EFFECTS OF LONG-TERM USE OF INSECTICIDE TREATED BEDNETS ON SPECIES COMPOSITION AND KNOCKDOWN RESISTANCE IN MALARIA VECTORS IN ASEMBO LOCATION OF WESTERN KENYA

BY

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Effects of long-term use of insecticide
DECLARATION

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

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To our children; Calvin, Faith and Oliver-Bryan, who endured my absence during this study.
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
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<td>DNA</td>
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Insecticide-treated bed nets (ITNs) for personal protection against malaria vectors have been in use for the control of malaria in most tropical areas where malaria transmission is intense. However, the emergence of insecticide resistance in *Anopheles* vectors of malaria may impair the effectiveness of this control measure. Insecticide resistance has been reported in several countries, Kenya included. Effects of long-term use of ITNs on species composition and knockdown resistance (kdr) were investigated in Asembo Location of Western Kenya where a large scale ITN program has been implemented since 1997. Mosquitoes were sampled from a 12km transect in February and May 2004 from Asembo ITN intervention area and the adjacent Seme non-ITN intervention area. Indoor collection of adult mosquitoes from human dwellings was done using mouth aspirators and larval collection from habitats around homesteads using dippers. The mosquitoes were identified morphologically as *Anopheles gambiae s.l.* and as *An. arabiensis* or *An. gambiae s.s.* by polymerase chain reaction (PCR). Analysis of the presence of knockdown resistance (kdr) allele was done using kdr diagnostic PCR. Results showed that there was significant reduction in the proportions of *An. gambaie s.s.* in Asembo with relatively higher proportion of *An. arabiensis* compared to Seme (Binomial regression GenMod procedure, $P<0.0001$). The kdr allele was present in *An. gambiae s.s.* only but absent in *An. arabiensis*. There was no statistical difference in kdr allele frequency trend along the transect (logistic regression, $df=1$, $X^2=2.7664$, $P=0.0963$). Overall, the frequency of kdr gene was 25% in Asembo and 16.7% in Seme despite lack of wide spread use of ITNs in Seme. Nevertheless, these kdr allele frequencies were not statistically different from each other (Fisher Exact test, $F=17.9614$, $df=2$, $P=0.05274$). The genetic differentiation based on the kdr allele was estimated but was also not significant with a low genetic variability ($F_{st}$ value) of 0.0098, which corresponded to migration index (Nm value) of 25.23. The difference in kdr phenotypes between Asembo and Seme mosquitoes was not significantly different (ANOVA, $F=8.71E-16$, $df=1.4$, $P=1.000$). These results suggest free interaction between the Asembo and Seme mosquito populations. The observations of this study suggest that widespread use of ITNs in Western Kenya has led to an increase in kdr frequency spreading to adjacent Seme non-ITN intervention area. Another impact of long-term use of ITNs was seen in altered species proportions (less *An. gambiae* proportion and more *An. arabiensis* in Asembo than Seme). Though the kdr allele responsible for knockdown resistance in mosquitoes was absent in *An. arabiensis*, this species is known to be exophilic hence may not be effectively controlled by use of ITNs.
CHAPTER ONE

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Malaria is a severe vector-borne disease causing death and development problems in the world. It is caused by a single-celled protozoan parasite of the genus *Plasmodium*. Of the four species of malaria parasites, *P. falciparum*, which is the most deadly species of malaria parasites (WHO/UNICEF, 2005), is common in Africa and causes more than 95% of all the malaria infections. Almost all of the remaining malaria infections are caused by *Plasmodium malariae* (Bloland *et al.*, 1999b) while infections due to *Plasmodium ovale* are rare and the fourth species, *Plasmodium vivax*, is almost absent from Africa. Anopheline vectors that transmit malaria flourish well in tropical Africa where the climate and ecology provide ideal conditions (WHO/UNICEF, 2005). Thus, the combination of the most deadly parasite species and most efficient vector in Africa increase transmission of malaria, the risk of disease and malaria related deaths.

It has been observed that countries experiencing endemic malaria are among the poorest in the world with low rates of economic growth (Sachs and Malaney, 2002). Thus, poverty and lack of good quality health care have hindered the control and treatment of malaria. It was estimated in 1993 that 2020 million people are at risk of the disease globally and 300-500 million are ill with malaria (WHO, 1997).
The use of drugs to attack the parasite and vector control to impair transmission, are the two main approaches involved in combating malaria. The treatment is complicated by the spread of strains of *P. falciparum* resistant to the commonly used and cheap anti-malarial drugs like chloroquin (Snow *et al.*, 1998). The next generation of anti-malarial drugs, artemisinin based combined therapies (ACTs), are effective and life saving but much more expensive than drugs that were previously effective against malaria parasites (WHO/UNICEF, 2005).

The other approach of combating malaria is through vector control. Therefore, various methods have been employed to control malaria vectors and these include use of chemicals (insecticides), biological control and prevention of breeding. Personal protection measures such as the use of repellents, insecticide vaporizers, house screening and protective clothing have also been found to be useful in limiting human-vector contact and thus reducing malaria transmission (WHO, 1997).

Although important advances continue to be made in the development of alternative vector control measures, use of insecticides remains a vital part of integrated control programs (Ferrari, 1996). Organochlorines (DDT), organophosphates, carbamates and pyrethroids are the four classes of insecticides approved by WHO for indoor residual spraying. Pyrethroid insecticides, which are the only insecticides approved by WHO for the impregnation of bed nets, are safer than these other insecticides for human health because they can be hydrolyzed and excreted by humans and other mammals (Ray, 1991, Casida *et al.*, 1983). Unfortunately, insect species have a remarkable ability to develop...
resistance to every class of insecticide that has been developed. The emergence of resistance to pyrethroids in the *Anopheles* vectors is likely to reduce the effectiveness of this control measure.

Pyrethroid resistance in *Anopheles gambiae* was first reported in West Africa (Chandre *et al.*, 1999a) and it is believed that this resistance might have been selected by the intensive use of DDT and pyrethroids for cotton crop production (Chandre *et al.*, 1999b). The resistance phenotype results from a single point mutation in the Para-sodium channel gene (Martinez-Torres *et al.*, 1998). This resistance was also noted in Kenya after large-scale use of permethrin impregnated bed nets. The leucine-serine mutation found in the Kenyan species offers less resistance to pyrethroid insecticides than the leucine-phenylalanine mutation found in the West African species of malaria vectors (Ranson *et al.*, 2000).

Despite the presence of the resistance gene in malaria vectors in Western Kenya, recent studies in the same area have shown that ITNs have high impact on the reduction of vector densities, sporozoite rates, morbidity and mortality in young children and malaria during pregnancy (Phillips-Howard *et al.*, 2003a, Gimnig *et al.*, 2003a, Ter Kuile *et al.*, 2003). These studies confirm that ITNs are still important in the reduction of malaria through vector control even in the presence of the kdr gene. There is need, however, for surveillance studies to allow early detection of the resistance genes in *Anopheles* mosquitoes as a step towards resistance management to ensure continued success of ITN strategy. The objective of this study was to investigate the effect of long-term ITN use
on *An. gambiae* and *An. arabiensis* species proportions and frequency of knockdown resistance (kdr) alleles.

### 1.2 LITERATURE REVIEW

#### 1.2.1 Malaria situation in Africa

The malaria which is caused by *P. falciparum* parasites exerts its greatest toll in sub-Saharan Africa. It is one of the causes of morbidity and mortality, creating a significant barrier to economic development (Rogers *et al.*, 2002). To date, a large population of the world, about 2020 million people (WHO, 1997), is still at risk of the disease and death due to malaria. Approximately 300-500 million clinical cases and 1-3 million deaths mostly of children under five years of age occur every year worldwide (WHO, 2000). In Africa, malaria has been ranked the third killer communicable disease after pneumococcal acute respiratory infections and tuberculosis (WHO, 1996). Malaria exerts a heavy economic burden due to medical costs associated with it, foregone income due to lost workdays and premature death of workers as a result of this disease (Sachs and Malaney, 2002). Most malarial countries are poor and have a slow economic growth (Luke and Jeffrey, 2001).

#### 1.2.2 Burden of malaria in Kenya

The magnitude of the problem posed by malaria in Kenya is high especially in Western Kenya and on the Kenyan Coast, which have been categorized as perennial high transmission areas (WHO/UNICEF, 2005). Whereas malaria is endemic in the Coast and Lake Region, other areas such as the Aberdare ranges and the areas around Mt. Kenya are
virtually malaria-free and are hence characterized as low transmission risk regions (WHO, 1996). Western highlands of Kenya, which have stable transmission with seasonal peaks, experience epidemics almost annually from May to July (Malakooti et al., 1998).

Overall, malaria kills 26,000 children each year in Kenya and accounts for 30% of all outpatient attendance and 19% of all admissions to the health facilities (Ministry of Health, 2001). At the sub-national level, surveillance systems in 2002 indicated that hospital attendance due to malaria was, for example, 26% and 15% for Kitale and Kericho district hospitals, respectively (WHO/UNICEF, 2005). Primary school pupils miss 11% of school days and secondary school students miss 4.3% because of malaria (Sachs and Malaney, 2002). Epidemics of *P. falciparum* malaria which have been observed in high altitude areas are attributed to changes in land use patterns and increase in malaria vector populations, breakdown in provision of health services and drug resistance (Shank et al., 2002).

1.3 Mosquito vectors of malaria

Mosquitoes are important vectors of several tropical diseases including malaria. There are about 3,000 species of mosquitoes, of which about 100 are vectors of human diseases. About 380 species of *Anopheles* mosquitoes occur around the world and 60 species are vectors of malaria (WHO, 1997). Female mosquitoes remain alive for two to three weeks under natural conditions (Service, 1976) and their longevity is important because an efficient vector must live long enough for the parasite to undergo complete development.
to enhance disease transmission. Thus, the epidemiology of malaria and the biology of its vectors are inextricably intertwined.

Factors responsible for sustainability of vector life cycles sufficient for parasite development and transmission to human hosts are basically ecological. Climate is the most important in limiting transmission and distribution of malaria on a large scale (Craig et al., 1999). Hence, the distribution of efficiently transmitted pathogens such as those causing malaria is generally limited by the distribution of competent vectors (Rogers et al., 2002). Lindsay and Martens (1998) have established that there is a direct association between malaria and warm weather in malaria endemic countries. It has also been observed that malaria prevalence in Kenya is determined by climatic and ecological conditions which favour survival and relative abundance of vectors (Mutero et al., 1998). White (1982) reported that all human malaria is transmitted by Anopheles mosquito vectors except for the special circumstances of infections acquired transplacentally or through blood transfusion. Therefore, malaria transmission is enhanced by the presence of competent vectors in an area.

1.3.1 The animal and human biting habit of mosquito vectors

Female mosquitoes must feed on blood from animals and/or humans to develop eggs as vertebrate blood contains essential proteins necessary for this process (Kogan, 1990). Most mosquito species show a preference for certain animals or for humans. Malaria is transmitted through the bite of an infected female mosquito during feeding, which frequently takes place at night although daytime biting may occur. Some species prefer
to feed in forests, outside or indoors (WHO, 1997). Those that bite in the early evening are more difficult to avoid than species that feed at night.

1.3.2 The resting behaviour of mosquitoes

Digestion of the blood meal and development of eggs takes two to three days at temperatures of above 23°C (White, 1982). Therefore, a blood-fed female looks for a safe resting place that is shaded and once eggs are fully developed, the gravid mosquito leaves its resting place to look for a suitable oviposition site. Some species prefer to rest in houses or cattle sheds, while others rest outdoors on vegetation or at other natural sites like crevices. According to Bhatt et al (1989), various factors affect the choice of mosquito resting places and these include temperature, humidity, and protection against sunlight, wind and predators. The resting behaviour of mosquitoes may indicate whether the mosquito species is important as nuisance insects or vectors of diseases. Non-vector mosquitoes are expected to rest mainly outdoors though during the wet season, vector species like An. gambiae and A. funestus may rest outside due to greater availability of shaded areas (Gillies and De Meillon, 1968). Resting behaviour is also important as it governs selection of control measures; mosquitoes that rest indoors are easier targets for the control by indoor residual spraying and use of ITNs, for example (Curtis, 1999).

1.3.3 Malaria vectors in Kenya

Entomological studies undertaken in Kenya have identified An. gambiae s.s., An. arabiensis and An. funestus as the main vectors of malaria in Western and Coastal Kenya regions (Taylor et al., 1990, Mbogo et al., 1995). These species occur throughout the
year with peak population coinciding with seasonal rains. In Mwea-Tebere irrigation scheme, \textit{An. funestus} and \textit{An. arabiensis} are the main vectors of malaria (Ijumba \textit{et al.}, 1990), the latter species being more predominant while in Western Kenya, \textit{An. gambiae s.s.} and \textit{An. arabiensis} are the only two members of the \textit{An. gambiae} complex represented.

In many settings, malaria parasite infection rates in \textit{An. funestus} are often lower than those in \textit{An. gambiae} (Gillies and De Meillon, 1968), making this species the second most important vector of malaria after \textit{An. gambiae}. However, in some areas, \textit{An. funestus} is responsible for the major part of malaria transmission, for example, in Madagascar where it breeds in rice fields (Laventure \textit{et al.}, 1996).

\textit{Anopheles funestus} is highly susceptible to insecticides hence is easily eliminated and is slow to re-colonize the same place (Gillies and De Meillon, 1968). In Western Kenya, this species has been reduced greatly in ITN intervention areas (Gimnig \textit{et al.}, 2003a) while in Mwea area of Central Kenya, Kamau \textit{et al.} (2003) found no \textit{An. funestus} mosquitoes and attributed this to the use of pesticides in agriculture and / or the use of insecticides in the form of aerosols or mosquito coils.

\textbf{1.3.4 Vectorial capacity}

Vectorial capacity is an index used to quantify the rate of disease transmission by estimating the potential rate of contact between infectious vectors and susceptible hosts. It is the product of the man biting rate, the human blood index and the mosquito
survivorship from the time of becoming infected to the time of becoming infectious. The vectorial capacity is widely adopted to estimate vector populations’ ability to transmit malaria and is expressed as $C = ma^2p^n/-\log_p$, where:

- $m =$ density of vectors in relation to man
- $a =$ No. of blood meals/vector/day
- $p =$ daily survival probability (proportion of vectors surviving/ day)
- $n =$ incubation period in the vectors in days (Wernsdorfer and McGregor, 1988).

Studies in Asembo on the western shores of Lake Victoria obtained $P. falciparum$ sporozoite rates of 9.6%, 6.4% and 0.4% for $An. gambiae$, $An. funestus$ and $An. arabiensis$ respectively (Taylor et al., 1990). $Anopheles arabiensis$ has a lower sporozoite rate probably because of its less longevity in nature (Gillies and Coetzee, 1987) and its zoophilic and exophilic tendencies (Molineaux and Grammicia, 1980). Although $An. arabiensis$ seems to be a less important vector than $An. gambiae$ s.s. or $An. funestus$, its zoophilic and exophilic behavior may make it less susceptible to residual insecticides and impregnated mosquito nets or curtains as intervention strategies for vector control. Furthermore, it is an important vector in the rice-growing regions of Ahero, east of Kisumu, and Mwea-Tebere in central Kenya. Nevertheless, malaria transmission due to this species could be reduced as this species may shift its feeding to other non-human hosts.
1.4 Malaria control strategies and constraints

The malaria situation in the tropical and sub-tropical regions calls for the development of new methods and improved utilization of available control strategies. This is because there is widespread resistance to drugs by parasites and evidence of the emergence of resistance to insecticides by vectors. In areas with intense transmission, control activities aim to stop preventable deaths and minimize suffering from the disease. Since malaria is a preventable and curable disease, Global Control Strategies include early detection, containment and prevention of the infection (WHO, 1996a). Malaria can be controlled by avoiding the bite of the anopheline mosquitoes (through protective measures against the vector), by administration of anti-malarial drugs (chemotherapy) and by use of prophylactic drugs to prevent development of the disease in case of an infective bite when a person visits malarious areas (chemoprophylaxis), (WHO, 1997).

In Kenya, a national malaria strategy was launched in 2001 involving mobilization of Districts experiencing endemic malaria. These Districts developed business plans with malaria control components that reflect four strategic approaches. The approaches involved are: a) access to prompt and effective treatment, b) management and prevention of malaria during pregnancy, c) use of ITNs and other vector control methods; and d) epidemic preparedness and response in sixteen epidemic-prone Districts (WHO/UNICEF, 2005).
1.4.1 Chemotherapy and chemoprophylaxis

Chemotherapy is the treatment of a disease using drugs. Prompt access to effective treatment is one of the key interventions promoted by Global Malaria Programme (GMP) and can be done by administration of anti-malarial drugs to persons showing signs of malaria in malaria-endemic areas. The anti-malarial drugs attack the *Plasmodium* malaria parasites in the blood. However, the control of malaria has been met with some challenges. One of the major obstacles has been the development of resistance to drugs by the malaria-causing parasite *P. falciparum*. Chloroquin, which is a cheap drug and one that has been most widely used, has lost its clinical effectiveness while sulfadoxine/pyrimethamine (SP) is also becoming ineffective (WHO/UNICEF, 2003). Artemisinin based combined therapies (ACTs) are the latest anti-malarial drugs which are highly effective but are much more expensive.

Prophylaxis is the prevention of disease or control of its possible spread. Chemoprophylaxis for malaria control, therefore, means prevention of the disease by use of anti-malarial drugs in case an infective bite by the anopheline mosquito is encountered and is usually administered to people visiting malarious areas. Intermittent preventive treatment (IPT) during pregnancy with SP is another key intervention recommended by GMP in countries with areas of stable malaria transmission (WHO/UNICEF, 2005) and this may lead to improvement of maternal and child survival (WHO/UNICEF, 2003). Recent national surveys in Kenya on antenatal clinic attendance and use of anti-malarial drugs for prevention of malaria among pregnant women have indicated that only 20% of the 80% that attend antenatal clinics once or twice receive any anti-malarial drugs
(WHO/UNICEF, 2005). This is because many malaria control programs are hampered by financial and operational problems (WHO, 1997). Prophylactic protection through the use of ITNs, a key control strategy also advocated by GMP, is another approach of malaria prevention. There is also the potential of anti-malarial vaccine although it has taken long before implementation (WHO/UNICEF, 2005).

1.4.2 Anti-vector measures

Vector control is one of the methods employed to reduce transmission of many vector-borne diseases and is ranked as one of the best methods of protecting a community against malaria (WHO, 1995). Vector control is important if adequate treatment is not available and diagnosis of a disease is difficult. Besides, treatment of malaria is complicated by the spread of strains of *P. falciparum* resistant to the commonly used anti-malarial drugs. Thus, the main objective of vector control is the reduction of malaria morbidity and mortality by reducing the levels of transmission. The particular vector control methods to be applied in a community depend on the local situation and the preferences of the population (WHO, 1997).

Various strategies have been adopted towards malaria vector control. These include chemical control by use of insecticides (Palchick, 1996), biological control agents (Lacey and Orr, 1994, Woodring and Davidson, 1996), environmental management (Mitchell, 1996) and genetic control (Karamjit, 1996). Of all the vector control strategies employed, chemical control methods have been widely used due to their effectiveness and rapid action. However, wide use of chemical control has led to resistance by disease
vectors. Thus, there is need for integrated vector management (IVM), which is a comprehensive approach that utilizes all available control strategies to reduce the status of mosquitoes as vectors to tolerable levels while maintaining a quality environment. Integrated vector management recognizes that no one control technique is the best for all situations, and that all have their advantages and drawbacks.

1.4.2.1 Larval control

Larval control, which is also referred to as source reduction is done through environmental management. Two forms of environmental management are recognized. Environmental modification which is long-term and this may be achieved through alteration of the breeding sites of the vectors by filling ponds and marshes on a permanent basis. Environmental manipulation which is short-term and this can be done by repeatedly removing vegetation from ponds and canals and clearing premises (WHO, 1997).

Another method of source reduction is the use of products called larvicides which can be applied to the breeding sites. Spraying breeding water surfaces with synthetic inorganic larvicides like temephos or petroleum oils is also done to kill the immature stages of mosquito vectors although this may lead to pollution of the environment and mosquitoes develop resistance to the chemicals used.

Biological control is suitable for the control of larvae without polluting the environment. In this method, other living organisms are used as predators of mosquito larvae or their
products used as toxins against the larval mosquitoes. The biological agents commonly
used to control mosquitoes include larvivorous fish, parasites and bacteria which all
attack mosquito larvae. The mosquito fish, *Gambusia affinis*, are widely used by public
health and mosquito control agencies throughout the world to help reduce mosquito
breeding. This species is preferred because of its adaptability, resistance to unfavourable
conditions and the ability to produce many young ones within a short period of time.
Larvae of *Toxorhynchites* mosquitoes and nymphs of dragonflies are also predatory
hence feed on mosquito larvae. Cyclopoid copepods are tiny crustaceans that attack first
and second instar larvae of mosquitoes and therefore control them effectively (Jack,
2003). Plant products like Neem oil extracts have larvicidal properties while Azolla,
which is a free-floating fern that covers the water surface completely, may kill the
mosquito larvae (WHO, 1997). Other biological control agents are parasitic nematode
worms, fungi that grow on bodies of mosquito larvae and bacteria that produce toxic
products. Bacterial larvicides such as *Bacillus thuringiensis*-*Bti* and *B. sphaericus*-*Bs* are
among the most widely used biological methods.

### 1.4.2.2 Control of adult mosquitoes

Current malaria control strategies emphasize domestic protection against adult
mosquitoes with insecticides, and improved access to medical services. However,
malaria prevention by killing adult mosquitoes is generally favored because moderately
reducing mosquito longevity can radically suppress community level transmission
(Killeen *et al.*, 2002). House designs in tropical areas have ventilations that allow easy
entry of mosquitoes. These ventilations are sometimes fitted with the insecticide-treated
screens that prevent entry while maintaining some ventilation (WHO, 1997). Since some malaria vectors enter houses to bite and rest, the use of insecticide treated nets (ITNs) for personal protection against *Anopheles* mosquitoes has become popular (Zaim *et al.*, 2000). Insecticide-impregnated bed nets have the advantage of acting as a physical barrier and also reduce mosquito densities by killing them.

### 1.4.2.2.1 House screening

House structures in the tropical countries are provided with ventilations. Openings such as windows and eaves allow easy entry of flying insects like mosquitoes. Thus screening of these openings prevents insects from entering while maintaining some ventilation (WHO, 1997). Treated screening or curtains provide a toxic barrier that prevent entry of mosquitoes into houses and may kill them at the same time. Mutinga *et al.* (1992) demonstrated that screens impregnated with permethrin can be effective against *An. gambiae s.s.* for about six months. However, the concept of house screening is not new. Towards the end of the 19th Century; Angelo Celli demonstrated that screening of houses against mosquitoes can protect people from malaria (Lindsay *et al.*, 2002). Screenings require less netting material compared to bed nets and are hence cheaper, in addition to requiring little or no attention from members of a household.

### 1.4.2.2 Repellents

Repellents are substances that drive away mosquitoes and other biting insects. Repellents like petroleum jellies are applied directly on the exposed skin or to clothing and other fabrics like bed nets and anti-mosquito screens to protect against mosquito biting (Curtis
et al., 1987). Insecticide vaporizers like mosquito coils and vaporizing mats have a deterrent effect hence prevent mosquitoes from entering a room. They also have excito-repellent effect which irritates and disturbs mosquitoes after contact. Commercial products may be too expensive for many communities so local plants and leaves are often burnt to produce smoke which repels mosquitoes (Seyoum et al., 2002a). As placing branches or whole plants inside houses to repel mosquitoes is another method of application practiced by communities in Western Kenya, Seyoum et al (2002b) showed that live and intact potted plants can reduce exposure to malaria vector mosquitoes.

1.4.2.2.3 Biological control

Biological control is the use of natural enemies to control pests and disease vectors. Organisms used include viruses, bacteria, protozoa, fungi, plants, parasitic worms, predatory mosquitoes, spiders and fish. A recent field study in Tanzania showed that an entomopathogenic fungus that infected and killed adult *Anopheles gambiae* would significantly reduce malaria transmission intensity even at moderate coverage (Scholte et al., 2005). Biological control methods have an advantage over chemical methods because they are non-toxic to fish, mammals and most other non-target organisms in the environment. Most biological control methods target the immature stages of the mosquito vectors.

1.4.2.2.4 Zooprophylaxis

Zooprophylaxis is the control of vector-borne diseases by attracting vectors to domestic or wild animals in which the pathogen cannot multiply (Kawaguchi et al., 2004). The
keeping of animals like cattle close to human habitation may reduce transmission of malaria by zoophilic and exophilic vectors like *An. arabiensis*. However, introduction of domestic animals may increase mosquito density thereby enhancing, rather than reducing, malaria transmission (Sota and Mogi, 1989). It is now known that presence of livestock increases mosquito fitness by supplying more blood, but reduces the basic reproductive ratio of the malaria parasite since the livestock act as a dead-end host for the parasite because the human malaria parasite *Plasmodium* species has a closed transmission cycle between humans and mosquitoes (Kawaguchi *et al.*, 2004).

Treating Zebu cattle with insecticide to control exophilic and zoophilic malaria vectors has been tried in Ethiopia (Habtewold *et al.*, 2004) and has shown promise. Combining zooprophylaxis and insecticide spraying may reduce insecticide resistance (Kawaguchi *et al.*, 2004), because the animal may act as a bait to attract mosquitoes and they will take a blood meal at the same time a large dose of the insecticides which are applied directly on the body surface of the animal. This may lead to the death of the mosquitoes rather than developing resistance to the insecticides.

Saul (2003) observed that zooprophylaxis may be inefficient with realistic values of host-searching by mosquitoes and the associated vector mortality although use of animals as bait to attract mosquitoes to insecticides is predicted to be a promising strategy. Furthermore, in Europe, changing of agricultural practices resulted in more effective zooprophylaxis and has been attributed to the disappearance of malaria (Bruce-Chwatt, 1985). However, zooprophylaxis alone may not be an effective intervention method
against malaria (Bogh et al., 2002). In an earlier study, Bogh et al (2001) observed that a passive zooprophylaxis using cattle does not alter the individual exposure to parasites in the Gambia. Therefore, the intervention of combining zooprophylaxis and insecticides may be a better option.

1.4.2.2.5 Chemical control

Chemical control is a strategy that involves use of chemical substances called insecticides to control or kill mosquitoes. The chemical control of mosquitoes can target the larval or adult stage of the mosquito’s life cycle using larvicides and adulticides, respectively. Adulticides are the products aimed at controlling the adult flying population. The common insecticides used to control vectors are DDT, malathion and synthetic pyrethroids. Insecticides can be used for indoor residual spraying, space spraying or for treatment of materials like curtains, bed nets and house screens.

Dichlorodiphenyltrichloroethane (DDT) is effective for residual indoor spraying but was banned from use due to its non-biodegradable effects (Curtis, 1994; Curtis, 1999). Malathion replaced DDT but has high refusal rate and shorter residual activity (White, 1999), hence could not be continued for spraying. Pyrethroids are quick acting and highly toxic to insects. They are also safe for humans and mammals at recommended dosages as well as being relatively safe for the environment because of their quick breakdown in the soil (WHO, 1997). However, many insects have developed resistance to most insecticides.
1.4.2.5.1 Residual and space spraying

Insecticides are used to spray space and walls inside human dwellings where they leave a residual effect. Indoor residual spraying is the most effective and feasible method of chemical control of malaria vectors and is the principal method of killing adult mosquitoes that rest indoors. However, control programs frequently lack well-trained field staff to apply the insecticides and to maintain the application equipment (WHO, 2002a). Besides, residual insecticides pollute the environment and may have adverse effects on human beings. Space spraying, on the other hand, is designed to provide a rapid knockdown and mortality of vectors with no or little residual effect, as part of the integrated vector management. This method aims at rapid reduction of flying insect populations to reduce or interrupt the transmission cycle of insect-borne diseases during emergency or epidemic situations (WHO, 2003). Nevertheless, space spraying is very costly and may not be economical in rural settings where homes are scattered.

1.4.2.5.2 Insecticide-treated materials (curtains and ITNs)

Insecticide-treated bed nets come in different shapes and colours and serve as human baited traps when people sleep under them by attracting and killing mosquitoes. They are, therefore, important tools in the control of malaria. Presently, synthetic pyrethroids are the only class of insecticides suitable for impregnation of bed nets because of their low toxicity to mammals and rapid action. Pyrethroids specifically have a powerful excito-repellent effect hence those lying next to individuals under the net are also protected from mosquito bites. The repellency effect also allows old, torn nets to remain relatively effective (UNICEF, 1991). Furthermore, permethrin-treated bed nets have a
community-wide suppression of mosquito population thus villages neighbouring intervention areas are also offered some protection (Gimnig et al., 2003b).

Recent studies in Western Kenya have shown that ITNs have high impact on the reduction of vector densities, sporozoite rates, morbidity and mortality in young children and malaria during pregnancy (Phillips-Howard et al., 2003a, Gimnig et al., 2003a, Ter Kuile et al., 2003). These results were consistent with reports from Gambia, Ghana, Burkina Faso and Coastal Kenya on the improvement of health in children following the use of ITNs (D’Alessandro et al., 1995, Binka et al., 1996, Habluetzel et al., 1997 and Nevill et al., 1996). Trial of ITNs in an area of Tanzania holoendemic for malaria also showed reduction in vector densities and sporozoite rates (Magesa et al., 1991). Other than bed nets, materials for curtains can be treated with insecticides and used to prevent mosquito house entry through openings like windows and eaves of houses in hot tropical countries. Mutinga et al (1993) observed that the insecticide-treated cotton clothes (ICIPE Mbu cloth), hung inside rural huts were effective in reducing malaria parasitemia in Marigat area of Baringo District, Kenya.

1.4.3 Vector resistance to insecticides

Resistance is the ability of an organism to tolerate doses of a toxicant that would be lethal to a majority of individuals in a normal population of the same species. It may arise as a result of mutations that alter normal physiological, morphological and behavioural attributes of a species (Ferrari, 1996). Resistance mechanisms include reduced penetration of the insecticide into the body of the vector due the changes in the cuticle,
metabolic detoxification of the insecticide by enzymes which convert them to harmless substances and target site insensitivity to insecticides due to mutations. Behavioural resistance involves reduced contact with the insecticide thus enhancing survival in a treated environment.

Insect populations have developed resistance to every class of chemical insecticide including microbial drugs and insect growth regulators (Ferrari, 1996, Brogdon and McAllister, 1998). Multiple resistances to all four groups of insecticides; organochlorines, organophosphates, carbamates and pyrethroids, have been reported in *An. sacharovi* in Southern Turkey (Kasap *et al.*, 2000). Cross-resistance to insecticides with similar mode of action aggravates the situation (Brooke *et al.*, 2000). Malaria vectors can develop resistance to insecticides by two main mechanisms both of which have a biochemical basis. These are metabolic detoxification and target site mechanisms. The target site for organophosphate and carbamate insecticides is acetyl cholinesterase in nerve synapse while that of organochlorines (DDT) and pyrethroids is the sodium channel of nerve sheath (Brogdon and McAllister, 1998).

### 1.4.3.1 Resistance of mosquitoes to pyrethroid insecticides

Widespread pyrethroid resistance in vectors of malaria has been reported in West Africa (Martinez-Torres *et al.*, 1998, Chandre *et al.*, 1999), in the Middle East (Enayati *et al.*, 2003) and Western Kenya (Vulule *et al.*, 1994). Thus, the monitoring and management of pyrethroid resistance is important in the use of pyrethroid-impregnated bed nets if this strategy of vector control is to succeed (Ranson *et al.*, 2000). The early detection of
resistance is a vital part of resistance management because it leads to the development of insecticide-use strategies that minimize the rate of evolution of resistance. Understanding insecticide resistance at molecular level is important as the resistance gene may be detected before it spreads to sibling species that are susceptible to pyrethroids (Ferrari, 1996, Weill et al., 2000). It has been noted that lack of insecticide use does not necessarily mean absence of insecticide resistance in an area (Brogdon and McAllister, 1998). For example, the movement of esterase resistance to organophosphates in Culex pipiens into certain areas of France has been reported (Rivet et al., 1994).

1.4.3.2 Knockdown resistance (kdr) in mosquitoes to pyrethroids

Among the resistant strains of mosquitoes that have been studied in West Africa, knockdown resistance (kdr) is the most common (Chandre et al., 2000). Knockdown resistance refers to the cases of resistance to DDT and pyrethroid insecticides in insects that result from reduced sensitivity of the nervous system (Soderlund and Knipple, 2003). This resistance mechanism results from a single point mutation (leucine-TTA to phenylalanine-TTT) in the Para-sodium channel (Martinez-Torres et al., 1998) and gives characteristic knockdown resistance. Such pyrethroid resistance is termed ‘kdr’ due to the knockdown resistant phenotype observed in house flies with this type of mutation (Williamson et al., 1996).

In Western Kenya, reduced susceptibility to permethrin was demonstrated in Anopheles gambiae two years after the first trial of permethrin–treated bed nets in 1990 (Vulule et al., 1994). A leucine-serine mutation was later found in the sodium channel of An.
*gambiae* from Western Kenya (Ranson *et al.*, 2000). The kdr mutation confers cross-resistance to pyrethroids and DDT because of the common mode of action of these insecticides. This supports the hypothesis that the heterogeneity observed in *An. gambiae* populations would have been caused by DDT even before the introduction of ITNs.

1.4.3.3 Gene flow and genetic differentiation

Gene flow is a collective term that includes all mechanisms which result in movement of genes from one population to another (Slatkin, 1985). Migration within established populations is one of the mechanisms of gene flow within a species and may determine genetic changes in local populations. Mutation and natural selection favouring adaptations to local environmental conditions lead to genetic differentiation of local populations and gene flow (movement of individuals or gametes), opposes that differentiation (Slatkin, 1987). This may lead to genetic homogeneity in adjacent populations of a species. There are two methods of estimating levels of gene flow: (1) direct methods by estimating dispersal distances due to mobility of a species and (2) indirect methods by use of allele frequencies (Slatkin, 1987).

Lehmann *et al.* (1996b) measured a low genetic differentiation between *Anopheles gambiae* populations from East and West Africa. This is because there is no barrier to gene flow between the two places though far apart. A study done in Asembo Bay in Western Kenya showed that genetic differentiation of *An. gambiae* mosquitoes among villages was low (Kamau *et al.*, 1998b). A similar study done in Mali also showed that
the gene flow among villages within each chromosomal form of *An. gambiae* was high while between species gene flow was low (Taylor *et al.*, 2001).

Kamau *et al.* (1999) observed no relationship between genetic differentiation and geographical distances hence concluded that there is high capacity for the spread of genes. It is, therefore, most likely that the gene responsible for insecticide resistance can spread since the area associated with a deme is more than 50 km in diameter (Lehmann *et al.*, 1996a). However, the Rift Valley complex has been shown to be a barrier to gene flow leading to genetic differentiation of mosquitoes in Kenya (Lehmann *et al.*, 2000).

The current study was concerned with the investigation of the effect of long-term use of pyrethroid-impregnated bed nets on the frequency and spread of the kdr alleles. The study also investigated species composition structure of *An. gambiae* s.s. and *An. arabiensis* following the long-term use of ITNs.

### 1.5 Problem Statement and Justification for the study

Insecticide resistance by vectors is a major obstacle to malaria control programs. When resistance arises, it leaves few insecticide choices due to cross-resistance to insecticides with similar mode of action. In many areas where malaria is a problem, mosquitoes are found throughout the year with density peaks coinciding with seasonal rains. Seasonal variation in insecticide resistance has also been reported in a number of insect species (Lenormand *et al.*, 1999). Therefore, it is important to understand the effects of the use
of the insecticide-treated bed nets (ITNs) on seasonal changes in species composition and on the development of insecticide resistance.

It is evident, from studies on the impact of ITNs that variation in mosquito densities and composition may have important consequences on the disease transmission in a given area (Gimnig et al., 2003a). Results from studies in Asembo, Rarieda Division of Western Kenya, have shown that the use of permethrin-treated bed nets in intervention villages has reduced vector densities greatly (Gimnig et al., 2003a). Previous work does not link the combined effect of seasonal changes and use of ITN to species composition and prevalence of knockdown resistance. Thus this study investigated the effect of long-term, intensive use of ITN on species composition and frequencies of the kdr allele in *An. gambiae s.l.* mosquitoes in a holoendemic malarious area of Western Kenya. The overall objective of this study was to assess the effect of the permethrin-treated bed nets on malaria vector species composition, the development and spread of insecticide resistance.

### 1.6 Null Hypotheses

1. Long-term use of ITNs does not affect changes in *An. gambiae s.l.* mosquito species composition during different seasons.

2. Long-term use of ITNs does not affect the prevalence of kdr gene in *An. gambiae s.s.* and *An. arabiensis*.

3. The prevalence of the kdr gene is not associated with changes in species composition in an area of long-term intensive ITN use.
4. There is no gene flow between an area of long-term ITN intervention (Asembo) and adjacent non-intervention area (Seme) mosquito populations.

1.7 Objectives of the study

1.7.1 General objective

To investigate the effects of long-term use of Insecticide Treated Nets (ITNs) on species composition and prevalence of the knockdown resistance (kdr) gene in *An. gambiae s.s.* and *An. arabiensis* in Asembo Location of Western Kenya during the rainy and dry seasons.

1.7.2 Specific objectives

1. To determine how the proportions of *An. gambiae* and *An. arabiensis* change with season in an area of intensive long-term ITN use.

2. To determine the effect of long-term ITN use on the prevalence of kdr gene in *An. gambiae s.s.* and *An. arabiensis*.

3. To establish whether the prevalence of the kdr gene in an area of long-term ITN use is associated with changes in mosquito vector species composition.

4. To measure gene flow between an area of long-term ITN intervention (Asembo) and an adjacent non-intervention area (Seme).
CHAPTER TWO
2 MATERIALS AND METHODS

2.1 Description of the study area

This study was conducted in East Asembo and South West Seme areas both on the Northern shores of Lake Victoria. Asembo is in Bondo District about 50km west of Kisumu Town while Seme is in Kisumu District about 40km west of Kisumu Town (Figure 1). Asembo and Seme study areas have similar climatic conditions and experience a bimodal pattern of rainfall, with the heaviest falling from March to May. A smaller peak occurs in November and December. The area is characterized by gently rolling hills that are drained by several small streams that empty into Lake Victoria. Approximately 16,190 people live in East Asembo location (one of the four locations of Rarieda Division whose total population is 56,883) and 13,624 in South West Seme location (one of the four locations of Kombewa Division whose total population is 60,183), (GoK, 2001).
Figure 1: Map of study areas showing the location of Asembo and Seme

NB: Scale in km.
2.2 Introduction and field evaluation of ITN efficacy in Asembo

Asembo is part of the initial study site in Rarieda Division where ITN trials started in 1992 (Bloland et al., 1999a, b). Fifteen villages were randomly allocated with ITNs then entomological and epidemiological outcomes followed longitudinally. Following the observed improvement on child health and reduction in malaria cases during pregnancy, large-scale allocation of ITNs was implemented in 1997 by public lottery to achieve a coverage ratio of 1.34 persons per net (Phillips-Howard et al., 2003b). Studies evaluating child morbidity and mortality, mosquito density and malaria incidences were conducted in this study area and ITNs proved to be effective in reducing vector densities and incidence of new infections. Monitoring of insecticide resistance has not been done regularly apart from the one done in 1991 when evidence for reduced susceptibility to permethrin was first noticed, and later on when mechanism of permethrin tolerance was documented (Vulule et al., 1994, 1999).

2.3 Housing structure and type of farming in the study areas

Most of the communities in both areas practice subsistence farming, main crops being maize, millet, cassava and groundnuts. Sheep, goats and cows are also present. The inhabitants are mainly of the Luo tribe living in highly dispersed homesteads surrounded by their crop fields. Their houses are constructed of mud, sticks and grass thatch. Some are made of brick walls with iron sheet roofs. Most houses have open eaves hence mosquitoes enter and exit unhindered. The homesteads consist of two to four houses (Plate 1).
Plate 1. A site in Seme showing a typical Luo homestead
2.4 Sampling scheme

Mosquito sampling was carried out along a 12km transect from the middle of Asembo (ITN implementation region) to the middle of Seme (control region) with 6km on either side of the border. Six sampling points were selected and from each of these points, ten family compounds (homesteads) were included. All houses in each family compound were sampled provided people had slept in them the previous night. Family compounds consisted of two to four houses therefore a total of about 220 houses were sampled once. Sampling was carried out from 8.00 –11.30 a.m. Larvae were also collected from breeding sites located within 100-200m from houses in each family compound.

2.5 Mosquito collection, transport and preservation

Sampling for mosquitoes in the selected villages was done in the month of February 2004 for dry season samples and in the month of May 2004 for rainy season samples. Indoor resting mosquitoes were collected by manual aspiration using mouth aspirator. Torches were used to illuminate the dark hiding places in the house. The mosquitoes were then transferred into a labeled paper cup for each house.

Larvae were collected using a dipper and a pipette. First the pool of turbid water was observed for larval movements then 4-10 dips were made depending on the size of the larval habitat. The dipper was lowered at 45° until one side was just below the surface of the water (WHO, 2002). Larvae were then searched for in the dipper by the eye (Plate 2). The larvae below or on the surface of the water in the dipper were picked by a pipette and transferred to a collecting tube labeled for the habitat and the compound number.
The types of larval habitats that were sampled included transient rain pools, hoof prints (Plate 3), permanent pits (Plate 4) artificially created by human activities, edges of dry seasonal swamps and from small pools of water on dry riverbeds. Since this study was not concerned with mosquito densities, collection was done once in each habitat type at each of the six sampling points to get samples for genetic analysis.

The paper cups containing adults and the collecting tubes containing larvae were put in a cool box cushioned with cotton wool and transported to the laboratory. Back in the laboratory, the larvae were air dried while the adults were inactivated by placing them in the refrigerator at 4°C for about 10 to 15 minutes. They were then preserved individually in 1.5ml microfuge tube using silica gel as the drying agent.
Plate 2. Searching for larvae in the dipper
Plate 3. Hoof prints filled with water as breeding site for *Anopheles* mosquitoes
Plate 4. Man-made pit as a breeding site for *Anopheles* mosquitoes; feasibility study
2.6 Mosquito identification based on resting position and morphology

*Anopheles* adult mosquitoes were distinguished from genus *Culex* by their resting position on the surface on which they rest. The mosquitoes of genus *Anopheles* rest with the abdomen held up at an angle from the surface on which it is resting, forming a straight line with the proboscis whereas genus *Culex* rest with the abdomen parallel with the surface on which it rests. *Anopheles* larvae, on the other hand, rest parallel to water surface while the *Culicine* larvae hang vertically from the water surface.

Adults and larvae of *Anopheles* mosquitoes collected from Asembo and Seme were further identified morphologically as *An. gambiae* complex under the light microscope using wing, leg and abdomen markings for adults while the mesopleural basal hook was used for identification of the larvae (Gillies and De Meillon, 1968). Presence of large curved and sharply pointed basal spine of pleural hairs and poorly developed inner shoulder hairs were used to distinguish the larvae of *An. gambiae* complex from other anophelines. On the other hand, adults have speckled legs, a pale interruption of the 3\textsuperscript{rd} main dark area of vein one and very scanty scaling on the abdomen confined to the 8\textsuperscript{th} or rarely 7\textsuperscript{th} terga (Gillies and Coetzee, 1987). The few *Anopheles funestus* mosquitoes collected were not used in this study.

2.7 Extraction of DNA from individual mosquitoes

Genomic DNA was extracted from the single field collected mosquitoes using the alcohol precipitation method according to Collins *et al* (1987). Briefly, adult mosquito abdomens or whole larva specimens were individually placed into a 1.5 ml microfuge tube
containing 100µl of grinding buffer (4 parts of homogenization buffer and 1 part of lysis buffer) and ground. The triturated homogenized specimen was incubated in a water bath at 65°C for 30 minutes to denature the nucleases. Fourteen microlitres of Potassium acetate (8M) was then added and the mixture cooled on ice for 30 minutes to precipitate the mosquito parts, other insoluble substances and denatured proteins. The mixture was spun in a cold centrifuge (4°C) at 14,000 revolutions per minute (rpm) for 15 minutes and the resulting supernatant transferred to a new sterile microfuge tube. Two hundred microlitres of cold 100% ethanol was then added and the mixture chilled overnight at -20°C to precipitate DNA and then centrifuged for 20 minutes to pellet the DNA. The excess ethanol was poured out and the DNA pellet rinsed with 200µl of 70% ethanol. The pellet was rinsed again with 200µl absolute ethanol (100%) and air-dried for 12 hours at room temperature after which the DNA was resolubilized in 100µl of sterile deionized water and stored at -20°C. One microlitre of this DNA was used for the polymerase chain reaction (PCR) to distinguish between *An. gambiae s.s.* and *An. arabiensis*.

### 2.8 Species identification by Polymerase Chain Reaction (PCR)

*Anopheles gambiae s.s.* and *An. arabiensis* were distinguished by PCR using the method of Scott *et al.* (1993). The DNA was amplified in a GeneAmp PCR system 9700 machine supplied by Applied Biosystems. For a single 15µl reaction, the following were added to the appropriate 0.2 ml PCR tube: 1.5µl of 10x MgCl₂ free buffer, 1.8µl of 25mM MgCl₂, 0.6µl of 10mM dNTP, 0.52µl of 0.02µg/µl of each diagnostic primers, 0.06µl of amplitaq polymerase, 1µl of template DNA and sterile deionized water added to final volume of
The three different specific primers used in the polymerase chain reaction (PCR) were; universal primer (UN), *An. gambiae* (GA) and *An. arabiensis* (AR). The primers were supplied by Sigma and the sequences were: GA- 5’ CTG GTT TGG TCG GCA CGT TT 3’; AR- 5’ AAG TGT CCT TCT CCA TCC TA 3’; UN- 5’ GTG TGC CCC TTC CTC GAT GT 3’. The PCR was performed for 30 cycles at a denaturation temperature of 94°C for 30 seconds, an annealing temperature of 60°C for 30 seconds and an extension temperature of 72°C for 30 seconds. The program included a pre-denaturation step of 5 minutes at 94°C and a final extension step at 72°C for 5 minutes. The amplified DNA was then analyzed by electrophoresis on agarose gel or stored at 4°C awaiting electrophoresis.

### 2.9 Agarose gel electrophoresis

To analyze the PCR products, 15μl of each amplified sample was mixed with a standard agarose gel loading buffer containing bromophenol blue dye and electrophoresis performed in 3% agarose Tri-Borate-EDTA (TBE) gels containing ethidium bromide for staining. The gels were run for 20-30 minutes at 5 to 10 v/cm for sufficient separation of PCR products. Control *An. gambiae* and *An. arabiensis* specimens, which give characteristic bands of 390bp and 315bp, respectively, were included on all gels (Cornel and Collins, 1995). The amplified fragments (DNA bands) were visualized by illumination with short wave ultraviolet light and photographed using a polaroid camera for documentation.
2.10 Diagnostic test for knockdown resistance alleles

Analysis of knockdown resistance alleles was done using the method of Martinez-Torres et al (1998) but according to the adaptation made by Ranson et al (2000). Three microlitres of extracted DNA from a single mosquito were used as a template in a 25 µl reaction volume together with the following: 2.5 µl of 10x buffer, 2.0 µl of 25mM MgCl₂, 2.0 µl 10mM dNTPs, 0.25 µl of primer AgD₁ & AgD₂, 0.42 µl of primer AgD₄ & AgD₅ and 0.1 µl of Taq Polymerase. The primers were supplied from International Livestock Research Institute (ILRI) in Nairobi-Kenya and the sequences were as follows:

AgD₁ - 5’ATA GAT TCC CCG ACC ATG 3’
AgD₂ - 5’AGA CAA GGA TGA TGA ACC 3’
AgD₄ - 5’CTG TAG TGA TAG GAA ATT TA 3’
AgD₅ - 5’T TT GCA TTA CTT ACG ACT G 3’

As amounts of primer in the stocks were not quantified by the supplier, serial dilutions were used to determine the primer amounts that gave satisfactory amplification of the expected products. Primers AgD₁ and AgD₂ amplify a 293bp fragment in the region where kdr mutation occurs while primers AgD₂ in combination with primer AgD₄ give a 137bp fragment which characterizes the susceptible allele. Primer AgD₁ in combination with primer AgD₅ give a 195bp fragment which characterizes the resistant allele. The cycling conditions were a pre-denaturation step of 5 minutes at 94⁰C, 30 cycles of 25 seconds at 94⁰C, 20 seconds at 56⁰C and 8 seconds at 72⁰C followed by a final extension step of 10 minutes at 72⁰C. One larva from each breeding site was included in the
analysis of knockdown resistance allele to minimize number of siblings, which are closely related genetically.

2.11 Ethical considerations and clearance

The study involved intrusion of privacy during indoor resting collections and interruption of house owners’ daily routine. However, long-term studies have been going on in the area hence a good rapport exists between the local people and the researchers and therefore, the residents gave consent. The bed net study was reviewed and approved by the institutional review boards of the Kenya Medical Research Institute (KEMRI, Nairobi, Kenya) and the Centers for Disease Control and Prevention (CDC, Atlanta, GA).

2.12 Statistical Analyses

The difference in proportions of *An. gambiae* and *An. arabiensis* was tested by Binomial Regression, using the GENMOD procedure in SAS, as a measure of effect of ITN. The significance of the trend in kdr allele distribution was analyzed by logistic regression. The differences in kdr allele frequencies between Asembo sampling points and Seme sampling points matched by distance from the border and the level of gene flow between Asembo and Seme were analyzed by genic differentiation measures available in Genepop version 3.1d. Mean allele frequencies in Asembo and Seme were compared by Fisher Exact test. Genetic differentiation between Asembo and Seme based on the kdr allele was estimated using Fst, according to Weir and Cockerham (1984) and its significance was assessed by P-value from contingency table. The migration index (Nm value), which indicates the effective number of migrants exchanged between the populations in one generation, was calculated from Fst values by using Wright’s formula: \( Fst=1/(1+4Nm) \), according to Slatkin (1987). Values of Nm greater than one suggest significant rates of gene flow (migration). The difference in kdr phenotypes between Asembo and Seme mosquitoes was analyzed by ANOVA.
3 RESULTS

3.1 Analysis of *An. gambiae* s.s. and *An. arabiensis* species proportions in Asembo and Seme

A total of 1033 *Anopheles gambiae* s.l. mosquitoes were collected from Asembo and Seme during the rainy season in May 2004 and 815 were identified to species level by the PCR assay. Of the 256 *An. gambiae* s.l. mosquitoes collected from Asembo, the proportion of *An. gambiae* s.s. was 59% while in Seme, this species accounted for about 79% of the 559 specimens collected. Collections from Asembo consisted of 94 larvae and 162 adults while those collected from Seme were 74 larvae and 485 adults. Overall, a greater proportion (79%) of adults compared to larval stage (21%) was collected (Table 1).
Larval species composition indicated a higher proportion of *An. arabiensis* than *An. gambiae s.s.* in both Asembo ITN region and Seme non-ITN area but statistical analysis showed that the distribution of these species was significantly different between the two areas (Binomial regression GenMod procedure, $P=0.0001$). The proportions of *An. gambiae s.s.* in Asembo and Seme in larval collections were 11.7% and 20.3%, respectively. Contrary to the observation made on the species proportions among the larval collection, the proportion of *An. gambiae s.s.* was greater than that of *An. arabiensis* in the adult mosquito collections in both Asembo and Seme. For the adult mosquito samples, 86.4% (140 out of 162) and 87.6% (425 out of 485) were *An. gambiae s.s.* in Asembo and Seme, respectively, while the percentages of *An. arabiensis* were 13.6% (22 out of 162) and 12.4% (60 out of 485) in Asembo and Seme, respectively. When data from the larval and adult mosquito collections were combined, mean percentages of *An. arabiensis* were 41% and 21.3% in Asembo and Seme respectively. Collections made from the first two sampling points in Asembo, furthest from the Asembo-Seme border, had the largest *An. arabiensis* proportion whereas the two sampling points in Seme furthest away from the Asembo-Seme border had the lowest proportions of *An. arabiensis*. *Anopheles gambiae s.s.* dominated in Seme at points furthest away from the border of Asembo and Seme. Figures 2 and 3 show the frequency distribution of *An. gambiae s.s.* and *An. arabiensis* along the Asembo-Seme transect for the larval and adult mosquito collections, respectively.
Figure 2. Frequency distribution of *Anopheles gambiae* s.s. and *An. arabiensis* along the Asembo-Seme transect among the larval mosquito collections. The sampling points; 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme.
Figure 3. Frequency distribution of *Anopheles gambiae* s.s. and *Anopheles arabiensis* along the Asembo-Seme transect for the adult mosquito collections. The sampling points; 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme.
During the dry season in February 2004, only 42 *An. gambiae* s.l. mosquitoes were collected from both Asembo and Seme (10 from Asembo and 32 from Seme), two of which were not identified to species level due to non-amplification by PCR. Out of the ten larvae collected from Asembo, 11% (1 out of 9) were *Anopheles gambiae* s.s. while 89% (8 out of 9) were *Anopheles arabiensis*. In Seme, larvae of *An. gambiae* s.s. accounted for 70% while 30% was *An. arabiensis*. These samples were collected from the second sampling point in mid Asembo and the last sampling point at extreme end of Seme away from the Asembo-Seme border (Figure 4). The rest of the sampling points had dry breeding sites or muddy dirty water without larvae. No adult mosquitoes were collected from Asembo during the dry season and only 22 were collected from Seme. The proportion of *An. gambiae* s.s. adults collected in Seme was 86% (18 out of 21) and that of *An. arabiensis* was 14% (3 out of 21).
Figure 4. Species proportions of *Anopheles gambiae* s.l. collected from Asembo and Seme during the dry season. The sampling points; 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme.
3.2 Analysis of the presence of knockdown resistance (kdr) gene by polymerase chain reaction (PCR)

The kdr diagnostic PCR amplified three different fragments of sizes 137bp, 195bp and 293bp in mosquitoes collected from Asembo and Seme. Plate 5 shows a typical agarose gel with sample results of the amplified fragments.

Plate 5. A typical gel showing the different fragments obtained with the kdr diagnostic polymerase chain reaction.

Fragment 293bp, shown in lanes 1, 4, 5, 6, 8, 10, 11, 13, 14 and 15, is the universal band present in all mosquito specimens and represented the region (sodium channel) that may contain the kdr mutation. Fragment 195bp, shown in lanes 1, 14 and 15 characterizes the kdr allele while fragment 137bp shown in lanes 4, 5, 6, 8, 10, 11, 13, 14 and 15, characterizes the susceptible allele. Lane M contains 100bp DNA marker.
3.2.1 Frequency distribution of the kdr allele and genotypes in *An. gambiae s.s.* from Asembo and Seme

Each gene at a particular locus consists of a pair of alleles that determine the genotype of the individual. Allele frequencies, were therefore, calculated from the various genotypes obtained with kdr diagnostic PCR. Homozygous susceptible (SS) mosquitoes contained two susceptible alleles while the homozygous resistant (RR) mosquitoes contained two resistant alleles. Mosquitoes that were heterozygous (SR) based on kdr gene had one resistant and one susceptible allele. A total of 402 *An. gambiae s.s.* specimens were scored for kdr genotypes. These were mainly adults from the two study areas. Majority of the larvae were *An. arabiensis* which were all homozygous susceptible hence not included in the analysis of the kdr frequency. For the few *An. gambiae s.s* larvae that were collected, one specimen from each breeding site was used to minimize inclusion of siblings which are closely related genetically.

Samples from both Asembo and Seme exhibited all possible genotypes, that is, homozygous susceptible (SS), heterozygous (SR) and homozygous resistant (RR). The distribution of susceptible, resistant and heterozygous genotypes is shown in Table 2 while the calculated kdr allele frequencies are shown in Table 3. The kdr allele frequency distribution in *An. gambiae s.s.* per collection point along Asembo-Seme transect is shown in Figure 5.
Table 2. Genotypic distribution of *An. gambiae s.s.* in Seme and Asembo

<table>
<thead>
<tr>
<th>Area</th>
<th>Zone</th>
<th>SS</th>
<th>RR</th>
<th>SR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asembo</td>
<td>1</td>
<td>9</td>
<td>5</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>57</td>
<td>7</td>
<td>11</td>
<td>75</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>66</td>
<td>14</td>
<td>24</td>
<td>104</td>
</tr>
<tr>
<td>Seme</td>
<td>4</td>
<td>87</td>
<td>9</td>
<td>16</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>46</td>
<td>11</td>
<td>15</td>
<td>72</td>
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<tr>
<td></td>
<td>6</td>
<td>92</td>
<td>8</td>
<td>11</td>
<td>111</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>227</td>
<td>29</td>
<td>42</td>
<td>298</td>
</tr>
</tbody>
</table>

50
Table 3. Frequency distributions of the kdr allele along Asembo-Seme transect for *An. gambiae s.s.*

<table>
<thead>
<tr>
<th>Area</th>
<th>Zone</th>
<th>Resistant</th>
<th>Susceptible</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asembo</td>
<td>1</td>
<td>21(42%)</td>
<td>29(58%)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6(75%)</td>
<td>2(25%)</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25(16.7%)</td>
<td>125(83.3%)</td>
<td>150</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>52(25%)</td>
<td>156(75%)</td>
<td>208</td>
</tr>
<tr>
<td>Seme</td>
<td>4</td>
<td>34(15.2%)</td>
<td>190(84.8)</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>37(25.7%)</td>
<td>107(74.3)</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>29(12.7)</td>
<td>199(87.3%)</td>
<td>228</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>100(16.8%)</td>
<td>496(83.2%)</td>
<td>596</td>
</tr>
</tbody>
</table>

NB: Table 3 was generated from table 2 i.e. alleles were calculated from genotypes.
Figure 5. The frequency distributions of the kdr susceptible and resistant alleles per collection point along Asembo-Seme transect. The sampling points; 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme. Only 4 mosquitoes were analyzed for the collection point 2 in Asembo; half of which were homozygous resistant and the other half were heterozygous.
The highest frequencies of the kdr resistant allele were observed for samples taken in Asembo away from the Asembo-Seme border while the lowest were for the samples from Seme away from the border. Frequencies of the kdr allele for the samples at points located at the border of the two study areas were similar. Overall, the trend indicated that the kdr allele frequency decreased from the Asembo ITN area towards the Seme control area but this trend was not statistically significant (logistic regression, $X^2=2.7664$, df=1, $P=0.0963$). The level of genetic differentiation between Asembo and Seme based on the distribution of the kdr allele was estimated using Fst which is the standardized variance in allele frequencies. An Fst value of 0.0098 was obtained for this comparison and corresponded to an Nm value of 25.3, suggesting that there are significant levels of gene exchanges between the two areas. Pair wise comparisons of allele frequency of points matched by distance from Asembo-Seme border showed that points furthest from the border were significantly different (Fst=0.2564, $P=0.00000$) but those near the border were not significantly different (Fst=-0.0075, $P=0.76806$). The mean kdr allele frequencies for Asembo and Seme were 25% and 16.7%, respectively, but these were not significantly different from each other (Fisher Exact test, $F=17.9614$, df=2, $P=0.05274$).

3.2.2 The analysis of phenotypes of An. gambiae s.s. mosquitoes collected from Asembo and Seme

The kdr allele is completely recessive and therefore, all mosquitoes that are homozygous for the susceptible allele or heterozygous are phenotypically susceptible to pyrethroid insecticides and only those that are homozygous for the resistance allele constitutes the resistant phenotype. Although the frequency of resistant phenotype appeared to be higher
in Asembo (13.5%) than Seme (9.7%), this was not statistically different (ANOVA, F=8.71E-16, df=1.4, P=1.000). Figure 6 shows phenotypic frequencies based on kdr allele for *An. gambiae s.s.* in Asembo and Seme.

Figure 6. The frequency of susceptible and resistant phenotypes in *An. gambiae s.s.* along the 17km Asembo-Seme transect. The sampling points 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme. Only 4 microplates were analysed for the collection point 2 in Asembo, of which 3 were heterozygous while the other were resistant.
Figure 6. The frequency of susceptible and resistant phenotypes in *An. gambiae* s.s. along the 12km Asembo-Seme transect. The sampling points; 1, 2 and 3 represent Asembo while 4, 5 and 6 represent Seme. Only 4 mosquitoes were analyzed for the collection point 2 in Asembo, of which 2 were heterozygous while the other 2 were resistant.
Dry season samples that amplified for kdr diagnosis were all 100% homozygous susceptible. The *An. arabiensis* mosquitoes were also all susceptible for the two-season collections.
CHAPTER FOUR

4 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

Significant differences were observed in the proportions of *An. gambiae* and *An. arabiensis* mosquitoes between the Asembo area where ITN use is intensive and in Seme where it is not intensive, suggesting that long-term ITN use has affected the distribution of these two species. This effect is most likely associated with behavioural differences between the two species with respect to blood feeding and resting. In the absence of the availability of ready human blood meals under conditions of ITN use, the more zoophilic *An. arabiensis* would be expected to have a competitive advantage over the highly anthropophilic *An. gambiae* (Gillies and De Meillon, 1968) as the former species would utilize a broad range of other alternative hosts. In addition, *An. arabiensis* has been shown to commence biting at dusk before people retire to bed (WHO, 1997), a behaviour that improves the chances of this species acquiring human blood meals over *An. gambiae*.

*Anopheles arabiensis* is also preferentially exophilic, with a high proportion of the population exiting houses after acquiring blood meals. This behaviour reduces the chances of mosquitoes of this species coming into contact with the insecticide-treated surface and acquiring lethal doses of the insecticide. Studies by Service *et al* (1970, 1978) have even shown that this exophilic behaviour of *An. arabiensis* is enhanced by house-spraying. Thus, *An. arabiensis* is the malaria vector of concern since it avoids contact with the insecticide-treated surface even though it is susceptible to pyrethroids that are used to treat the bed nets. Therefore, a different strategy is needed for the control
of *An. arabiensis* whose proportion seems to increase in the ITN area. In an attempt to formulate an alternative approach of increasing insecticide efficacy to control this species, a recent study done in Ethiopia suggested that *An. arabiensis* may be controlled by applying insecticides to Zebu cattle that are an alternative host of this species (Habtewold *et al.*, 2004).

On the other hand, the proportion of *An. gambiae s.s.* which has declined in the ITN intervention area is paradoxical in that, it is expected to be greater than that of *An. arabiensis* since the former species has been noted to have reduced susceptibility to pyrethroid insecticides (Vulule *et al.*, 1994). However, it has been speculated that resistant females of *An. gambiae s.s.* are killed by prolonged contact with the insecticide-treated surface due to diminished sensitivity to the irritant effects of pyrethroids (Chandre *et al.*, 2000). Longer contact with the insecticide-treated surface allows them to pick lethal dose of the insecticide through their tarsi, and this may explain the declining proportion of *An. gambiae s.s.* in areas with widespread use of ITN.

Very few mosquitoes were collected during the dry season and it was not possible to get samples from all the sampling points along the established sampling transect. Furthermore, no adults were collected from the Asembo ITN area during the dry season and this is consistent with an earlier study which observed a decline in mosquito densities in intervention houses (Gimnig *et al.*, 2003a). Comparison of the proportion of *An. arabiensis* in dry season and rainy season showed that this species was more abundant during the dry season particularly in Asembo ITN area.
Analysis of knockdown resistance in this study revealed a three-fold and four-fold increase in the frequency of the kdr allele in Asembo and Seme respectively when compared with the results reported by Stump et al (2004) for a 2000/2001 study in the same area. The study involved use of pyrethrum spray collection method and it is possible that kdr allele frequencies were under-estimated as the homozygous resistant genotype (the resistant phenotype) could have been under-sampled using this method. When the data was reanalyzed whilst excluding this genotype, the frequencies of the kdr alleles were 13.3% and 7.9%, representing 1.7-fold and 2-fold increases, in Asembo and Seme, respectively. This suggests that there has been a real increase in the frequency of the kdr allele in this area within the intervening four-year period between the two studies and that the spread of this mutation between the two areas has been maintained. This also implies that although the two areas experience significant levels of gene exchange, the spread of the kdr alleles from Asembo ITN area to Seme non-ITN area is recent and ongoing.

There was no trend in the frequency of the kdr allele from the area of intensive ITN use towards the area where ITN use was not intensive. This finding can be explained in terms of the spill over effects between the two areas given that this study covered a 12 km transect. To estimate the degree of genetic differentiation between the Asembo and Seme mosquito sub-populations using kdr allele as a genetic marker, the variance in allele frequencies standardized by the mean frequency among populations (Fst) alongside migration indices (Nm) were calculated between the paired points equidistant from the Asembo-Seme border on both sides. The Nm values indicate the relative strength of gene
flow and genetic drift, where values greater than one are expected to result from gene flow but those less than one are associated with genetic drift (Slatkin, 1987). Genetic differentiation in terms of kdr allele was found to be relatively high away from the Asembo-Seme border but low at the border. This supports the suggestion that the rising level of kdr frequency observed in Seme is due to the spill over effects from Asembo ITN area. This also implies that although the two areas experience significant levels of gene exchange, the spread of the kdr alleles from Asembo to Seme is recent and on going. Furthermore, pooled allele frequency for Asembo versus Seme showed low genetic differentiation between the two areas with population exchanging an average of 25.3 individuals per generation and suggesting that resistance alleles are spreading from Asembo ITN region to Seme non-ITN region. These findings on genetic variation are consistent with previous studies on population genetic structure in Western Kenya, which showed that the minimum area associated with a deme is about 50km (Lehmann et al., 1996a) and there is no barrier to gene flow at microgeographic level (Kamau et al., 1998b). Therefore, the spread of kdr gene seems to be an on going process in the absence of barriers to the gene flow and this may threaten the effectiveness of ITN use.

Though the homozygous resistant mosquitoes may be killed due to reduced sensitivity to pyrethroids, the frequency of the kdr allele in the heterozygous state remains the same and may be a resource for natural selection. Thus, the gene flow between Asembo and Seme might have decreased diversity among these two populations in terms of kdr allele frequency, causing their gene pools to be similar. Consequently, natural selection may
not be limited to Asembo area where intense widespread use of ITNs was implemented in 1997 but also most likely in the adjacent Seme non-ITN area.

Brogdon and McAllister (1998) have argued that the level of resistance to insecticides must be high enough to affect a control method for resistance to be a concern. The question that remains to be answered, however, is what levels of resistance are high enough to compromise the efficacy of intervention programs employing insecticides for vector control? The current study found the percentages of the resistant phenotypes to be 13.5 and 9.7% in Asembo and Seme respectively, suggesting that a bigger part of the mosquito population is still susceptible to pyrethroids. In addition, the frequency of heterozygous mosquitoes compared to homozygous resistant was higher in the two study areas. The knockdown resistance (kdr) allele is known to be recessive (Hemingway et al., 1995) and this further supports the suggestion that a greater proportion of the population is still susceptible to pyrethroids in Western Kenya. These findings are consistent with those obtained with field samples from Ivory Coast where frequency of resistant homozygous individuals was extremely low in adult females even for samples where frequency of heterozygosity was about 75% (Weill et al., 2004).

In Ghana and Burkina Faso, high kdr frequencies have been reported ranging from 98-100% (Yawson et al., 2004) while in Mali, the kdr frequency of 89% has been reported (Fanello et al., 2003). Despite the presence of the kdr allele in malaria vectors, various studies conducted in West African countries have shown that ITNs are effective in controlling malaria. However, the degree of anthropophily of An. gambiae decreases
from West to East Africa. This implies that the impact of ITN intervention may be higher in West Africa where the malaria vectors must get a blood meal from humans (Costantini et al., 1999). Nevertheless, the kdr frequency in East Africa is much less than West Africa and a greater proportion of the mosquito population are still susceptible to pyrethroids. Besides, the leucine-serine mutation found in East Africa offers less resistance to pyrethroids than the leucine-phenylalanine mutation found in West Africa (Ranson et al., 2000). Therefore, ITNs are effective in controlling malaria even in East Africa.

Insecticide resistance studies conducted in Ghana and Nigeria found exposure to deltamethrin to result in more than 95% mortality despite widespread prevalence of the kdr mutation in West Africa (Kristan et al., 2003). In Côte d’Ivoir, nets impregnated with permethrin or deltamethrin were found to provide good levels of protection where the frequency of the kdr allele was 94% (Chandre et al., 2000). These findings are somewhat paradoxical when considered in the light of the fact that the kdr mutation is one of the main mechanisms of resistance to pyrethroids. However, because the kdr mutation is associated with reduced irritant effects to pyrethroids, it has been speculated that this allows resistant mosquitoes to stay longer on treated surfaces and thus acquire lethal doses of the insecticide (Chandre et al., 2000). This may also explain the declining percentages of An. gambiae, associated with the widespread ITN use, which was observed in this study. It seems then, that pyrethroid-impregnated nets may continue to be effective even among An. gambiae populations with relatively high frequencies of the kdr gene. But contrary to the frequent observations of the presence of the kdr mutation
associated with insecticide use, this mutation has not been detected in the Bioko Island in Equatorial Guinea four years after the onset of an ITN program for malaria control (Berzosa et al., 2002). This emphasizes the focal nature of insecticide resistance and thus the need for continued surveillance.

All Anopheles arabiensis mosquitoes were found to be homozygous susceptible although they were collected from same houses and breeding sites as An. gambiae s.s. This finding is similar to those of other studies in several west African countries where little or no resistance has been found in An. arabiensis and the M form of An. gambiae s.s. despite the presence of high frequencies of the kdr mutation in the S form of An. gambiae s.s. (Chandre et al., 1999, Kristan et al., 2003, Fanello et al., 2003, Diabate et al., 2002, Yawson et al., 2004). The exophilic and zoophilic behaviour of An. arabiensis may likely explain the lack of the kdr mutation in this species: because mosquitoes exit houses soon after feeding, chances of prolonged contact with the insecticide treated surfaces are greatly reduced thus reducing the selection pressure for the mutation. In addition, An. arabiensis will utilize other available alternative hosts, reducing the need to rely on human host and the associated risk of prolonged contact with the insecticide treated surfaces in attempts to acquire such blood meals. Two cases of the occurrence of the kdr mutations in An. arabiensis have, however, been reported, one in Burkina Faso (Diabate et al., 2004) and the other in western Kenya (Stump et al., 2004). The occurrence of kdr allele in An. arabiensis may be due to an independent mutation event as was found in the Burkina Faso case, since the exact point of mutation was different from that of An. gambiae s.s., but could also arise by a transfer from An. gambiae s.s. through
introgression. Both of these events would be expected to be rare since the partially zoophilic and exophilic behaviour of An. arabiensis reduces selection pressure due to house spraying and use of ITNs and because interbreeding between wild populations of An. gambiae complex sibling species is infrequent, hybrids existing at frequencies of only between 0.1% and 0.2% (White, 1971). Kamau et al (1998a) have also estimated low Nm value between the two sibling species signifying extremely low or no interchange of genes. On the other hand, Hargreaves et al (2003) have proposed that the absence of pyrethroid resistance in An. arabiensis suggests that kdr gene does not play a role but increased levels of non-specific esterases and glutathion- S- transferase enzyme activity could account for resistance in this species.

Comparison of rainy and dry season samples showed that resistance occurs during the rainy season. This has been shown to be the case in Burkina Faso (Diabate et al., 2002) but this is in rhythm with use of agricultural pesticides. In Southern France, seasonal variation in resistance in Culex pipiens to organophosphates has also been demonstrated (Lenormand et al., 1999). Very few adults were collected from Seme during dry season and these were all susceptible unlike those collected from the same sampling point during the rainy season. These results remotely suggest that resistance is higher during the rainy season. Since the rainy season may be associated with low temperatures, and dry season with high temperatures, there could be a relationship between resistance and season because effects of permethrin-impregnated nets have been found to be greater at 37°C than at low temperatures (Hodjati and Curtis, 1999). However, since the dry season samples were few in this study, the conclusion that resistance is higher during the rainy
season is conservative at this stage. It has been observed that mosquito densities resting indoors are greatly reduced in ITN area (Gimnig et al., 2003a). Therefore, it is difficult to get many indoor-resting mosquito samples in the ITN area during the dry season to compare resistance with the rainy season samples.

4.2 Conclusions
The findings of this study show that intensive use of ITNs has altered species proportions, that of *An. arabiensis* being significantly higher than that of *An. gambiae* in Asembo. The rising proportion of *An. arabiensis* suggests that this species avoids contact with the insecticide-treated surface. Thus, this species may continue transmitting malaria in the presence of ITNs when it comes in contact with exposed humans.

The decrease in density of the more serious vector, *An. gambiae s.s.*, in the ITN intervention area is important for the community in reducing disease incidences. However, the finding that *An. arabiensis* has increased in proportion necessitates integration of other control approaches.

Increase in kdr allele frequency following widespread use of ITNs points to possible future impairment of pyrethroid impregnated bed nets as a control strategy for malaria. Thus, identification of resistance alleles in areas where insecticides have been used is necessary to enable the monitoring of resistance status and hence early resistance management. Knowledge on the frequency of resistance genes may help in formulating application regimes that will reduce further increase in resistance.
There is high rate of gene flow (kdr allele) between Asembo and Seme hence spread of kdr allele to non-ITN areas is an ongoing process. Therefore, lack of insecticide use does not preclude immigration of resistance genes within distances such as those of between the two study areas.

The findings of this study may lead to a better understanding of insecticide resistance level in mosquito vectors to improve the efficacy of impregnated bednets. Besides, the prediction of conditions under which insecticide resistance occurs is necessary in order to minimize the evolution of resistance in insect vectors. On the other hand, surveillance of the presence, frequency and distribution of resistance genes may help during emergency use of insecticides.

### 4.3 Recommendations

1. A different strategy, other than ITNs, is needed for the control of *An. arabiensis* (partially exophilic vector) whose proportion seems to increase in the ITN area. Thus, studies should be carried out to identify other vector control approaches that can be integrated with ITNs for the control of *An. arabiensis*.

2. There is need for adequate surveillance data to guard against interference of disease control by vector resistance to insecticides.

3. There is need to explore alternative methods of insecticide application to reduce evolution of resistance and prevent spread of resistance genes. Such a possible strategy
for blocking the spread of resistance genes would be to give an insect growth regulator alongside the pyrethroid to reduce the fecundity of the adult female mosquitoes or zooprophylaxis to interfere with the parasite life cycle. Therefore, in view of the findings that resistance genes are spreading to non-ITN areas and that frequency of kdr alleles has increased, ITN manufacturers should explore other forms of control which could be long-lasting and stronger to overcome resistance by vectors.
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