with one or more holes) that had at least one mosquito resting inside were 37% and 48%, respectively.

The mean number of *Anopheles* mosquitoes found within nets increased in nets with hole sizes above 50 cm^2 (Table 6).

Table 6: Mean number of mosquitoes found in ITNs in Bungoma grouped by the hole index category

Hole index category	Sample size	Mean number of *Anopheles* per net	Lower 95% Confidence intervals	Upper 95% Confidence intervals	Nets with Mosquitoes inside
No holes	163	1.01	0.67	1.34	37%
<50 cm^2	55	1.40	0.73	2.07	24%
50-500 cm^2	103	2.06	1.3	2.78	41%
>500 cm^2	53	1.91	0.94	2.87	40%

The number of mosquitoes collected from nets with total hole areas less than 50 cm^2 was not significantly different from nets with no holes. For nets with hole areas 50–500 cm^2 and >500 cm^2, there were generally twice as many mosquitoes observed resting inside the nets compared to the nets with no holes (RR=2.08, P = 0.001 for 50–500 cm^2; RR=1.89, P=0.012 for >500 cm^2) (Table 7). There was no increase in the number of mosquitoes found resting in nets with hole areas >500 cm^2 compared to nets with hole areas between 50–500 cm^2 suggesting a threshold effect. There was no significant difference in the number of mosquitoes found resting in the different net types, nor in nets in different age categories (Table 7).

Table 7: **Results of a Poisson regression model of the number of *An. gambiae* s.l. inside ITNs in Bungoma County including the model estimates, risk ratios and *P*-values.**

Parameter	Risk Ratio	*P*-value
Net type		
Olyset	0.85 (0.50, 1.46)	0.563
PermaNet	0.74 (0.38-1.47)	0.394
SupaNet	Ref.	Ref.
Net age		
Less than 3 years	1.01 (0.54-1.82)	0.94
Older than 3 years	Ref.	Ref.
Hole index category		
>500 cm^2	1.89 (1.148-3.111)	**0.012**
50 to 500 cm^2	2.08 (1.38-3.12)	**<0.001**
<50 cm^2	1.34 (0.8-2.36)	0.243
No holes	Ref.	Ref.

The 95% CI for the estimate and risk ratios are given in parentheses. Significant *P*-values are given in boldface. The SupaNet was used as reference since it was a common local brand of retreatable net and does not fall into the long lasting insecticidal net category.

4.2.2 Bioefficacy of Long Lasting Insecticide treated Nets (LLINs) against pyrethroid resistant *Anopheles gambiae* s.s. mosquitoes from western Kenya

4.2.2.1 Net bioassays

When exposed to brand new, unused, unwashed nets, *An. gambiae* s.s. from Bungoma had 57.5% knockdown and 37.5% mortality to Olyset (N = 40) and 54% knockdown and 22% mortality (N = 50) to PermaNet 2.0 nets.

4.2.2.2 Susceptibility of mosquito samples to insecticides

Within Bungoma, mosquitoes reared from females collected inside nets had lower susceptibility (5% to deltamethrin and 34% to permethrin) compared to those from larval

collections (43% to deltamethrin and 53% to permethrin). Mosquitoes from Bungoma, whether collected from inside nets or as larvae generally showed lower susceptibility compared to mosquitoes collected as larvae from Gem (75% to deltamethrin and 65% to permethrin). In Gem, there were no mosquitoes collected from inside nets (Figure 8). Based on the current WHO insecticide susceptibility guidelines where: mortality >=98% is susceptible, Mortality <98% but>= 90% is probable resistant while mortality <90% is confirmed resistant , populations from Bungoma and Gem would be classified as resistant to the two insecticides (WHO, 2013a).

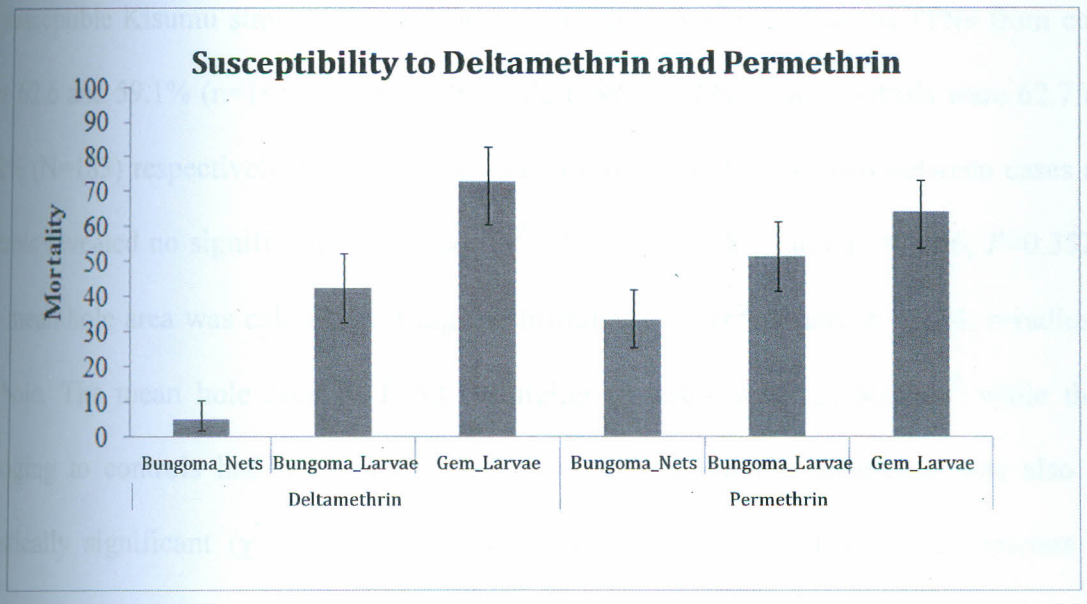


Figure 8: Susceptibility status of mosquito populations. Mortality of *An. gambiae* s.l. mosquito samples when exposed to deltamethrin and permethrin. Bungoma_Nets represents mortality of *F1* offspring of mosquitoes collected resting inside nets in Bungoma, Bungoma_larvae represents mortality of samples collected as larvae in Bungoma and reared to adults for exposure while Gem larvae mortality represents mortality of samples collected as larvae in Gem and reared to adults for exposure.

4.2.3 Molecular assays of mosquitoes collected inside nets

All the mosquitoes collected inside nets were identified as *An gambiae* s.s. (N = 512), while 40 samples did not amplify and were not identified. All the *An. gambiae* s.s. samples

collected from inside the nets were homozygous for *kdr* 1014S (N = 512) and 1.8% of the *An. gambiae* s.s. collected inside nets tested positive for *Plasmodium falciparum* sporozoites (N = 552).

4.3 To assess the association between sleeping under a holed ITN and the presence of malaria infection in children under five years in Bungoma

A total of 448 children under 5 years of age, 224 cases and 224 controls, were recruited from villages around Kimaeti Health Centre and their ITNs brought back to KEMRI/CDC bioassay laboratory for evaluation. Of these ITNs, 71 were disqualified from analysis since they were either untreated conventional ITNs or were round nets. The mean knockdown and mortalities for susceptible Kisumu strain *An. gambiae* s.s. mosquitoes exposed to the ITNs from cases were 62.6 and 59.1% (n=195) respectively while those of ITNs from controls were 62.7 and 59.2% (N=183) respectively. Statistical comparison of mean knockdown between cases and controls revealed no significant differences (χ^2 =0.763, *P*=0.3822 and χ^2 =0.866, *P*=0.3521). The mean hole area was calculated using the formula: $A = \pi r^2$, where π =3.14, r=radius of the hole. The mean hole area of ITNs belonging to cases was 13,650.5cm^2 while those belonging to controls had a mean of 13550.76 cm^2, though the difference was also not statistically significant (χ^2 =0.72, *P*=0.4648). The results from different parameters are presented in Table 8. A total of 640 *Anopheles* mosquitoes were collected by PSC inside the participant houses giving a mean vector density of 1.36 and 1.47 in the case and control houses, respectively. This difference was however, not significant (χ^2 =0.57, *P*=0.48).

Table 8: **Comparison of different parameters likely to be associated with malaria infection between cases and controls.**

Parameter		N	Case (%)	Control (%)	$\chi 2$	*P*-Value
Net type	Olyset	347	52.4	47.6	0.7637	0.3822
	Permanet	31	38.7	61.3		
	Conventional	71	35	65		
Net Bioassays	Knockdown @1hr	374	62.6	62.7	0.7637	0.3822
	Mortality @24 hr	374	59.1	59.2	0.866	0.5321
Mosquito density		640	48.1	51.9	0.57	0.48
Average Hole Size		448	50.7	49.3	0.7221	0.4684

4.4 To assess age modulated resistance in natural *Anopheles* populations of Bungoma, Teso, Asembo and Ahero

4.4.1 Susceptibility to insecticides

Adult *An. gambiae* s.l. and *An. funestus* s.l. adults were exposed to deltamethrin and permethrin separately. Results are presented in tables 9 and 10.

Table 9: Comparative mortality of *An. funestus* versus *An. gambiae* to deltamethrin in the study sites

Month	District	Insecticide	Species	N	% Mortality	χ2	*P*-value
2013 Jan	Ahero	Deltamethrin	*An. funestus*	27	0	41.9	Null
2013 Jan	Ahero	Deltamethrin	*An. gambiae*	60	75		
2013 Jan	Asembo	Deltamethrin	*An. funestus*	95	36.8	18.91	<0.0001
2013 Jan	Asembo	Deltamethrin	*An. arabiensis*	32	81.3		
2013 Jan	Bungoma	Deltamethrin	*An. funestus*	56	32	0.106	0.744
2013 Jan	Bungoma	Deltamethrin	*An. gambiae*	60	35		
2013 Jan	Teso	Deltamethrin	*An. funestus*	34	50	3.45	0.063
2013 Jan	Teso	Deltamethrin	*An. gambiae*	11	81.8		
2012 Oct	Asembo	Deltamethrin	*An. funestus*	51	43	1.96	0.16
2012 Oct	Asembo	Deltamethrin	*An. arabiensis*	14	64		
2012 Oct	Bungoma	Deltamethrin	*An. funestus*	12	33.3	0.2	0.65
2012 Oct	Bungoma	Deltamethrin	*An. gambiae*	23	26.9		
2012 Oct	Teso	Deltamethrin	*An. funestus*	35	31.4	0.7843	0.3758
2012 Oct	Teso	Deltamethrin	*An. gambiae*	31	41.9		
2012 July	Ahero	Deltamethrin	*An. funestus*	20	80	19.93	<0.0001
2012 July	Ahero	Deltamethrin	*An. arabiensis*	164	98		
2012 July	Asembo	Deltamethrin	*An. funestus*	62	41.94	3.98	0.046
2012 July	Asembo	Deltamethrin	*An. arabiensis*	14	71.43		
2012 July	Bungoma	Deltamethrin	*An. funestus*	22	22	0.4335	0.51
2012 July	Bungoma	Deltamethrin	*An. gambiae*	29	31		
2012 July	Teso	Deltamethrin	*An. funestus*	20	25	12.47	0.0004
2012 July	Teso	Deltamethrin	*An. gambiae*	32	75		
2012 May	Ahero	Deltamethrin	*An. funestus*	21	71.4	5.1	0.02
2012 May	Ahero	Deltamethrin	*An. arabiensis*	132	89.4		
2012 May	Asembo	Deltamethrin	*An. funestus*	18	22	0.62	0.43
2012 May	Asembo	Deltamethrin	*An. arabiensis*	196	31		
2012 May	Bungoma	Deltamethrin	*An. funestus*	0	0	-	-
2012 May	Bungoma	Deltamethrin	*An. gambiae*	104	15.3	-	-

Table 10: **Comparative mortality of *An. funestus* versus *An. gambiae* to permethrin in the study sites**

Month	District	Insecticide	Species	N	% Mortality	$\chi 2$	*P*-value
2013 Jan	Ahero	Permethrin	*An. funestus*	47	8.5	38.4	P<0.0001
2013 Jan	Ahero	Permethrin	*An. gambiae*	48	70.8		
2013 Jan	Asembo	Permethrin	*An. funestus*	48	38.8	1.694	0.193
2013 Jan	Asembo	Permethrin	*An. arabiensis*	50	56		
2012 Oct	Teso	Permethrin	*An. funestus*	22	81.82	26.17	<0.0001
2012 Oct	Teso	Permethrin	*An. gambiae*	118	25.42		
2012 July	Ahero	Permethrin	*An. funestus*	73	90.4	8.72	P=0.003
2012 July	Ahero	Permethrin	*An. arabiensis*	182	98.4		
2012 July	Asembo	Permethrin	*An. funestus*	38	50	1.6	0.206
2012 July	Asembo	Permethrin	*An. arabiensis*	5	80		
2012 July	Bungoma	Permethrin	*An. funestus*	22	27.3	0.23	0.63
2012 July	Bungoma	Permethrin	*An. gambiae*	28	21.4		
2012 July	Teso	Permethrin	*An. funestus*	15	46.7	0.004	0.949
2012 July	Teso	Permethrin	*An. gambiae*	42	47.6		
2012 May	Ahero	Permethrin	*An. funestus*	35	97	0.7	P=0.4
2012 May	Ahero	Permethrin	*An. arabiensis*	105	93		
2012 May	Teso	Permethrin	*An. funestus*	7	100	19.968	<0.0001
2012 May	Teso	Permethrin	*An. gambiae*	107	22.4		

4.4.2 Age grading using NIR technique

Mosquito ages were predicted using the NIR technique. Data was separated according to species and time of collection. Tukey Kramer HSD test was used in conjunction with ANOVA to find means that were significantly different from each other. Both *An. funestus* and *An. gambiae* samples collected in October were significantly older than those collected in all the other time points (q*=2.57, *P*=0.05) (Table 11).

Table 11. **Mean ages of *An. gambiae* and *An. funestus* over the four time points.**

	An. funestus		*An. gambiae*	
Level	Group	Mean	Group	Mean
Oct-12	A	7.7	A	7.8
Jan-13	B	6.6	B	5.7
May-12	B	5.9	B	5.5
Jul-12	B	5.6	B	5.2

4.4.3 Age modulated resistance as determined by logistic regression

Simple logistic regression was performed modelling the outcome (live/dead) based on age. Mosquito ages were categorized into two groupd; those ≤8 days and >8 days. Mosquitoes > 8 days of age had 2.5 times higher frequency of being resistant to insecticides as compared to those ≤8 days of age (P<0.0001) (Table 12).

Table 12: The association between *Anopheles* age and insecticide resistance

Parameters	Live n(%)	Dead n(%)	Total n(%)	Odds Ratio	P value	95 % confidence interval	
Age category							
≤8 days	310(27.10)	834(72.90)	1144(79.50)				
>8 days	142(48.14)	153(51.86)	295(20.50)	**2.50**	**<0.0001**	**1.9195**	**3.2480**

Odds Ratio (OR) was determined using simple logistic regression analyses.

CHAPTER FIVE: DISCUSSION

This study identified widespread and heterogeneous insecticide resistance with mortality estimates ranging from 45-100% for deltamethin and 29-100% for permethrin. There was no correlation in cluster specific mortality of *An. arabiensis* between permethin and deltamethrin ($Z = 2.9505$, $P = 0.2483$). Three Cytochrome P450-dependent monooxygenase genes among them CYP6M2 were found to be over expressed in the resistant Bondo population compared to the other two populations. Live mosquitoes were found resting inside ITNs in an area of high pyrethroid resistance but not in an area of mild resistance. No differences were observed in net condition and bioefficacy of ITNs used by children diagnosed with malaria and those without. ITNs that repel and kill susceptible mosquitoes are not as effective against pyrethroid resistant mosquito populations. The average ages of mosquitoes were found to be highest in the dry seasons as compared to wet seasons.

5.1. To compare variations in insecticide resistance and identify genes over expressed in pyrethroid resistant versus susceptible Anopheles mosquitoes from Bondo, Rachuonyo, Teso and Nyando

This study identified heterogenous species specific phenotypic resistance in multiple clusters of western Kenya. There was no correlation in cluster specific mortality of *An. arabiensis* between permethin and deltamethrin ($P = 0.248$). Three Cytochrome P450-dependent monooxygenase genes among them CYP6M2 were found to be over expressed in the resistant Bondo population compared to the other two populations. The four districts in which this study was conducted are currently sites of widespread vector control using insecticide-based tools, ITNs and IRS. The lack of correlation in cluster-specific mortality of *An. arabiensis* between permethin and deltamethrin indicates a difference in insecticidal efficacy between trans (in this case permethrin) and alpha-cyano pyrethroids (in this case deltamethrin). For the small number of clusters where sufficient *An. gambiae* and *An.*

arabiensis were obtained it was possible to determine 7 of the 17 tests that reached a conventional significance threshold ($P<0.05$). Four tests showed higher resistance in *An. arabiensis* and three in *An. gambiae* s.s., suggesting no consistent marked difference in resistance between species.

Malaria vector control has relied heavily on the use of pyrethroids due to their efficacy and relatively low toxicity to non-target organisms (Zaim et al., 2000; Zaim and Guillet, 2002). The restricted and rather narrow range of insecticides for use in vector control necessitates strict monitoring and management of insecticide resistance within the control program to ensure sustained control (Kleinschmidt et al., 2009; Chanda et al., 2011; WHO, 2012a). Resistance to pyrethroids has emerged and is spreading at an alarming rate (Corbel et al., 2007; Nauen, 2007; Ranson et al., 2011). In this light, the Global Plan for Insecticide Resistance Management (GPIRM) document released by WHO in 2012 impresses upon the need to setup insecticide resistance monitoring programs where none exists and to scale-up monitoring efforts in areas that have implemented monitoring programs (WHO, 2006; IRAC, 2010; WHO, 2012a). In Kenya, several studies have already documented insecticide resistance in multiple sites (Vulule et al., 1994; Stump et al., 2004; Mathias et al., 2011; Ochomo et al., 2013).

Nyando and Rachuonyo had, at the time of sample collection, yearly IRS programs in addition to the distribution of ITNs. Despite sustained vector control efforts employing pyrethroids since early 2000s, vectors in Nyando demonstrated widespread susceptibility with only 5 of the 18 clusters tested against deltamethrin and 2 of the 7 clusters tested against permethrin having <90% mortality (WHO threshold for resistance). The observation of low resistance despite long term insecticide use for public health had been made in previous studies in western Kenya and in several sites in Tanzania (Vulule et al., 1996; Mathias et al., 2011; Kabula et al., 2012). Curiously, vectors in Teso and Bondo districts, where only ITNs

are the main malaria intervention, had the highest levels of insecticide resistance, suggesting that additional sources such as agriculture (Chouaibou et al., 2008), may be contributing to the selection pressure for insecticide resistance.

Whereas most resistance studies present susceptibility results representative of large administrative areas, usually districts, this study presents cluster specific susceptibility data. Data from previous studies has shown sharply contrasting results between smaller sites within the administrative districts. A study of *An. culicifacies* susceptibility to DDT in Baluchestan in Iran in 1972, revealed mortality between 16.4 and 42% in 5 villages within the same district (Manoucheri et al., 1975). In yet another study in two villages in Burkina Faso, Valle de Kou 5 and Valle de Kou 7 reported different mortalities of 73 and 100% to permethrin respectively (Diabate *et al.,* 2002) though these results could have been confounded by the variation in the individual bioassay experiments as well. In contrast, a study conducted in two villages in Apac district in Uganda, showed consistently similar mortality to permethrin over 3 sampling time points in ADA and ADB villages with 99 and 98% mortality in January 2005 respectively, 93 and 92% mortality in September 2006 for *An. funestus* and 81 and 80% mortality in September 2006 in *An. gambiae* s.s. (Verhaeghen *et al.,* 2010). The range of mortality observed within and between districts in this study is thus not without precedent. What remains to be seen is whether there is temporal stability in the estimates of mortality which may allow us to determine the main drivers of the heterogeneity.

The distribution of *An. gambiae* species in Teso further depicts the need for cluster-specific insecticide resistance monitoring. Clusters within the same district have varying distributions of *An. arabiensis* and *An. gambiae* and consequently varying insecticide resistance status. Given the differences in resting and feeding behaviour of the two sibling species, different transmission dynamics are expected to be observed in these clusters (Githeko et al., 1994; Githeko, 1996). Previous resistance work in Western Kenya have shown higher resistance in

sites west, closer to the border with neighbouring Uganda where high resistance to pyrethroids has previously been detected (Ramphul et al., 2009; Verhaeghen et al., 2010; Mathias et al., 2011; Ochomo et al., 2013). Gene flow of resistance genes into bordering districts may thus be a reason for the observed resistance phenotypes but this needs further genetic studies. Resistance has previously been linked to insecticide use in agriculture in several parts of the continent; Uganda (Brogdon and Barber, 1990; Verhaeghen et al., 2010), Burkina Faso (Diabate *et al.,* 2002) and other sites in sub-Saharan Africa (Lines, 1988; Chouaibou et al., 2008; Müller et al., 2008). In addition, resistance may be attributed to an increase in possession of ITNs and the implementation of IRS programs within the study districts. For example, permethrin impregnated ITNs have been previously linked to a reduced susceptibility in *An. gambiae* s.s. though this elevation declined over time (Vulule et al., 1994; Vulule et al., 1996; Mathias et al., 2011) this necessitates the search and introduction of novel, non-insecticide based control regimen to boost the current insecticide based tools. This baseline study provides a background for the study of insecticide resistance mechanisms in mosquito populations in the different clusters to enable effective management of insecticide resistance and at the same time facilitate continued vector control efforts (Kabula *et al.,* 2012; WHO, 2013a).

The microarray analyses compared the gene expression profiles of resistant *An. arabiensis* from Bondo against the parapatric susceptible Nyando samples and lab susceptible Dongola strain. Three Cytochrome P450-dependent monooxygenase genes among them CYP6M2 were found to be over expressed in the resistant Bondo population compared to the other two populations. The P450 monooxygenases are an important metabolic mechanism by which insects species become resistant to insecticides (Scott, 1999). Of particular interest is CYP6M2 which has been reported in different parts of Sub-Saharan Africa, Ghana (Muller et al., 2007; Mitchell et al., 2012), Benin and Nigeria (Djouaka *et al.,* 2008), Ivory Coast (Edi *et*

al., 2014) and now Kenya. Recent reports have shown through transgenic expression of CYP6M2 in Drosophila that CYP6M2 can in combination with other genes, result in resistance to carbamates (bendiocarb), organochlorines (DDT) and pyrethroids (Edi *et al.*, 2014). Previous reports had implicated CYP6M2 in DDT and pyrethroid resistance (Muller et al., 2007; Djouaka et al., 2008; Mitchell et al., 2012). The finding of CYP6M2 in the Kenyan vector population is the first report of the gene in East Africa. Given the observation of its involvement in resistance to multiple insecticide classes, there is need to screen vectors of other species from multiple sites to map the extent of spread, in order to co-ordinate mitigation efforts. Apart from the P450 monooxygenases, 4 glutathione-S-transferase (GSTs) genes were also over expressed in the resistant populations. GSTs have also been implicated in insecticide resistance to all major classes of insecticides (Ranson and Hemingway, 2005) thus the 4 genes observed in these microarrays could also be involved in insecticide resistance to the different insecticides. It is important that the interaction of the genes over expressed in the resistant populations is understood. The observation of higher frequencies of L1014S *kdr* in Nyando populations compared to Bondo indicated that *kdr* may not be a reliable marker of phenotypic insecticide resistance in *An. arabiensis* populations of western Kenya but does suggest the involvement of other genes in insecticide resistance phenotypes observed in western Kenya.

5.2 To assess the association of insecticide resistance with the ability of *Anopheles* mosquitoes to rest in long lasting insecticide treated nets in Bungoma and Gem

In Bungoma, an area with high levels of pyrethroid resistance (Mathias et al., 2011; Ochomo et al., 2013), live *Anopheles* mosquitoes were routinely observed resting inside nets. This may have been due to declining bioefficacy of the nets, reduced susceptibility of the mosquitoes to pyrethroid insecticides or both. Susceptibility testing using females reared from field-collected larvae or from the *F1* generation derived from mosquitoes collected

Pyrethroid resistance has been spreading rapidly in sub-Saharan Africa and has been documented in 23 countries (Ranson et al., 2011). This may partly be in response to agricultural application and run-off of insecticides into mosquito breeding sites (Brooke et al., 1999; Chandre et al., 1999; Diabate et al., 2002), but increasingly in response to selection pressure resulting from the scale up of insecticide-treated nets and indoor residual spraying as malaria prevention tools (Vulule et al., 1994; Hargreaves et al., 2000; Henry et al., 2005; WHO, 2005b; WHO, 2006; Czeher et al., 2008; Mathias et al., 2011). Regardless of the source of insecticide pressure, insecticide resistance in malaria vectors has been predicted to eventually undermine control programmes that are solely reliant on insecticides such as indoor residual spraying (IRS) and ITN programmes (Ranson et al., 2011). While pyrethroid resistance has been documented in malaria vectors throughout sub-Saharan Africa, there is surprisingly little information on the impact of resistance on the effectiveness of vector control efforts.

An experimental hut trial in two sites in Benin, one with susceptible mosquitoes and the other with mosquitoes resistance to pyrethroids, showed blood-feeding was reduced by 96% at the site with susceptible vector population, but was largely unaffected at the site with high levels of pyrethroid resistance, while the mortality of mosquitoes entering huts at the susceptible site was nearly three times as high as that at the site with high levels of pyrethroid resistance (N'Guessan et al., 2007). Household trials in other parts of Benin also showed that sleeping under an ITN in an area with resistant mosquitoes was no more protective than sleeping under an untreated net, regardless of its physical condition (Asidi et al., 2012). During a longitudinal study of inhabitants of Dielmo village, Senegal, a rise in the incidence of malaria following the distribution of LLINs was attributed to increasing pyrethroid resistance in the

local vector populations (Trape *et al.,* 2011). In contrast, a study in Ivory Coast found no reduction in the protective efficacy of ITNs in an area with high levels of pyrethroid resistance (Henry *et al.,* 2005), while in Malawi, increasing pyrethroid resistance in *An. funestus* was not associated with an increase in malaria transmission in areas with LLINs although in areas with IRS, no additional impact was observed (Wondji *et al.,* 2012). In Benin, mosquitoes were collected from inside nets with 12 holes that were 4 cm x 4 cm. Insecticide treated nets reduced the number of mosquitoes entering compared to an untreated net but an average of 5 mosquitoes were collected each night under LLINs (Gnanguenon *et al.,* 2013).

A modelling study to measure the effect of pyrethroid resistance on the cost effectiveness of LLINs showed strong, positive correlations between insecticide susceptibility status and predicted population level insecticidal effectiveness of and protection against blood feeding by LLIN intervention programmes (Briet *et al.,* 2013). With the most resistant mosquito population, LLIN mass distributions would avert up to 40% fewer episodes of malaria compared to areas with a fully susceptible population (Briet *et al.,* 2013). An on-going study in western Kenya shows prospects of generating evidence within the next year or two on the impact of insecticide resistance on the efficiency of malaria control interventions.

Several factors associated with the number of mosquitoes inside nets were explored. As described above, the location was a strong determinant of the presence of mosquitoes inside nets, presumably due to the composition of the local vector population, and further analyses included only Bungoma. In that site, neither net brand nor the age of the nets was associated with the number of mosquitoes inside nets. Although the nets were not stratified by age, the study demonstrated high mortalities of susceptible mosquitoes exposed to nets collected from

the field indicating that most nets had adequate levels of insecticide. An increase in the number of mosquitoes inside nets with increasing levels of physical damage was however, observed. Nets with estimated hole areas of >50 cm^2 had more mosquitoes than nets with no holes. Although the sample sizes were limited, the data suggested that a threshold is reached beyond which increasing damage does not lead to increasing numbers of mosquitoes. This may indicate that beyond a certain amount of damage, nets are equally likely to be penetrated by mosquitoes. However, the possibility that increasing damage also allows for more mosquitoes to escape from nets, which may also account for the apparent threshold effect, cannot be ruled out. Interestingly, nets with no holes had an average of just over one mosquito per net. Presumably, this was due to improper usage and residents should be instructed on how to tuck their nets in to prevent mosquitoes from entering them.

It has been suggested that the physical integrity of the LLINs may be compromised before the insecticidal activity falls below established thresholds indicating the need for replacement (Asidi *et al.*, 2012; Gnanguenon *et al.*, 2013) and multiple reports have documented physical damage to nets under conditions of routine use. In a previous study, it wasnoted that 39, 24 and 63% of all the nets in use in Bioko Island, continental Equatorial Guinea and Malawi, respectively, were holed within two years of distribution (Rehman *et al.*, 2011). During a long-term assessment of a polyester-based LLIN in Uganda, more than 70% of nets had holes after only one year and more than 85% after two years (Kilian et al., 2008). Wills *et al.*, reported 54.5% of nets having holes after just six months of distribution in Ethiopia (Wills *et al.*, 2013). In Kenya, in an on-going net durability study in western Kenya, it was observed that up to 40% of some net types had holes within six months of deployment in Siaya County (Bayoh, pers. comm.) while some recent surveys reported that up to 74% of the bed nets in use in Kwale County had holes (Githinji et al., 2010; Mutuku et al., 2013a). The WHOPES

guidelines on monitoring the durability of LLINs outline methods to estimate the hole sizes on the net fabric (WHO, 2011a). However, the guidelines do not provide criteria for physical damage that is indicative of net failure and requiring the replacement of the nets. A previous study proposed a pHI of 88 corresponding to approximately 500 cm^2 of damage while in yet another investigation, it was observed that mosquitoes entering nets with 12 holes 4 cm x 4 cm corresponding to a pHI of 276 (Gnanguenon et al., 2013; Mutuku et al., 2013a). Several authors have suggested criteria based upon proportionate hole indexes (pHI) and the probability that owners will discontinue use due to the owners' perception that the nets are no longer effective (Kilian et al., 2011; Batisso et al., 2012). The cut-off for an unacceptable net ranged from a pHI of 300 in Chad corresponding to a hole area of approximately 1,000 cm^2 to a pHI of 764 in Ethiopia which corresponded to a hole area of approximately 1,200 cm^2. In studies in Bioko Island and Malawi, the risk of malaria increased with deteriorating condition of nets with untreated nets with at least one hole providing the least protection (Rehman *et al.*, 2011) although specific thresholds for net replacement were not presented. The data suggest that in areas with high levels of pyrethroid resistance, the threshold for a net requiring replacement may be at the lower end of the spectrum. While there is complex relationship between hole area and insecticidal activity of the nets and insecticide resistance and behaviour of the vector population, specific criteria for the physical integrity of nets should be developed to assist national malaria control programmes in determining the appropriate replacement strategies for LLINs.

5.3 To assess the association between sleeping under a holed ITN and the presence of malaria infection in children under five years in Bungoma

Universal coverage with LLIN is deemed successful if significantly high proportion of the community is covered (Sexton, 2011). Prior to universal coverage emphasis had been put on children under 5 years of age and pregnant women (Fegan et al., 2007; Mathanga and Bowie,

2007; Mathanga et al., 2009). The observation that 16% of the sampled population, all children under 5 years of age, were still using conventional ITNs despite these programs indicates a need for thorough monitoring and evaluation programs to ensure LLINs reach these priority groups. There were no differences in the average hole sizes and bioefficacy of LLINs used by participants from both case and control arms of the study. In addition, the knockdown and mortality rates of susceptible mosquitoes exposed to these nets was below the stipulated 80% cut-off for bioefficacy (WHO, 2011a). These results seem to suggest that the ITNs evaluated in this study were so damaged that it did not make a difference and other variables were more important predictors of malaria in children. It is clear that the ITN efficacy in these populations was compromised. Previous studies of net status in western and coastal Kenya had observed that 40% and 78% of the ITNs used by the respective populations studied had holes (Githinji et al., 2010; Mutuku et al., 2013b) thus the observations from this study largely support the evidence that a majority of populations in malaria endemic areas are still exposed to infectious bites. Sleeping in a house with a grass thatched roof had a slightly elevated risk of malaria compared to iron roof houses. A previous study in Kenya showed that metal roofing would seem to be protective against mosquito house entry by association with closed eaves thus reduced mosquito entry (Gamage-Mendis *et al.*, 1991). Similarly, a study in North-west Burkina Faso found that occupants of iron-roofed houses had decreased risk of infection compared to residents of thatched-roof and mud-roof houses (Ye *et al.*, 2006). The finding of more cases in houses with a greater number of occupants is likely associated with the fact that odours emanating from individuals attract mosquitoes towards a human host (Takken and Knols, 1999) or the fact that with more the people in a household, the more likely that ITNs would not be sufficient for everyone. More occupants is likely to increase the attractiveness to anophelines and this factor has been

associated with higher anopheline densities in other studies (Mbogo et al., 1999; Ernst et al., 2006).

The spread of pyrethroid resistance combined with increasing evidence that it may compromise malaria vector control programmes highlights the need for new insecticides and new tools for malaria prevention. Currently, LLINs are treated with pyrethroid insecticides only and their loss as an effective tool would seriously undermine malaria control programmes throughout sub-Saharan Africa. IRS with non-pyrethroid insecticides is an option that is immediately available. However, IRS is expensive relative to LLINs, particularly when spraying is done with non-pyrethroids, and is unlikely to be widely implemented without a significant increase in the amount of funding available for malaria control programmes. Two new LLIN products are currently available that incorporate a synergist to mitigate the effects of pyrethroid resistance. The Permanet 3.0 is treated with deltamethrin on the sides and deltamethrin plus piperonyl butoxide (PBO) on top (WHO, 2009a). PBO is a synergist and increases the potency of the pyrethroid insecticides by inhibiting oxidase enzymes that have been implicated as one mechanism of resistance (Kakko et al., 2000). Evidence that the PermaNet 3.0 is more effective than the PermaNet 2.0 which is treated with deltamethrin alone, however, is limited and occasionally mixed, presumably due to the presence of other resistance mechanisms that are unaffected by PBO and the WHO Pesticide Evaluation Scheme did not recommend this product for use as a resistance management tool (WHO, 2009a). The Olyset Plus is another bi-treated net with permethrin plus PBO throughout the net (WHO, 2012b). However, there is limited data on the efficacy of this net against wild populations of mosquitoes that are resistant to pyrethroid insecticides. Larviciding is an alternative vector control tool with a wide range of activity that is recommended for use against malaria vectors. However, as with IRS, larviciding can be expensive, and is currently only recommended, for specific settings. Other insecticides such

as chlorfenapyr (Raghavendra *et al.*, 2011), indoxacarb (N'Guessan *et al.*, 2007), and diafenthiuron (Paul et al., 2006) are being investigated as options for IRS but it is likely to be several years before commercially available formulations will be available. Spatial repellents (Achee *et al.*, 2012) and toxic sugar baits (Beier et al., 2012) have also been proposed for malaria prevention but these too require several years of evaluation and refinement before they can be considered viable tools for malaria control programmes

While this study has demonstrated that pyrethroid resistant mosquitoes are entering and surviving exposure to LLINs, the results should not be interpreted to indicate that LLINs are no longer useful in malaria control programmes. First, while pyrethroid resistance is widespread, the intensity of resistance in many areas is likely low and in these areas LLINs may still be effective. The lack of mosquitoes collected inside nets in Gem demonstrates that LLINs are not compromised everywhere and differences in the intensity of resistance, as well as the effectiveness of LLINs, may vary over relatively short distances. Second, this study was a cross-sectional survey and differences in the age of mosquitoes may affect their susceptibility to pyrethroid insecticides as older mosquitoes have been shown to be more susceptible. Additionally, mosquitoes may repeatedly encounter insecticides over their life and, although this has not been demonstrated, the cumulative exposure may eventually result in the death of the mosquitoes. However, the finding of *P. falciparum*-infected mosquitoes inside nets suggests that, at least in Bungoma, older mosquitoes are able to survive exposure to treated nets. Lastly, intact untreated nets still provide some protection and there may be community-wide effects where malaria transmission is reduced when most people in the population regularly sleep under nets. Without baseline data on the effectiveness of LLINs before the rise of pyrethroid resistance, the impact of resistance on the effectiveness of LLINs cannot be reliably measured. However, the data strongly suggest that the efficacy of

pyrethroid-treated nets may be compromised in areas with high levels of pyrethroid resistance.

5.4 To assess age-modulated insecticide resistance in natural *Anopheles* populations of Bungoma, Teso, Asembo and Ahero in western Kenya

When wild *Anopheles* mosquitoes are exposed to deltamethrin and permethrin insecticides, varying degrees of susceptibility is observed between the two vector species *An. gambiae* and *An. funestus*. In Ahero *An. funestus* had markedly higher survival rates compared to *An. arabiensis* consistent with an earlier report of increasing insecticide resistance in *An. funestus* in western Kenya (McCann *et al.,* 2014). This finding depicts the importance of insecticide resistance monitoring in all malaria vector populations in a geographical locality, contrary to previous studies that had solely focused on the *An. gambiae* s.l. group (Mathias et al., 2011; Ochomo et al., 2013). In other sites, there was not a marked difference in resistance to the pyrethroids in the local vector populations. An interesting observation was the increased tolerance to insecticides in fed and gravid *Anopheles* mosquitoes in comparison to the unfed mosquitoes. This may be a result of increased metabolic enzyme activity resulting in detoxification of insecticides thus the protective effect against the toxicity due to insecticides (Hemingway et al., 2004).

The NIR technique is an efficient tool for use in the approximation of mosquito ages and has he advantage of being able to scan many samples within a short time thus convenient for a arge entomological survey (Mayagaya *et al.,* 2009; Sikulu *et al.,* 2014). The ability to letermine the age of mosquitoes in a population is an important factor to determine the fficacy of an intervention expected to reduce the transmission potential of malaria vectors. rior to NIR technique, only ovarian dissection for observation of dilatations in skeins could e used to approximate mosquito ages. (Polovodova, 1949; Detinova, 1962). This technique

is however, time consuming and relies on the expertise of the person conducting the dissections. NIR technique is thus more convenient and easy applicable in evaluation of programs and is easily implementable to collect the very important entomology index, mosquito age.

More than 75% of the mosquitoes collected were estimated to be less than 8 days old. Upon ingesting an infectious bloodmeal, the *Plasmodium* parasite undergoes an extrinsic incubation period in the mosquito that lasts between 8 and 10 days depending on host as well as parasite factors (Beier, 1998; Koella et al., 1998). This therefore means that most mosquitoes collected in the households had not yet developed the capacity to transmit malaria parasites. There was however, an increasing survival rate with age, where mosquitoes above 8 days of age were better able to survive exposure to pyrethroids. This could be because this group of mosquitoes were likely to be fed or gravid and therefore having a higher proportion of circulating metabolic enzymes capable of detoxifying the insecticides or because of selection. The older ones could have survived because they are resistant, with the younger ones not yet having been though the selection. A study of the effect of bloodmeals on the levels of cytochrome P450 monooxygenases in *Culex pipiens* observed increased levels of circulating P450 monooxygenase enzymes following a blood meal (Baldridge and Feyereisen, 1986). The observation of increasing tolerance with mosquito age indicates that these mosquitoes may be able to withstand higher insecticide doses thus obtain blood meals even in the presence of insecticide based control interventions.

Regression analysis modelling age against insecticide resistance revealed higher resistance in the mosquitoes older than 8 days of age when compared to those younger than 8 days. This is a deviation from what is known where resistance has been thought to be higher than younger mosquitoes (Chouaibou et al., 2012; Kulma et al., 2013). This is likely because older

mosquitoes may have interacted with insecticide labelled control tools during their previous attempts to find a blood meal and therefore have greater tolerance to them.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of findings

1. Variations in insecticide resistance between the study districts of Bondo, Rachuonyo, Nyando and Teso were largely heterogenous and not comparable within and between districts. Two non-specific cytochrome P450 genes, a gene for GSTE4, 2 genes for CYP6M2, and 3 for GSTD 1_5 were found to be elevated in resistant mosquito populations from Bondo.
2. Insecticide resistance was observed to be associated with the ability of *Anopheles* mosquitoes to rest inside long lasting insecticide treated nets in Bungoma but not in Gem.
3. Sleeping under a holed ITN was not found to be associated with higher malaria infection compared to sleeping under an intact ITN in children under five years of age in Bungoma.
4. Insecticide resistance was identified to be modulated by mosquito age in natural Anopheles populations of Bungoma, Teso, Asembo and Ahero.

6.2 Conclusions

1. Insecticide resistance to pyrethroids is heterogeneous and non-comparable in the study sites with varying frequencies within and between districts indicating that different selection pressures are in play within the different districts of western Kenya. The genes found to be expressed in resistant populations can be used markers of resistance thus guide the selection of effective insecticides for vector control.
2. Insecticide resistance is protecting mosquitoes against insecticides used in long lasting insecticide treated nets and as such, enable them to rest on the nets without getting killed.

3. In an area of high insecticide resistance, children sleeping under a holed ITN bears no increased risk of malaria infection as compared to sleeping under an intact ITN.
4. Mosquito ages influence their insecticide resistance status and as such, the resistance status of a natural mosquito population is likely to be modulated by the average ages of the mosquitoes.

6.3 Recommendations

1. The finding of marked heterogeneity within and between districts is indicative of a need for resistance monitoring at administration levels much lower than the district. The identification of novel resistance markers associated with insecticide resistance shows that these genes have the potential of being used as markers for insecticide resistance. Further studies on their frequencies and validation in other local mosquito populations are needed to understand the level of involvement of the genes in the insecticide resistance phenotypes observed in Kenya.
2. Identification of mosquitoes resting inside long lasting nets highlight the need for new insecticide classes for use in bed nets as well as prompt replacement of ITNs once they acquire holes in areas of high resistance in the mosquito vectors.
3. Since holed and intact nets have the same efficacy in areas of high insecticide resistance, more tools are needed to integrate in vector control programs in such areas of high insecticide resistance.
4. The observation of older mosquitoes having higher insecticide resistance means such mosquitoes may survive insecticide exposure in ITNs and therefore insecticide resistance mitigation strategies need to be effected.

6.4 Recommendations for further research

1. This study generated a resistance map for only 4 districts in western Kenya. Further studies are needed to expand this map to other areas and include non-pyrethroid insecticides. In addition, studies are needed to identify the frequency of the resistance genes identified in this study.
2. Further research is needed to evaluate the ability of *Anopheles* mosquitoes to rest in combination nets which are improved to include a synergist thus mitigating insecticide resistance.
3. There is a need for further studies to investigate other factors apart from ITN that could be predisposing certain children in Bungoma to malaria compared to others.
4. Further studies are needed to identify the overall effect insecticide resistance has on malaria transmission, having observed higher resistance in older, potentially infected malaria vectors.

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