IMPACT OF INTENSIVE LARVAL CONTROL AND MONITORING ON MALARIA VECTORS IN AN AREA OF HIGH INSECTICIDE TREATED NET COVERAGE IN RURAL WESTERN KENYA

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DECLARATION

I, Ally Adinani Omary, hereby declare that this is my original work and has not been presented for a degree in any other University.

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DEDICATION

I wish to dedicate this thesis to my immediate family, to my wife Rayah, my parents Adinani and Aziza, my sisters, Zalihina, Stina, Razia, Stepha, Hawati, Afidha and to my brothers Mwamfula, Shui and Hemedi. They have always offered support and encouragement in my all endeavors.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

Bti	*Bacillus thuringiensis* var *israelensis*
Bs	*Bacillus sphaericus*
CDC	Centers for Disease Control and Prevention
CG	Corn Granular
DDT	Dichlodiphenyltrichloroethane
DNA	Deoxyribonucleic Acid
ELISA	Enzymes Linked Immunosorbant Assay
EIR	Entomological Inoculation Rate
GPS	Global Positioning Systems
IPEP	International Pops Elimination Project
IGR	Insect Growth Regulators
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
ITU	International Toxic Unit
IGS	Intergenic Spacer Regions
KEMRI	Kenya Medical Institute
LLITNs	Long Lasting Insecticide Treated Nets
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMI	President's Malaria Initiatives
PSC	Pyrethrum Spray Collection

UMCP	Urban Malaria Control Project
UNICEF	United Nations for Children's Fund
RBM	Roll Back Malaria
RR	Relative Ratio
WDG	Water Dispersible Granular
WHO	World Health Organization

ABSTRACT

Integrated control of vectors of human malaria in sub-Saharan Africa relies on strategic targeting of mosquito vectors. An anti-larval control study was implemented in an area of high insecticide treated bed net (ITN) coverage in rural western Kenya with perennial malaria transmission. The goal was to determine whether larval control would provide added benefit to ITNs in reducing malaria transmission. Larval control using *Bti* was implemented in a 2 x 2 km^2 zone in Asembo, western Kenya. A neighboring 2 x 2 km^2 zone was identified as non-intervention zone. Larviciding and monitoring was done once weekly in all potential habitats within the intervention zone. Larval and adult *An. gambiae* populations were sampled fortnightly in both zones for a period of 7 months. *An. gambiae* larval density in the intervention zone was reduced by 79% for all instars and 97% for the late instars (L3, L4 and pupae). Fewer female *Anopheles* mosquitoes (39.5%) were observed in the intervention zone after controlling for household ownership of bednets (Relative ratio (RR) = 0.605, p<0.001). However, in limited pre- and post-intervention collections, data suggest a pre-existing trend in lower mosquito numbers in the intervention zone and therefore, new study is needed to determine whether reduced densities were due to pre-existing differences in the two zones. This study concludes that intensive larviciding and ITNs use can further reduce human exposure to malaria vectors in endemic rural settings.

CHAPTER 1.0: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

In spite of mankind's longstanding struggle to control malaria mosquitoes, malaria remains a foremost threat to individual health (Minakawa *et al.*, 2005). There were approximated 881,000 malaria deaths in 2006, of which 91% were in Africa and 85% were of children under 5 years (WHO, 2008). *Plasmodium falciparum* is the most serious human malaria parasite and the most common one in Africa south of the Sahara (Snow *et al.*, 2005).

Malaria control is difficult and cannot be accomplished by a single intervention (Shiff, 2002). The existing tools such as insecticide treated nets (ITNs) and indoor residual spraying (IRS) that target adult malaria vectors have become the main tools in the global fight against malaria vectors. Regular use of ITNs by children can reduce their overall risk of dying by 20% and the occurrence of clinical malaria by almost 50% (RBM, 2002). IRS was once widely used as a malaria control measure but fell out of favor after the collapse of the Global Malaria Eradication campaign. Currently, about half of all African countries include IRS as part of their malaria control policy (RBM and WHO/UNICEF, 2005).

Early studies reported that IRS and ITNs sharply reduce malaria transmission (Maboso *et al.*, 2004; Gimnig *et al.*, 2003a; ter Kuile *et al.*, 2003); but they do not eliminate it entirely; additional complimentary tools are urgently needed, in particular ones that target other mosquito stages and which, when implemented, synergize with IRS or ITNs in their effects in reducing vector density and transmission intensity.

The use of microbial larvicides for mosquito larval control have been demonstrated to be an effective tool for the control of malaria vectors in Africa and could be incorporated into integrated vector management (IVM) strategies for reducing malaria transmission (Majambere *et al.*, 2007). While four insecticide classes (carbomates, pyrethroids, oganophosphates and organochlorides) are available for IRS and only pyrethroids for ITNs (Najera and Zaim, 2002) , a wide range of microbial larvicide (Fillinger *et al.*, 2003) and insect growth regulators (Yapabandara *et al.* 2001; Yapabandara and Curtis 2004) are available for the control of immature stages. Furthermore, resistance to many larvicides may be slow to develop. *Bacillus thuringiensis* var *israelensis* (*Bti*) has been shown to have a low probability of developing resistance (Wirth *et al.*, 2000; Mittal, 2003).

Larval control is more challenging to implement than adult control (Killeen *et al.*, 2002a) due to the presence of a wide range of potential larval habitats and the transient, unpredictable nature of many of these habitats (Gillies and DeMeillon, 1968). However there has been growing interest in the feasibility of controlling malaria vectors by targeting immature stages (Killeen *et al.*, 2002b). This interest has been attributed to the fact that adulticides may have negative impacts on human health and the environment while vectors targeted with these insecticides often developed resistance (IPEP, 2006). Historically, control programs targeting the immature stages of malaria vectors, including *An. gambiae*, have been very successful. Malaria was eliminated from parts of Zambia using larval control integrated with other methods (Utzinger *et al.*, 2001) and larval control was the main strategy employed in the elimination of *An. gambiae* from Brazil (Soper and Wilson, 1943) and Egypt (Shausha, 1948).

Initial studies in western Kenya indicate that *Bti* may be effective even in rural Africa (Fillinger and Lindsay, 2006). However, recent unpublished study employing larval control in an area of

high ITN coverage (i.e. Asembo bay, western Kenya) demonstrated that there was effective control of the larval stages and emergence rates in the surveyed area but there was no apparent effect on the adult mosquito population (CDC unpublished data). The poor performance against adult mosquitoes may have been due to incomplete coverage of larviciding, less monitoring of staff or short duration of intervention rather than efficacy of the control tool itself.

The findings reported in this thesis were designed to demonstrate the added impact of intensive larval control in an area with high insecticide treated net usage. Larviciding was implemented using *Bacillus thuringiensis* var *israelensis* (*Bti*) at weekly intervals (Fillinger and Lindsay, 2006) in all breeding habitats in a single 2 x 2 km intervention zone. Monitoring to assess larvae mortality was done one day after larviciding had been completed. Larval, pupal and adult mosquito densities were evaluated at fortnightly intervals in the intervention zone and a neighboring 2 x 2 km area without larviciding. All laboratory processing of collected samples was done in the CDC/KEMRI laboratories in Kisumu, western Kenya.

1.2. LITERATURE REVIEW

Mosquito control for the prevention of malaria has traditionally relied upon the application of residual insecticides to the walls of dwelling structures. Many of these insecticides are no longer available due to concerns about their adverse impact on human health or the environment, or due to resistance in the mosquitoes. Indoor residual spraying and insecticide treated nets are associated with substantial reductions in malaria morbidity and mortality, but may have adverse human health and environmental outcomes (Turusov *et al.*, 2002). Furthermore, these tools do not completely interrupt transmission. Larval control is widely used for the control of nuisance mosquitoes in the United States and Europe and, in the past, has been used to target vectors of

malaria (Soper and Wilson, 1943; Kitron and Spielman, 1989; Hays, 2000). However, larval control has been considered unlikely to contribute to malaria prevention in Africa where transmission is intense, occurs year round and is transmitted largely by mosquitoes which breed in small, transient bodies of water that are difficult to target effectively. However, a wide range of environmentally friendly mosquitocides have been developed and applied in field trials and have been shown to be effective in larval control (Fillinger *et al.*, 2003). While considered by many to be an ineffective tool for malaria prevention in Africa, larval control has not been thoroughly evaluated and it could be particularly valuable in combination with adult control strategies, such as the use of ITNs (Fillinger *et al.*, 2003).

1.2.1. Biology of *Anopheles gambiae*

Understanding the biology and behavior of *Anopheles* mosquitoes can facilitate understanding malaria transmission dynamics and can assist in designing suitable control strategies. Factors that affect a mosquito's ability to transmit malaria include its natural vulnerability to *Plasmodium* (Habtewold *et al.*, 2008), its host choice (Dekker *et al.*, 2001) and its longevity (Benedict *et al.*, 2009). Factors that should be taken into consideration when designing a control program include the preferred feeding and resting behavious of adult mosquitoes (Mahande *et al.*, 2007). The main malaria vectors in western Kenya are *Anopheles gambiae s.l* and *An. funestus* (Minakawa *et al.*, 2002). *An. gambiae* and *An. funestus* bite more often indoors than outdoors with peak biting activity occurring between 23-3hrs (Mathenge *et al.*, 2001).

The different species within the *Anopheles gambiae* complex (or *Anopheles gambiae sensu lato*) are identical morphologically and can only be identified to species level by the polymerase chain reaction (PCR) technique (Scott *et al.*, 1993). The *Anopheles gambiae* complex comprises

of the most world's effective malaria vectors (Coetzee *et al.*, 2004).This complex was considered to have seven morphologically indistinguishable species (Service, 2004). The seven sibling species are: *Anopheles gambiae sensu stricto, Anopheles arabiensis, Anopheles quadriannulatus, Anopheles quadriannulatus species B, Anopheles bwambae, Anopheles melas* and *Anopheles merus*.

In general terms seasonal variations in *Anopheles gambiae* complex have a tendency to follow the seasonal pattern of rainfall. The density begins to increase almost immediately after the main rainfall, reaching a peak in the middle of the rains and declining gradually thereafter as water levels become stabilized and vegetation and predators become established (Gillies and DeMeillon, 1968).

Female *Anopheles gambiae s.s* prefer to rest indoors (endophilic) and is extremely anthropophilic (Githeko *et al.*, 1996) while *Anopheles arabiensis* prefer to rest outdoors (exophilic) and is highly zoophilic (Githeko *et al.*, 1996; Mahande *et al.*, 2007). Biting by nocturnal endophagic *Anopheles* mosquitoes can be obviously reduced through the use of ITNs (Hawley *et al.*, 2003) or through mosquito proofing houses (Lindsay *et al.*, 2002; 2003). Endophagic/endophilic mosquitoes are readily controlled by IRS and ITNs (Nevill *et al.*, 1996; Mabaso *et al.*, 2004). However exophagic/exophilic vectors are best controlled through source reduction (Kitron and Spielman, 1989).

1.2.1.1. *Anopheles gambiae* Larval Habitats

There are six immature stages during mosquito growth; eggs, L1, L2, L3, L4 and pupal. Eggs are laid directly on the surface of water or on mud and even on moist sand (Huang *et al.*, 2005).

5

After hatching of the eggs, anopheline larvae float parallel just below the air-water interface making them exceptional from other species such as *Culex* spp which suspend downward from the air-water interface. The larvae of *An. gambiae s.l.* breath atmospheric oxygen through two spiracular openings on the eighth abdominal fragment and feed by moving brush-like structures on their mouthparts that generate a current of water (Merrit *et al.*, 1992). They sift out microbes, and other particulate organic matter or debris (Merrit *et al.*, 1992). The duration from egg to adult varies considerably among species and is strongly influenced by climatic conditions. *Anopheles gambiae s.s* can develop from egg to adult in as little as 5 days but usually take 10-12 days in tropical conditions (Gimnig *et al.*, 2002).

Typical *An. gambiae s.s* and *An. arabiensis* habitats include puddles, shallow ponds, and man-made habitats such as borrow pits, brick pits, tyre tracks, ditches, human foot and animal hoof prints (Minakawa *et al.*, 1999; Gimnig *et al.*, 2001; Fillinger and Lindsay, 2004). Another common characteristic of the breeding habitat of *An. gambiae s.l.* is their shallow nature and the presence of algae (Gimnig *et al.*, 2001). These habitats are open containing no, or little aquatic vegetation (Gimnig *et al.*, 2001; Fillinger and Lindsay, 2004). However, larvae are usually absent from large areas of uninterrupted water such as lakes, especially if they have large numbers of fish and other predators (Service, 2008). They are also usually absent from large rivers and rapidly flowing water.

1.2.2. Adult Mosquito Control Strategies

Recent malaria control policies have emphasized household protection against adult mosquitoes with insecticides, and improved access to medical services (Greenwood and Mutabingwa, 2002). Malaria prevention by killing adult mosquitoes was considered more effective than larval control because it decreases both adult numbers and adult longevity and can drastically curb

malaria transmission (Killeen *et al.*, 2002a). Adult mosquito control measures include indoor residual spraying (IRS), space spraying and insecticide treated nets (ITNs /LLITNs).

1.2.2.1. Indoor Residual Spraying (IRS)

Indoor residual spraying was the primary malaria control tool employed during the global malaria eradication campaign in the 1960s. Since then, it has been recommended more for interrupting malaria transmission during epidemics and other emergency situations provided it is well timed with high coverage (RBM and WHO/UNICEF, 2005). More recently, the use of IRS for routine prevention has been advocated and IRS is increasingly being implemented in areas with high, perennial transmission. However, the high cost of IRS and the emergence of insecticide resistance may limit the long-term application of IRS for malaria vector control (WHO, 2006).

The main purpose of IRS was to reduce the survival of malaria vector(s) entering the houses. However, it is of little use for control of malaria vectors which rest outdoors (exophilic) particularly if they also bite outdoors (exophagic) and if they do not enter sprayed houses. To achieve maximum impact, all possible resting surfaces of vectors should be sprayed with an appropriate insecticide, at a prescribed amount sufficient to remain effective throughout the transmission season (Najera and Zaim, 2002). This requires teams of trained spray operatives equipped with hand operated spray pumps and an organizational structure with strong coordination to ensure that all targeted structures are sprayed within a short period of time.

The most widely used insecticide for indoor residual spraying was dichloro-diphenyltrichloroethane (DDT). Dichlorodiphenyltrichloroethane has already been replaced by pyrethroid, organophosphate or carbamate insecticides such as malathion because of its negative

impacts on the environmental, human health, and the development of resistance in target mosquitoes (IPEP, 2006). The shift to carbamate insecticides mitigated the concerns of increasing insecticide resistance although these insecticides are generally more costly and less safe for humans. Pyrethroids such as deltamethrin and lambdacyhalothrin are effective at lower doses than DDT and are often more acceptable to householder owners because they leave no deposits on walls and can kill other nuisance insects. Most vector control specialists recommend the use of different classes of insecticides in rotation as a method to reduce the development and spread of insecticide resistance to any one class of insecticide (WHO, 2004).

1.2.2.2. Insecticide Treated Nets (ITNs)

Mosquito nets have long been used to protect against mosquitoes, including malaria vectors (Najera and Zaim, 2002).On the other hand, nets are often torn or not well hung, thus allowing mosquitoes to penetrate or feed on the sleeper (Najera and Zaim, 2002). Therefore, it is recommended to treat mosquito nets with a fast acting insecticide that will repel or kill mosquitoes before or shortly after feeding (Lines *et al.*, 1987).

Insecticide treated bed nets work as a physical barrier, preventing contact by vector mosquitoes and thus afford personal protection against malaria to the individual using the nets. The insecticide provides added protection by irritating or repelling mosquitoes that attempt to feed or by killing those that successfully feed. The widespread implementation of insecticide treated nets has been shown to have a community effect whereby the entire mosquito population is depressed and even those who do not use nets are afforded some protection (Hawley *et al.*, 2003; Gimnig *et al.*, 2003b).

Insecticide treated bed nets are an essential component of the Roll Back Malaria campaign to reduce morbidity and mortality due to malaria in Africa. Studies in Ghana, Gambia, Kenya and Tanzania demonstrated that use of ITNs decreased child illness by 29% to 63% and childhood mortality by 17% to 63% (IPEP, 2006; Alonso *et al.*, 1991). The present challenge is to scale-up and sustain coverage with ITNs (Lines *et al.*, 2003). The development of long-lasting insecticide treated nets which do not require regular retreatment has removed one important barrier to the implementation of ITNs (Najera and Zaim, 2002). Both WHO and PMI call upon National Malaria Control Programs and their partners involved in insecticide-treated net interventions to purchase only long-lasting insecticidal nets (LLITNs) due to their biological efficacy against vector mosquitoes for at least three years (Pacqué, 2007).

The use of artemisinin based combination therapy (ACT) and the revival support of IRS, present a new opportunity for malaria control which have resulted to significant reduction in malaria incidence (WHO, 2008). Three African countries reported dramatic reduction in malarial deaths by 50 per cent or more. Eritrea, Rwanda and Sao Tome and Principe achieved this result between 2000 and 2006/2007 (WHO, 2008). However, several challenges remain. First, there has been much debate about who should pay for nets. Currently, free distribution of nets is advocated but if donor funding is not sustained, market based approaches will be needed. In the past, cost has been a major barrier to obtaining nets among target populations. Second, while net ownership is rapidly increasing throughout Africa, net use is lagging (Baume *et al.*, 2005). New approaches to ensure regular use by persons at risk of malaria are needed. It is likely that these approaches will vary depending upon local cultural or behavioral practices. Lastly, insecticide resistance looms as a serious threat to the viability of ITNs and IRS as malaria prevention strategies. Insecticide resistance is a particular concern for ITNs as

pyrethroids are the only group of insecticides currently recommended for use on nets (WHO, 2006).

The large bednet study in Asembo documented that both *An. gambiae* and *An. funestus* bite primarily indoors late at night but their biting is reduced by use of ITNs (Hawley *et al.*, 2003). It is emphasized that the chosen study area has had a successful ITN program in place since 1990s with regular net replacement and retreatment schedules involving community leaders and other researchers (Hawley *et al.*, 2003). The goal has been to maintain high coverage and use of ITNs, the result of which has been dramatic suppression in transmission, morbidity, and mortality (Gimnig *et al.*, 2003b; Gimnig *et al.*, 2003a; Hawley *et al.*, 2003; ter Kuile *et al.*, 2003). Enough ITNs were allocated to allow every individual in every house to sleep under mosquito net (Hawley *et al.*, 2003). However transmission continues at a reduced level and thus has not been completely abated in the proposed study area (Lindblade *et al.*, 2004).

1.2.2.3. Space Spraying

Space spraying is the application of non-residual insecticides to rapidly reduce adult mosquito populations. Space spraying includes aerial application of insecticides as well as the use of vehicle mounted or hand-held foggers. The value of this approach is controversial: space spraying is most effective if applied when mosquitoes are actively flying, though this is rarely done in practice. Furthermore, the residual activity of space spraying is on the order of a few hours. Its primary benefit is that it can be more rapidly implemented compared to other adult vector control approaches. Therefore, space spraying has been recommended only for emergency or epidemic situations (IPEP, 2006) .

1.2.3. Larval Control Strategies

Larval control measures to alleviate malaria burden have been successfully used in different regions (Shousha, 1948; Soper and Wilson, 1943; Utizinger *et al.*, 2001; Fillinger and Lyndsay, 2006; Majambere *et al.*, 2007; Fillinger *et al.*, 2008). Malaria was successfully eliminated in the United States, Europe and the Middle East using larval control measures integrated with other approaches (Hays, 2000). However, it is argued that while vector control in Africa can only succeed by targeting all stages of the mosquito life cycle, it has historically focused on adult control using IRS and ITNs (Curtis, 1994; Robert *et al.*, 2000).

The historical literature demonstrates that anti-larval mosquito measures may be powerful tools for controlling malaria vectors (Killeen *et al.*, 2002a; Kitron and Spielman, 1989; Utizinger *et al.*, 2001; Fillinger and Lindsay, 2006). The major advantages of targeting the larval stages are that mosquitoes are killed before they disperse to human dwellings (Yohanes *et al.*, 2005) only if they are anthropophagic and endophilic/endophagic. Another advantage is that mosquito larvae cannot change their behaviors to avoid control activities directed to them (Killeen *et al.*, 2002b). In addition mosquito larvae are concentrated in specific places and thus can easily be killed. Studies conducted in Mbita western Kenya which covered an area of $4.5km^2$ with 8,000 people demonstrated that larval control may even be effective in rural Africa (Fillinger and Lindsay, 2006).

On the other hand larval control activities are more susceptible to faults in coverage and to invasions of flying adult mosquitoes from outside the intervention area than are ITNs and IRS (Killeen *et al.*, 2002a). Therefore it is suggested that larval control programmes should be piloted on small spatial scale in order to develop efficient larviciding application and monitoring

systems as well as to evaluate the feasibility of larval control in a given setting. The intervention can then be expanded and tested on a larger scale (Killeen *et al.*, 2002a).

1.2.3.1. Microbial Larvicides

Formulations of *Bacillus thuringiensis* var *israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) for mosquito larval control have the potential to be effective components of integrated vector management (IVM) strategies for reducing malaria transmission (Majambere *et al.*, 2007). *Busilus thurngensis israelensis* and *Bacilus sphaericus* are aerobic, gram positive spore forming bacteria (Zahiri and Mulla, 2004). Upon ingestion by susceptible larvae, the killing proteins are solubilised in the mid gut by a combination of an alkaline pH and proteolysis (Aronson *et al.*, 1986). Spore germination and proliferation of the vegetative cells into the haemocoel may result in a septicaemia, contributing to the cause of death (WHO, 1999). However, *Bti* is only effective against actively feeding larvae, and does not have an effect on pupae because they stop eating. Results from a field trial with *B. thuringiensis* water dispersible granules (WDG) in western Kenya demonstrated that, a minimum dosage of 200g/ha (2700 International Toxic Unit/mg) is adequate to completely curb appearance of late instars and the resulting pupae, despite its low residual effect when applied at weekly intervals (Fillinger *et al.*, 2003).

Biological larvicides (*Bti* and *Bs*) have been proven to be useful weapons for mosquito larval control in a variety of breeding habitats (Karch *et al.*, 1992; Majambere *et al.*, 2007). The performance of the microbial agent may be affected by water quality parameters such as organic content, salinity, pH, and water temperature. All these parameters vary with ecology and type of the breeding habitat (Shililu, 2001). *Busilus thurngensis israelensis* effectiveness has been

limited to clean water while *Bs* has been found to be effective in both polluted and clean water sources.

Both *Bti* and *Bs* are highly effective against mosquitoes, black flies and chironomid midges but have no known adverse effects on non-target organisms (Charles and Nielsen-Le Roux, 2000). Both are safe for humans, they can be dispensed by hand with no need for special protective equipment (WHO, 1999). *Bucilus thuringiensis* and *Bucilus sphaericus* are characterized by the ease of handling and cost effectiveness (Fillinger *et al.*, 2003). Another major advantage of *Bti* and *Bs* is the low probability of developing resistance (Charles and Nielsen-Le Roux, 2000). The equipment needed for larviciding is simple, inexpensive, and the skills needed for larviciding can be acquired easily (Becker, 1998; Mukabana *et al.*, 2006)

There are an increasing number of countries in the tropics interested in integrating microbial larvicides (*Bti*) into their mosquito control programmes (Rodgues *et al.*, 1998). More encouragingly, recent large scale larval control operational trials using microbial larvicide (*Bti*) have proved successful in urban Dar es Salaam, Tanzania, where the Urban Malaria Control Programme (UMCP) covers 55 km^2 , reducing malaria transmission intensity by approximately one third (Fillinger *et al.*, 2008) and infection risk by half (Geissbüehler, *et al.*, 2009). Also in the highlands of Kenya, smaller scale trials demonstrated an even stronger impact on transmission, infection prevalence and incidence of re-infection (Fillinger *et al.*, 2009).

1.2.3.2. Insect Growth Regulators (IGRs)

Insect growth regulators refer to a variety of chemical compounds that are highly active against mosquito larvae and other insects (Poopathi and Tyagi, 2006). Insect growth regulators mimic the natural hormonal mechanisms involved in growth and development of target organisms.

These larvicides often do not cause immediate mortality of immatures but rather disrupt molting or metamorphosis resulting in different types of morphological, anatomical and physiological deformities and ultimately preventing mosquito larvae from developing to the adult stage (Amalraj *et al.*, 1988a). A large number of IGRs have been evaluated for vector control but only very few of these were found effective and commercially feasible eg. diflubenzuron, methoprene, fenoxycarb (Amalraj *et al.*, 1988b) and pyriproxifen (Yapabandara and Curtis, 2002). Ecdysone agonists are hormonally active IGRs that were found to be active against *An. gambiae*, *Ae. aegypti* and *Cx. quinquefasciatus* (Beckage *et al.*, 2004). Insect growth regulators are harmless to most non-target organisms including invertebrates, fish, birds and other wildlife. They are also safe to man and domestic animals (Poopathi and Tyagi, 2006). It is probable that IGRs could play a significant role in future larval control (Mulla, 1995).

Mosquito larval control using an IGR was evaluated in Sri Lanka where hand-dug gem pits and river bed pools are important breeding habitats for the malaria vectors *An. culicfacies* and *An. subpictus* (Yapabandara and Curtis, 2002). All the gem pits and river bed pools were treated with a granular formulation of the insect growth regulator (pyriproxyfen) at a target dose of 0.01mg a.i/litre in eighty villages in central Sri Lanka. The application of pyriproxifen caused a significant reduction in the adult populations of *An. culicifacies* and *An. subpictus* (Yapabandara *et al.*, 2001). The incidence of malaria was reduced by 20 – 29% in the intervention villages compared to the control villages while the prevalence of parasitaemia also declined significantly. Pyriproxyfen required re-application only twice a year (Yapabandara and Curtis, 2002) thus reducing labour cost.

1.2.3.3. Larvivorous Fish

Fish are notorious predators of mosquito larvae and mosquitoes rarely breed in larger bodies of water where fish are present. Larvivorous fish have been used in mosquito control in water bodies for over 100 years (Bay, 1967). Fish have been found effective as a mosquito control agent (Prasad *et al.*, 1993). *Gambusia affinis* is a surface feeding omnivore, eating different types of aquatic organisms including eggs of native fish (Hurlbert and Mulla, 1981). A study of fish gut contents conducted in Colombia demonstrated that mosquito larvae were an important part of the diet (Torrente *et al.*, 1993).

Studies conducted in the western Kenya highlands where larvivorous fish *Oreochromis niloticus* (formally *Tilapia niloticus*) was introduced in ponds and caused a more than 94% decrease in both *An. gambiae s.l.* and *An. funestus* in the treated ponds, and more than 75% reduction in culicine mosquitoes. There was a highly significantly reduction in *Anopheles gambiae s.l* numbers when compare to pre-treatment level (Howard *et al.*, 2007). These suggest that fish can play part in reducing mosquito adult populations. While the use of larvivorous fish may be effective against mosquitoes in some settings, their applicability may be limited by a number of factors. First, careful consideration should be given to the potential for unintended environmental consequences of introducing non-native fishes as some species, particularly *Gambusia affinis*, have been shown to out compete and displace native fish (Lindsay *et al.*, 2004). *Gambusia affinis* is an opportunistic predator which feed on various foods such as algae, young of fish and other aquatic organisms which may be beneficial to the environment. Furthermore, *Gambusia affinis* is very small in size, thus may also be suitable to control mosquito larvae in small water bodies such as those that *Anopheles gambiae* mosquitoes tend to breed in.

1.2.3.4. Environmental Management

Means planning, organization, implementation and evaluation of deliberate changes of environmental factors with the aim of preventing the proliferation of vectors and reducing human vector contact (Shiff, 2002). Environmental management includes activities such as filling, drainage of swamps, vegetation clearing and modification of river boundaries. Filling of abandoned ditches, borrow pits and pockets of water with soil, stones, refuse or ash eliminates potential mosquito breeding sites (Lindsay *et al.*, 2004). These are particularly important if situated close to human habitation (Lindsay *et al.*, 2004). In India, filling of potential mosquito breeding sites with ash has been used to reduce malaria cases (Dua *et al.*, 1997). Intermittent irrigation is done in rice growing fields to allow drying out in India, China and East Asia, and has proven successful in reducing vector population (Lacey and Lacey, 1990). Impoundment involves flooding of large areas through construction of dams. Dams provide a large, permanent and well defined surface that is more amenable to supervision and control (Lindsay *et al.*, 2004). These impounded waters are commonly stocked with fish which are good mosquito larvae predators.

Interventions focusing on environmental management to control the larval stages of *Anopheles gambiae* and *An. funestus* have been implemented since 1929 for two decades at the copper mining community in Zambia where by activities implemented includes modification of river boundaries, drainage of swamps, vegetation clearance, oil application to open water bodies and house screening. Within three to five years malaria was reduced by 70% to 95% (Utzinger *et al.*, 2001). In Tigray, Ethiopia activities such as filling, draining and shading of potential mosquito breeding sites reduced adult *An. arabiensis* by 49% (Yohanes *et al.*, 2005). The passage points of humans and livestock along the riverbeds were filled with stones and gravel to

prevent the formation of mosquito breeding habitats in foot and hoof prints. Simple drainage ditches were constructed to allow fast water movement. Puddles and neglected water ponds excavated in the river or streambed for human use were filled with earth (Yohanes *et al.*, 2005). Source reduction has been utilized productively in diverse parts of the world particularly before the introduction of dichlorodiphenyltrichloroethane (Kitron and Spielman, 1989).

1.2.4. Monitoring of Mosquito Larval Control Activities

Management, monitoring, and evaluation system for implementing routine larviciding of malaria vectors in Africa has shown considerable potential for continued, rapidly approachable, data driven and affordable application (Fillinger *et al.*, 2008). Monitoring of larval control activities have been found useful in various regions (Soper and Wilson, 1943; Fillinger and Lindsay, 2006; Fillinger *et al.*, 2008).

In Dar es Salaam, community owned resource persons (CORPs) assigned to a single sub- ward to visit and apply *Bti* to all potential breeding habitats in each ten cell unit (TCUs), is monitored by larvae surveillance CORPs one day after the application of *Bti* to find out indicators of operational weaknesses, such as presence of late stage of mosquito larvae to be responded to by immediate re-introduction of the larvicide to avoid emergence of adult mosquitoes (Fillinger *et al.*, 2008). All larval control activities are supervised by one supervisor in a ward level. Studies in Mbita western Kenya and Dar es Salaam Tanzania, reported monitoring by spot check took place in randomly selected sites 24 hours after treatment to evaluate larval mortality (Fillinger and Lindsay, 2006; Fillinger *et al.*, 2008).

In the elimination of *An. gambiae* in Brazil, the work of inspectors in their working zones was rigorously inspected and supervised by a District Chief Inspector (Soper and Wilson, 1943), while in Dar es Salaam the work of inspectors are supervised by Municipal Malaria Control Coordinator (Fillinger *et al.*, 2008). In Brazil each inspector was monitored on an approximately hourly basis and could be held explicitly answerable for any discrepancies. Remarkably, the activities of the anti-larval and anti-adult control teams were separately reported so that inconsistencies could be identified (Soper and Wilson, 1943; Fillinger *et al.* 2008).

Following these successes of mosquito larval control in Dar es Salaam, Mbita and Brazil the objective of the current study was to demonstrate and quantify the added benefit of *An. gambiae* larval control for malaria prevention in a smaller area with intensive ITN usage, using an appropriate number of sprayers, a longer duration of time and increased monitoring in western Kenya where the previous study showed suppression of larvae but no impact on adult mosquito populations.

1.3. Justification and Significance of the Research

Insecticide treated nets and indoor residual spraying are effective tools for the prevention of malaria. However, in Africa, these tools have failed to completely eliminate malaria morbidity and mortality as called for in the Millennium Development Goals (World Bank, 2002). Additional complementary tools are urgently needed, especially those that target other mosquito stages and when implemented synergize with IRS or ITNs in their effect of reducing vector density and vectorial capacity.

Larval control study implemented in an area of high ITN coverage (i.e. Asembo bay western Kenya) demonstrated effective control of the larval stages and its emergence rates in the surveyed area but there was no apparent effect on the adult population (CDC unpublished data).

The poor performance against adult mosquitoes may have been caused by incomplete coverage of larviciding, less monitoring by staff, or short duration of intervention (3 months). It is for this reason that further study were carried out in the same area but on a geographically smaller scale. This involved more intensive supervision, long duration of larviciding (5 months) and more efforts which targeted the larval habitats to determine whether larval control would provide added benefit to ITNs in reducing malaria transmission.

Recent studies have shown that larval control approaches are even effective in endemic areas of sub Saharan Africa (Fillinger and Lindsay 2006). Despite numerous challenges, larval control offers several advantages over other approaches. Mosquitoes are targeted easily because they are restricted to limited water bodies and before they can transmit diseases.

The information obtained from this study of larval control of malaria vectors will be used: (1) by the Ministries of Health in sub-Saharan Africa to adjust malaria control policies so that they can employ interventions that target both the adult and immature stages, (2) to give the way forward to the development of large-scale trials and sustained program of larval control in rural Sub-Saharan Africa, (3) to provides information on the best available biological larvicide and the duration of application within the lowland areas of western Kenya and (3) in conjunction with on-going ITNs and IRS programmes, effective surveillance and treatment may be of the most effective strategies to finally achieve malaria elimination in many parts of sub Saharan Africa.

1.4. Objectives:

1.4.1. Overall Objective

To demonstrate and quantify the added impact of an intensive larval control operation on malaria transmission in an area with high ITN usage.

1.4.2. Specific Objectives

1. To estimate the added impact of combining larval control and ITN usage on malaria vector density and Entomological Inoculation Rate (EIR) in lowland western Kenya.
2. To determine the efficiency of larval control monitors by independent assessment of covered areas.

1.5. Hypothesis

Larval control in an area of high ITN coverage can further reduce malaria transmission potential.

CHAPTER 2.0. MATERIALS AND METHODS

2.1. Study area and population

The study was carried out at Asembo (Rarieda District) approximately 50km west of Kisumu city along the shores of Lake Victoria. The study area covers approximately 8 km^2. The topography of the area is characterized by gentle rolling hills and many temporary streams that are flooded during the rain seasons and flow into Lake Victoria. Rainfall is seasonally bimodal, with the heaviest rains falling from March through May and the shorter rains occurring in November and December. The average total annual rainfall is 1200mm per year while average daily temperature range from 19 -29° C. The main malaria vectors in this area are *An. gambiae* Giles and *An. funestus* Giles (Gimnig *et al.*, 2003b).

Most residents are members of the Luo ethinic group. The population of the study area is scattered among family compounds which are defined as one or more houses (usually 3-5 houses). Houses are grouped into compounds of related family members and separated by farmland. Most inhabitants practice subsistence farming with maize being the staple crop. Other crops are sorghum, millet, vegetables. Many residents keep animals such as goats, cattle and chicken. Asembo is an area where ITNs randomized trial programme took place (since late 1990s) with regular net replacement and retreatment schedule involving community leaders, and researchers (Hawley *et al.*, 2003; ter Kuile *et al.*, 2003; Gimnig *et al.*, 2003a; Gimnig *et al.*, 2003b; Lindblade *et al.*, 2004).
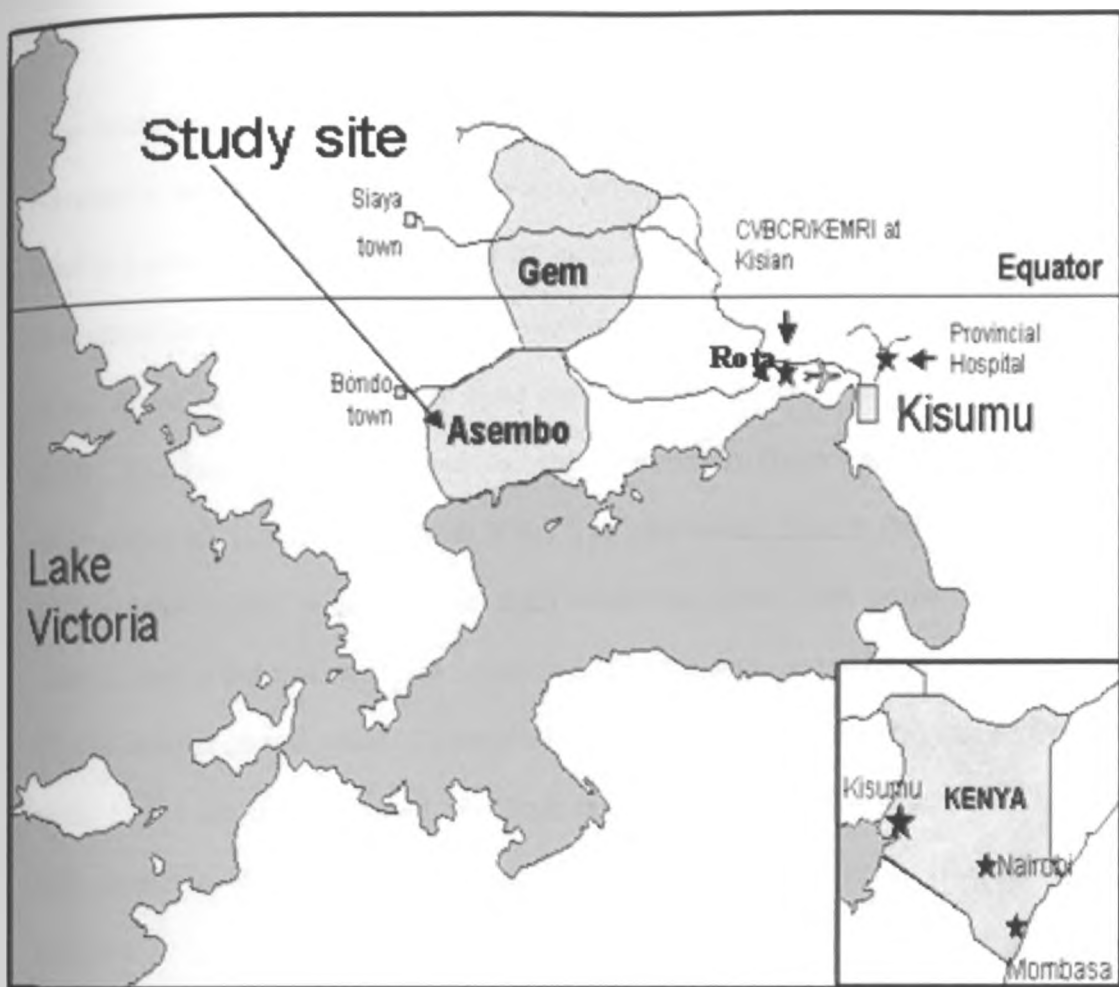
Figure 1: Map showing location of Asembo.

2.2. Study design

The study was a side-by-side comparison in which two (2 x 2 km) zones were randomly assigned to intervention and non-intervention areas. Both zones were part of a grid of 20 zones used in a previous study. The original 20 zones were grouped based on homogeneity of certain characteristics e.g. topography, presence of streams, human activities, and distance from the lake shore. Based on the similarities of these categories, two zones were randomly selected for the study. The two were randomized for the intervention (larviciding with ITNs) and non-intervention (no larviciding but with ITNs). The intervention zone in the current study was also an intervention zone in the previous study while the control zone in the current study was a control zone in the previous study. Approximately 9 months had passed between the end of the 1st trial and the current study. To simplify the work in the field, the area was divided into sub-zones (1 x 1 km) and plots (0.5 x 0.5 km) using GPS and obvious land marks such as roads, hills, trees, stones and houses. Most people living in both study zones own and regularly use bed nets.

A 1 x 1 km area was selected at the center of each zone and used as evaluation sub-zones. The evaluation areas were selected to provide a buffer zone and limit the influx of adult mosquitoes into the intervention zone.
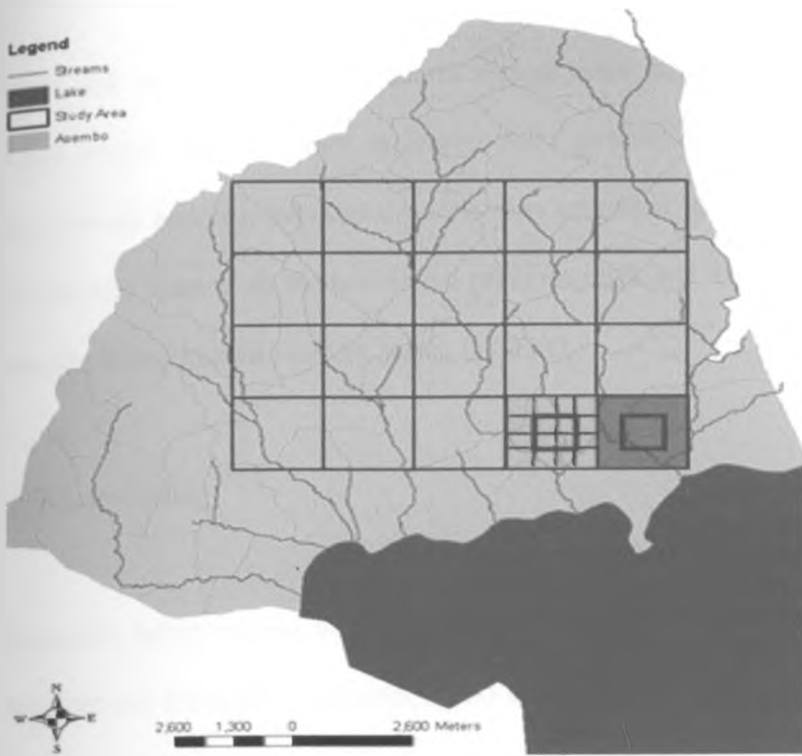
Figure 2: Map showing study design of the zones. The two zones in the southeast corner were used in the current study with the green zone representing the intervention and the pink non intervention zones. The evaluation areas (1 km x 1 km) are marked with a red rectangle in the center of each zone while the 0.5 km x 0.5 km plots in the intervention zone are marked with black lines.

2.3. Adults and immature mosquito densities

Adult and larval densities of malaria vectors were estimated twice per month in each zone. Adult vector densities were estimated from pyrethrum spray collections (PSC). EIR for intervention and non-intervention zone was estimated using adult vector density, number of people who slept in all the houses the previous night and the sporozoite rates as determined by enzyme linked immunosorbent assays (ELISA).

2.4. Larviciding

Mosquito larval control was implemented using the microbial larvicide VectoBac® (Valent BioSciences Corporation, Illinois, USA) containing the active ingredient *Bacillus thuringiensis* var *israelensis* (*Bti*) in one intervention zone. Two formulations were used: water dispersible granule formulation of *Bti* (VectoBac® WDG, potency 3000 international toxic units per mg [ITU/mg]) was applied in open, clear, accessible habitats with at most short or emergent vegetation using 5 liter hobra hand pump sprayers. A corn granular formulation of *Bti*, serotype H-14 (VectoBac® CG, potency 200 international toxic units per mg [ITU/mg]) was applied by hand in inaccessible habitats or habitats with tall vegetation. To ensure complete larviciding in inaccessible habitats, some shrubs were cut down to accesses the breeding habitats.

Two employees were assigned to spray in one 1 km x 1 km subzone each day. The entire intervention zone was covered in 4 days and subzones were revisited in a seven day interval (Figure 3). In each working day, sprayers were given sachets of *Bti* WDG and *Bti* CG with the instruction to return empty sachets each afternoon. The amount of larvicide taken, used and the balance was recorded in note books. The concentration of *Bti* applied was calculated based on

the surface area of each habitat and an amount equivalent to $0.05gm/m^2$ (500g/hectare) and 4 granules per 10 cm^2 (10kg /hectare) for *Bti* WDG and CG, respectively, was used.

Figure 3: Field application of a water dispersible granule formulation of *Bti* (VectoBac®). Application was done using hobra sprayers.

2.5. Larval Search

A team of two larval searchers was deployed in the intervention zone to monitor the efficiency of the spray effort throughout the spray period (5th, May 2008 to 24th September 2008). They collected data sheets and *Bti* CG each morning from the office and were instructed to return data sheets and any unused *Bti* CG to the office for inspection, discussion and recording each afternoon. On each visit, they carried recent data sheets of the particular plots as a reference and all open habitats found in each plot was described using standardized forms.

The larval searcher visited each subzone a day after it had been sprayed. To inhibit unwanted emergence of adult mosquitoes, the team of larval searchers applied *Bti* (CG) only to those habitats colonized by late *Anopheles* larval stages (L3 and L4 instar) which were presumably missed by the spray team on the previous day. All habitats containing water were recorded by visual inspection carried out on foot on a weekly basis. Entrance to all family compounds was made after consent was given by the residents. Each habitat was given a unique identification number per visit. Habitat characteristics such as type, perimeter and depth were recorded in data sheet during monitoring. Notes were taken on vegetation types associated with habitats in order to develop suitable control approaches for each habitat. The presence or absence of larvae and pupae was scored after a minimum of 10 dips per site with a standard 350ml capacity mosquito dipper. Anopheline and culicine larvae were distinguished macroscopically in the dipper according to whether they float horizontally just below the air water interface (anopheline) or hanging down from the air water interface (culicine). Morphological differentiation of pupae from different species was very difficult for employees with basic training. Therefore pupae were not distinguished among *Anopheles* and other species. Only anopheline larvae and pupae were recorded in the data sheet and then discarded. For the most part, dips were taken at the

water boundaries and near to tufts of vegetation where larvae can be anticipated using displacement suction dipping technique.

Figure 4: Larval monitoring after 24 hrs of spraying.

2.6. Independent Assessment of the Monitors

Routine spot checks to monitor the performance of the larval searchers were conducted using the recent data sheets from larval control monitors as a reference to follow up. One plot (0.5 x 0.5km) from the intervention zone was randomly selected each week and surveyed using the same method as larval monitoring. The only difference was that, new habitats that were not recorded by the monitors the previous/same day were recorded and the presence of immature mosquito stages documented. The difference between the independent monitoring data and the data from the larval monitors with respect to the number and status of all habitats provided information on the sensitivity of the larval monitors. All habitats colonized by late instar (L3 and L4) larvae were treated with *Bti* CGs.

2.7. Evaluation of Impact on Immature Mosquitoes

The density of immature mosquitoes was monitored every two weeks in a 1 x 1 km area in the centre of each zone. Monitoring began one month before and continued one month after the intervention phase in both zones to estimate pre- and post-intervention densities. An area sampler consisting of a plastic cylinder 12cm in diameter and 18cm in height (113.04 cm^2) was used to estimate larval densities in each habitat (Mutuku *et al.*, 2006b). Area samplers were taken at approximately one meter intervals along the perimeter of the breeding habitats. The area sampler was pushed into the substrate in each habitat such that it could support itself or, if this was not feasible, it was held tightly into the mud or sand until sampling was made (figure 5).

All larvae and pupae enclosed in the area sampler were transferred by pipette into larval trays where they were counted, sorted into their individual stage and documented before pupae were

transferred to plastic tubes with water for transport to the laboratory at CDC/KEMRI-Kisumu while larvae were discarded in both zones. In the laboratory pupae were held in plastic dishes within paper cups to permit emergence and the adults were identified to species morphologically. Emerged adults were killed by freezing at 4°C and identified based on morphological characters. *An. gambiae s.l.* mosquitoes were saved for PCR identification while *An. funestus* were discarded because there was no primer for this species. All culicine were also discarded.

Figure 5: Sampling immature mosquito stages in a breeding habitat.

2.8. Evaluating the Impact of Larval Control on Adult Mosquitoes

Indoor resting populations of mosquitoes were sampled within the same evaluation subzones at fortnightly intervals including two pre- and two post-intervention rounds of sampling. One household compound was randomly selected as a reference compound in each evaluation subzone and mosquito collections were done in 26 adjacent houses to the reference compound using pyrethrum spray catch (PSC) method between 6.00 and 11.00 hours.

Pyrethrum spray catch was conducted by spreading white sheets on the floor, over the furniture and all horizontal surfaces in the house. Care was taken to ensure all foods and water were removed from the house or covered. Four PSC staffs were deployed for each zone. Spraying was done around the eaves, inside the house on walls and under the roof using 0.025% pyrethrum emulsfiable concentrate with 0.1% pipyronyl butoxide in kerosene (Figure 6).

The house was closed for 10-15 minutes after which the door and windows were opened and the sheets collected. The sheets were lifted with care by holding four corners and moving them gently so that the mosquitoes were collected in the middle. The sheets were then placed on a flat space devoid of wind and examined for mosquitoes in daylight. Mosquitoes were identified morphologically as anopheline or culicine and the number of each was recorded. All anophelines were collected with entomological forceps, placed on moist filter paper inside labeled petri dishes, and transported to the laboratory. The number of mosquitoes collected and the number of people that slept in each house the previous night and presence or absence of bed nets was recorded.

Figure 6: Panel **A** and **B** indicates spraying around the eaves and evaluators looking for adult mosquitoes after PSC, respectively.

2.9. Laboratory processing of collected mosquitoes

All adult anophelines collected from the field were transported to the laboratory and stored at 4°C in the refrigerator. They were identified morphologically to species level using taxonomic keys (Gillies and Coetzee, 1987). Each individual mosquito was placed in a 1.5 ml tube, provided an identification number, and desiccated over drierite or silica gel for 48 hours and kept at room temperature for further processing. The species, sex, and abdominal status (fed, unfed, gravid or half-gravid) was recorded for each anopheline mosquito.

2.9.1. Polymerase chain reaction and Enzyme Linked Immunosorbent Assays

All *An. gambiae* mosquitoes were identified using a molecular identification protocol (Scott *et al.*, 1993). This method utilizes the polymerase chain reaction (PCR) to distinguish between members of the *An. gambiae* species complex based on differences in the intergenic spacer region (IGS) of ribosomal DNA (rDNA) in anopheline mosquitoes (see description below). Sporozoite infection was determined for all female *Anopheles gambiae s.l.* and *An. funestus* by Enzyme Emmunosorbent Assays (Wirtz *et al.*, 1987). The head and thorax were separated from the rest of the body of all female anopheline samples and assayed for the existence of the circumsporozoite protein antigen in the salivary glands using monoclonal antibodies specific for *Plasmodium falciparum*.

2.9.1.1. The Polymerase Chain Reaction

Deoxyribonucleic Acid (DNA) from per specimen of *An. gambiae s.l.* mosquitoes was extracted (Collins *et al.*, 1987) and then stored at -20°C until used as template for PCR (typically within 24 hours). Amplification of the IGS region involved a forward primer based on a DNA sequence that is conserved among all members of *An. gambiae* complex and a reverse primer that is species-specific.   The resulting PCR amplicons were electrophoresed on an agarose gel. Variations in length depended on which sibling species was present. For example, the two members of the *An. gambiae* complex that co-exist in western Kenya are *An. gambiae* s.s. and *An. arabiensis*. The PCR amplicons resulting from these species were 385 and 315 base pairs in length, respectively, and were easily distinguishable by visual inspection of Ethidium bromide stained agarose gel.

Reagents used in each PCR were Taq DNA polymerase, 10 x concentrated polymerase buffer, species-specific PCR primers for *An. arabiensis* (AR) as a reverse primer for *An. arabiensis* (AR- 5'-CTGGTTTGGTCGGCACGTTT), *An. gambiae s.s.* (GA) as a reverse primer for *An. gambiae s.s* (GA- 5'- AAGTGTCCTTCTCCATCCTA), a universal forward primer (UN- 5'-GTGTGCCCCTTCCTCGATGT), $MgCl_2$, PCR-grade water and DNA template. The total volume of the composition was 20µl for a single reaction mix and each number of items was multiplied according to the number of samples tested including controls (Appendix 1).

The PCR conditions for this protocol involved an initial melting step of 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute. Following amplification, all PCR samples were electrophoreses on 3% agarose gels.   Prior to casting each gel, 1µl of ethidium bromide was added per 50 ml of

agarose/TBE solution. A maximum of 88 samples could be assayed at a time (88 samples plus 8 controls on a 96-well plate).

2.9.2. Enzyme Linked Emmunosorbent Assays

To measure the infection of female anopheline mosquitoes, specimens were analyzed using the Enzyme-Linked Immunosorbent Assay (ELISA) sandwich indirect type. A monoclonal antibody (Mab) that recognizes a repetitive epitope on the circumsporozoite protein of *Plasmodium falciparum* was used to determine sporozoite infection in *An. gambiae s.l.* and *An. funestus*.

The head and thorax of all female mosquitoes were separated from the body using a sharp forceps and held in labeled 1.5ml eppendorf tubes. A blocking buffer (50µl) was added to each vial containing a mosquito sample. After one hour each sample was ground with its own sterilized pestle attached to a mortar driven grinder. Each pestle was then rinsed with 200µl blocking buffer and the rinse collected in the same tube making a total volume of 250µl of mosquito saturate in each tube.

A plain phosphate buffered saline (5ml) was mixed with 20µl of the monoclonal antibody (Mab) for *Plasmodium falciparum*. Then 50µl of Mab solution was added to each well of a 96 well plate so that the final concentration per well was 0.20µg Mab/50µl PBS solution and incubated at room temperature for 30 minutes for antibody solution to bind on the plate. The antibody was then poured out and excess liquid was removed by inverting the plates and banging on paper towels. Then 200µl of blocking buffer was added to each well and the plates were incubated for 1 hour at room temperature. The blocking buffer was dumped in the sink and 50µl of mosquito homogenate was added in each well. The first column was used as a negative control while the

second column was used as a positive control. The plates were incubated for 2 hrs at room temperature. After 2 hours the plates were washed twice using 200µl of Tween. Fifty microliters of conjugate (peroxidase labeled) was added in each well and incubated for 1 hour then the plates were washed four times with PBS Tween. Ten microliters of the mixture of peroxidase substrate solution A and B was added in each well then the plates incubated for 30 minutes at room temperature before the plate was read by visual inspection. A sample was considered positive if the well turned dark green.

2.10. Entomological inoculation rate (EIR)

Entomological inoculation rate, the number of infective bites that an individual receives during a determined period of time was calculated using the overall sporozoite rate, number of bites per person derived from the indoor density of blood-fed mosquitoes and the number of people who slept in the houses the previous night for each subzone. Sporozoite rate was calculated using number of infected mosquitoes divided by total number of tested mosquitoes. The entomological inoculation rate (EIR) was obtained by multiplying the human biting rate by the sporozoite rate. Biting rate was calculated using number of blood fed/half gravid mosquitoes divided by number of people multiplied by number of nights. The EIR was expressed as the number of infective bites per person per night. Human blood index was not considered and all female *Anopheles* mosquitoes were assumed to feed on human blood.

2.11. Ethical considerations

The study permit for the use of microbial larvicides from Valent BioSciences Corporation was granted by the Kenya Medical Research Institute KEMRI/CDC. Community consent for the

implementation of operational mosquito larval control was sought through community meetings initiated by the local area chief, district and divisional authorities. Verbal consent was sought from every household where access was needed for adult collection and/or larval habitat monitoring and control.

2.12. Data analysis

The average number of adult mosquitoes per house was calculated by dividing the total number collected in a zone by the number of houses visited during the PSC. The immature densities per sampling area were calculated by dividing the total number of immature stages by the total number of the sampling events using the area sampler (including repeated measures in the same habitat).

The coverage sensitivity was determined by dividing positive habitats with all potential habitats identified and sampled by the monitors for each immature stage. The monitor's sensitivity was calculated by dividing the number of positive identified habitats by the monitors for each instar by the number of positive identified habitat by spot check for each instar.

Statistical analysis to compare average number of mosquitoes in intervention and non-intervention zones was done using repeated measures poisson regression (Proc Genmod, SAS v9.1 for Windows). Most of adult collections were done in 53 houses every 2 weeks since there were few houses in the evaluation area. Therefore, models were controlled for repeated measures to adjust for clustering within houses. For larval collections, multiple area samplers were taken from habitats larger than one square meter and models were adjusted for repeated measures within the same habitat.

CHAPTER 3.0. RESULTS

The data presented in this thesis was performed within a period of 7 months (April – October 2008)

3.1. Overall immature mosquito densities

During the study period from 16th April to 21st October 2008 (including pre-intervention and post-intervention collections), a daily average of 44 habitats in intervention and 33 in none intervention areas were identified and sampled repeatedly totalling up to 1080 sampling events; 620 were in the intervention zone and 460 were in the non-intervention zone. Of these, 51.1% (n=552/1080) were found to harbour *An. gambiae* larvae; 66.8% (n=369/552) and 33.2% (n=183/552) habitats in the intervention and non-intervention zones, respectively. There were nine habitats types identified puddle/tyre truck, animal hoof prints, drainage ditches, stream beds, borrow pits, swamps, ponds and others (springs and water storage containers). All were observed in both zones except the pond habitat which was found only in the non-intervention zone. The most common habitat types were drainage ditches (n=429), followed by borrow pits (n=232), stream beds (n=213), puddle/tire tracks (n=66), animal hoof prints (n=58), swamps (n=29) and others (n=41). All identified habitats were classified into 8 groups according to vegetation characteristics which were condensed in to four main classes; habitats with no vegetation (none), with vegetation below the knee (short), with vegetation above the knee (tall), and floating. The most dominant habitats are characterised by short vegetation (n=799) followed by habitats with no vegetation (n=241), tall (n=36) and floating (n=4).

The average number of larvae collected per area sampler in the intervention and non-intervention zones are shown over time in Figures 7A, 7B and 7C. These figures include pre- and post-intervention numbers. The larvae density started to increase during post intervention period, thus must be sustained all through to lessen mosquito population. Since there were few collections during these periods, data from the pre- and post-intervention periods are not included in the remaining analyses. During the intervention period, the average number of total larvae per area sampler was 0.14 in the intervention zone and 0.61 in the non intervention zone, a 79% reduction (Relative ratio (RR) = 0.21; p<0.0001). For early instar larvae (L1 and L2), the average number per area sampler was 0.14 in the intervention zone and 0.45 in the non intervention zone, a 71% reduction (RR =0.29; p<0.0001). For late instars larvae (L3 and L4 plus pupae), the average number collected per area sampler was 0.005 in the intervention zone and 0.16 in the non intervention zone, a 97% reduction (RR = 0.03; p<0.0001).
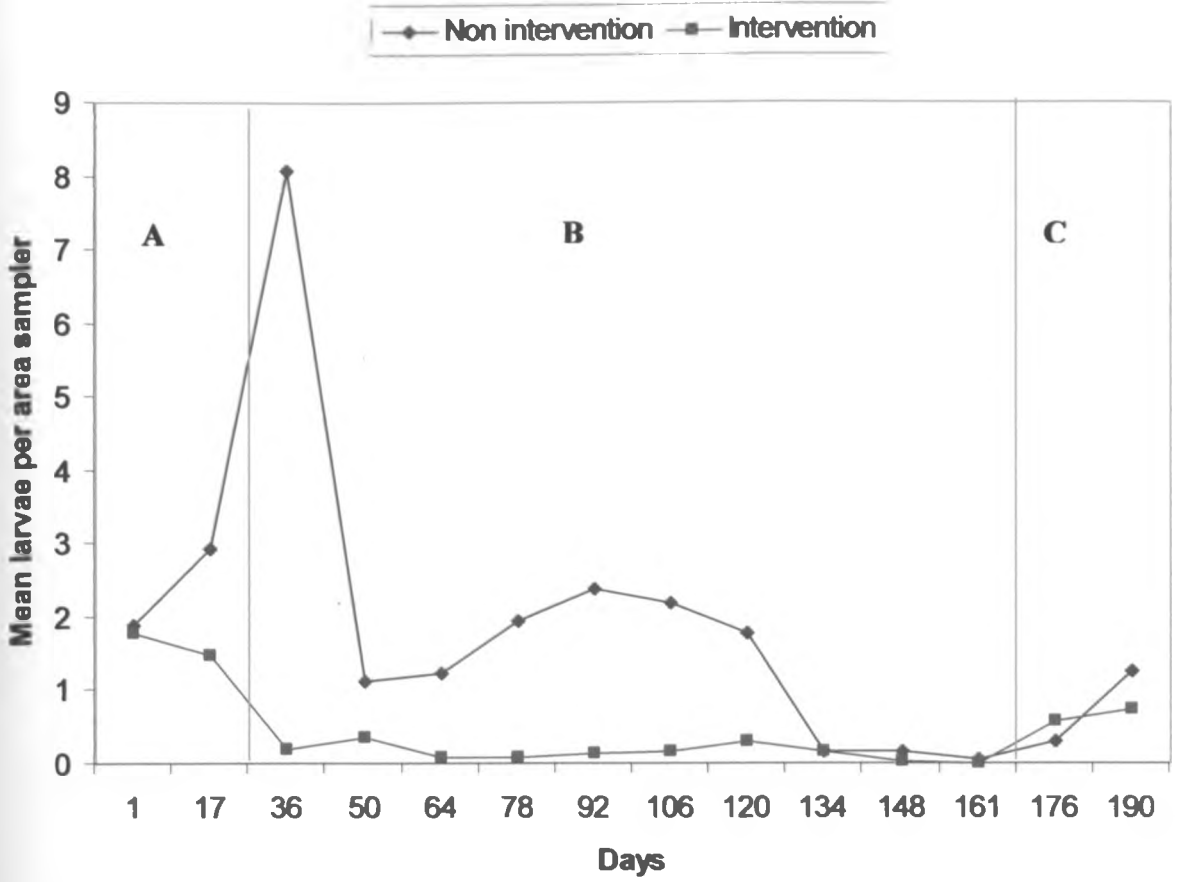
Figure 7A: Densities of anopheline larvae over time. Pre-intervention period **(A)**, Intervention

period **(B)** and Post- intervention period **(C).**
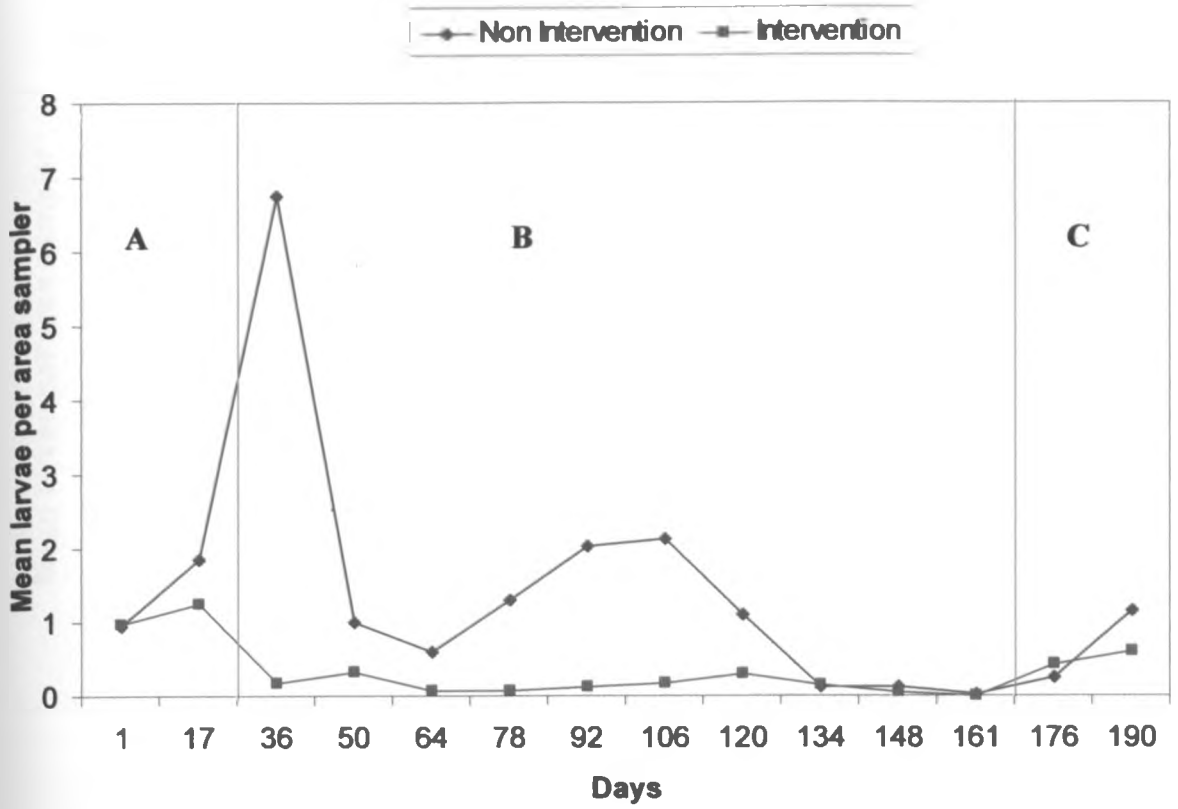
Figure 7B: Densities of early instar anopheline larvae over time. Pre-intervention period **(A)**, Intervention period **(B)** and Post- intervention period **(C)**.
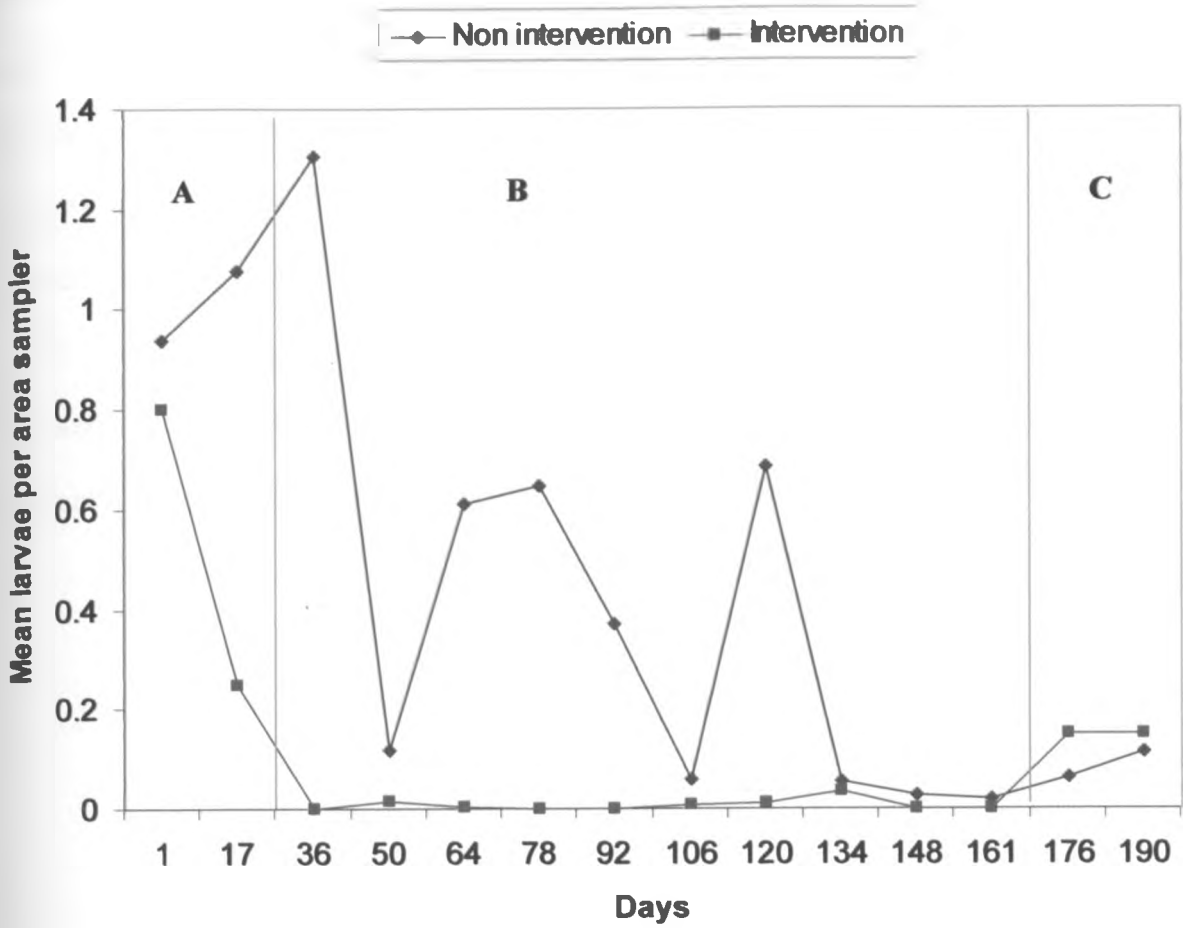
Figure 7C: Densities of late instar anopheline larvae over time. Pre-intervention period **(A)**, Intervention period **(B)** and Post- intervention period **(C)**.

The efficacy of the intervention in reducing immature anopheline mosquitoes was compared among the different stages as well as habitat types and the different vegetation types. Summary of the data is presented in Table 5. Overall, the density of *An. gambiae* in habitats observed in the intervention zone was reduced by 79%. For the L1 and L2 instar *An. gambiae*, densities were reduced by 71% while the late instars (L3 and L4 instar larvae plus pupae) were reduced by 97%. Among the different habitat types, the impact of larval control was reduced significantly for the animal hoof print, drainage ditch and borrow pit habitats but there was significant negative impact in swamps and no significant for other habitat types.

Table 1: Comparing total immature mosquito density by zones, stage, habitat and vegetation types during intervention period.

Variable	Factor	Intervention			Non intervention			Univariate rate ratio 95%			
		Total area sampler	Total immature	Mean/area sampler	Total area sampler	Total immature	Mean/area sampler	RR	Lower CI	Upper CI	P
	Total	8460	1207	0.142	5604	3418	0.609	0.21	0.13	0.33	<0.001
Stage	Early instars[1]	8460	1163	0.137	5604	2532	0.452	0.29	0.19	0.44	<0.001
	Late instars[2]	8460	44	0.005	5604	886	0.158	0.03	0.01	0.08	<0.001
Habitat type[3]	Puddle/tire truck	63	18	0.29	435	72	0.17	1.04	0.21	5.28	0.961
	Animal hoof print	48	88	1.83	26	530	20.38	0.11	0.04	0.30	<0.001
	Drainage/ditch	3188	162	0.05	3320	1126	0.34	0.14	0.06	0.34	<0.001
	Stream bed	4069	859	0.21	314	8	0.03	2.53	0.64	10.11	0.189
	Borrow pit	819	37	0.05	701	773	1.10	0.02	0.001	0.20	0.002
	Swamp	245	43	0.18	196	1	0.005	32.44	2.87	366.43	0.005
	Pond[4]	NA	NA	NA	610	908	1.49	NA	NA	NA	NA
	Others[5]	28	0	0	2	0	0	NA	NA	NA	NA
Vegetation type[3]	None	1214	268	0.22	652	729	1.12	0.09	0.03	0.34	<0.001
	Short	7041	938	0.13	4211	2421	0.57	0.23	0.14	0.39	<0.001
	Tall	191	1	0.005	727	268	0.37	0.04	0.01	0.18	<0.001
	Floating[5]	14	0	0	14	0	0	NA	NA	NA	NA

[1]Early instars include 1st and 2nd instar larvae.
[2]Late instars include 3rd and 4th instar larvae plus pupae.
[3]Analyses for each habitat and vegetation type were for total anopheline immatures.
[4]Pond habitat type was not observed in the intervention zone.
[5]For the other habitat types and the floating vegetation, several cells had zero values and therefore no statistical analysis was done.

3.2. Emerged pupae

During the intervention period, 61 *An. gambiae s.l.* pupae were allowed to emerge. All pupae collected during the intervention period were from the control zone. Polymerase chain reaction was done on all *An. gambiae s.l.* and 41 were successfully amplified. Of these, 95.1% were identified as *An. arabiensis*.

3.3. Adult densities

Mosquitoes were collected by PSC twice per month. The mean number of *Anopheles* females collected per sampling round is shown in figure 8. Average densities for the pre-intervention, intervention and post-intervention periods are given in table 3.
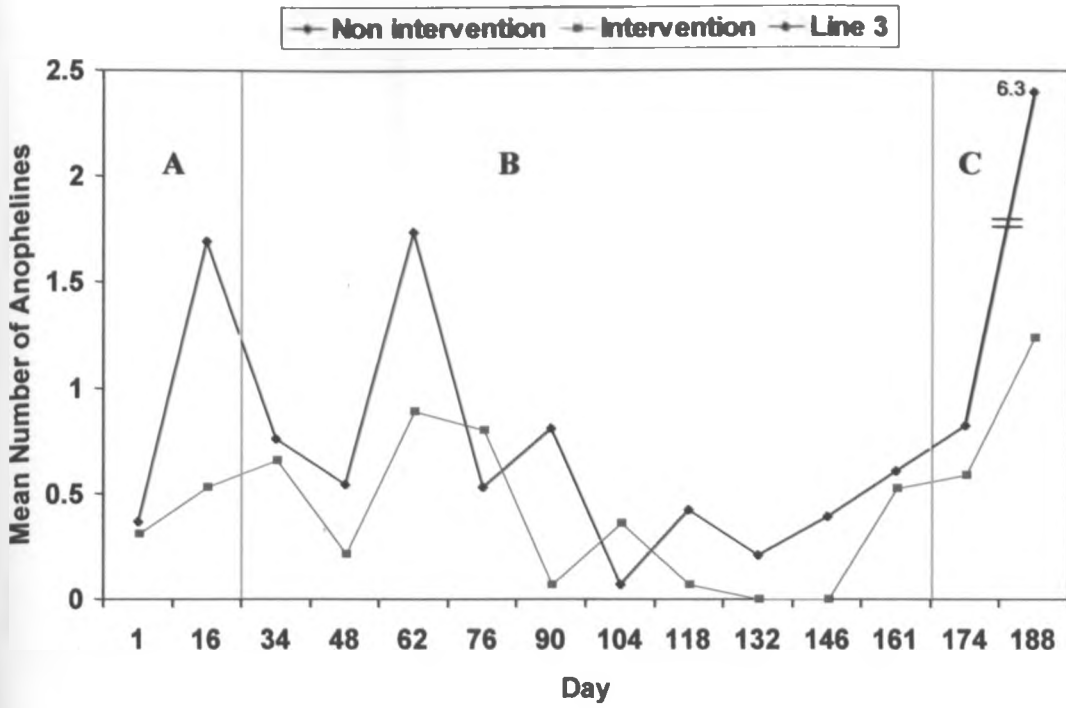
Figure 8: Densities of adult female *Anopheles* mosquitoes over time. Pre-intervention period

(**A**), Intervention period (**B**) and Post- intervention period (**C**). Line 3 was reduced its length

to shape the figure since the mean number was high (6.3).

Table 2: Average number of female mosquitoes in non intervention and intervention zones during the pre-intervention, intervention and post-intervention periods.

Period	Zone	Number of houses	Anophelines	Culicines
Pre-Intervention	Intervention	69	0.41 (0.20-0.61)	7.65 (4.68-10.61)
	Non intervention	64	0.91 (0.51-1.30)	3.94 (2.83-5.05)
Intervention	Intervention	259	0.35 (0.25-0.45)	3.38 (1.47-5.29)
	Non intervention	266	0.61 (0.45-0.78)	1.17 (0.92-1.42)
Post-Intervention	Intervention	34	0.91 (0.47-1.35)	1.03 (0.65-1.41)
	Non intervention	32	3.41 (1.06-5.75)	6.34 (1.26-11.43)

During the intervention period, there were 0.35 female anophelines per house in the intervention zone compared to 0.61 per house in the non-intervention zone. By poisson regression, there were 39.5% fewer mosquitoes in the intervention zone after controlling for household ownership of bednets (Relative ratio=0.605, p<0.001). Although fewer houses were sampled, there were fewer mosquitoes collected in the intervention zone before and after larval control was implemented. For culicine mosquitoes, there were consistently more females collected in the intervention zone than the non-intervention zone through all three time periods.

3.3.1. Species Composition of Adult Mosquito Populations

During the intervention period, a total of 385 anophelines were collected as adult mosquitoes, 125 in the intervention zone and 260 in the non intervention zone. Within the intervention zone, 14 of the 125 anophelines were identified as *An. funestus* (11.2%) while 111 were identified as *An. gambiae s.l* (88.8%). Within the non intervention zone, 53 of 260 anophelines were identified as *An. funestus* (20.4%) while 207 were identified as *An. gambiae s.l* (79.6%). A total of 350 *An. gambiae s.l.* were subjected to species identification by PCR. Of these 99 failed to amplify or were lost to follow-up. Of the 251 which were successfully amplified, 94.3% (83/88) in the intervention zone were identified as *An. arabiensis* while 90.8% (148/163) were identified as *An. arabiensis* in the non intervention zone.

The number of mosquitoes in each abdominal stage and estimated sporozoite rates are listed in table 4. For estimation of biting rates, fed and half-gravid mosquitoes were assumed to have fed within the last 24 hours. Dividing this number by the number of

52

people residing in each house gives the daily human biting rate and multiplying this number by the sporozoite rate gives the daily entomological inoculation rate (EIR). The EIR was then annualized by multiplying 365. Counting all anophelines, the annual EIR was estimated at 1.28 infectious bites per person per year in the non intervention zone. Most of this was contributed by *An. funestus* which had a relatively high sporozoite rate (6.90%). The sporozoite rates in the intervention zone were 0 for both *An. gambiae* and *An. funestus*. Therefore, the EIR for this zone was estimated at 0. The interpretation of these results, need to be approached with conscious as the densities of mosquitoes were very few.

Table 3: Impact of larval control on biting, sporozoite and EIR rates in a bed net area during intervention period.

	Zone	Number of houses	Number of people	Fed/H-gravid	Gravid	Unfed	Biting rate/night	Sporozoite rate (%)	Daily EIR	Yearly EIR
Total	Intervention	259	748	78	8	3	0.104	0	0	0
	Non intervention	266	645	125	26	17	0.193	1.81	0.0035	1.28
An. gambiae	Intervention	259	748	68	8	3	0.091	0	0	0
	Non intervention	266	645	100	23	14	0.155	0.75	0.0012	0.424
An. funestus	Intervention	259	748	10	0	0	0.013	0	0	0
	Non intervention	266	645	25	3	3	0.039	6.90	0.0027	0.982

3.4. Monitoring of spray team

The monitors managed to identify and sample 3256 breeding habitats. Of these, 19.5% (n=634/3256) were colonized with immature anopheline stage, 81.4% (n=516/634) had L1 and L2 stage larvae, while 15.3% (n=97/634) had L3 and L4 stage larvae and 3.2 % (n= 20/634) had pupae.

3. 5. Independent assessment of the monitors

Spot checks were conducted in 16 plots during intervention period to assess monitor's activity in terms of their sensitivity to detect immature mosquito stages.

Out of 131 potential habitats identified by the monitors, 9.9% (n=13/131) were colonized with *Anopheles* larvae stages; 9.9% (n=13/131) L1 and L2 stage larvae, 0% (n=0/131) for L3 and L4 stage larvae and 0 %(n=0/131) pupae. Spot check identified 158 potential habitats of which 27.22% (n=43/158) were colonized with *Anopheles* larvae stages, 26.58% (n=42/158) L1 and L2 stage larvae, 0.63% (n=1/158) Ł3 and L4 stage larvae and 0 % (n=0/158) pupae (table 5). The monitors sensitivity in detection of L1 stage larvae was 78% less, relative ratio (RR) = 0.22 (0.10-0.47, P = 0.0001) and 66% less, RR = 0.34 (0.17-0.69, P= 0.003) for L2 stage larvae. The numbers of L3 and L4 stage larvae were very few to perform statistical test.

Ta' 'e 4: Comparing efficiency of monitors by independent assessment.

Category	Immature stages					
	Early		Late		Pupae	
	Positive	Negative	Positive	Negative	Positive	Negative
Monitors	9.92%(n=13/131)	90.08%(n=118/131)	0%(n=0/131)	100%(n=131/131)	0%(n=0/131)	100%(n=131/131)
Spot check	26.58%(n=42/158)	73.42%(n=116/158)	0.63%(n=1/158)	99.37%(n=157/158)	0%(n=0/158)	100%(n=158/158)

CHAPTER FOUR

4.0. DISCUSSION

In this study, *An. gambiae* larvae density in the intervention zone was reduced by 79% for all instars and 97% for the late instars (L3, L4 and pupae). A 39.5% fewer female *Anopheles* mosquito was observed in the intervention zone. *An. arabiensis* was the dominant species accountable for 90%. The annual EIR was estimated at 1.28 infectious bites per person per year in the non intervention zone. *An. funestus* contributed higher sporozoite rate (6.90%) then *Anopheles gambiae* (0.75%). The sporozoite rates in the intervention zone were 0 for both *An. gambiae* and *An. funestus*. Therefore, the EIR for this zone was estimated at 0.

These results on adult densities obtained in the study area are higher then that observed in Dar es Salaam where a 31% reduction in *Anopheles gambiae* mosquitoes was observed (Fillinger *et al.*, 2008). However, the results differ from two studies conducted in western Kenya. In Mbita point, adult *Anopheles* densities were reduced by over 90% by a larvae control program (Fillinger and Lindsay, 2006). Nevertheless, in a previous study conducted in the same area as the current study, no effect was observed on the density of adult anophelines (CDC unpublished data).

The reasons for the different outcomes in these different studies are not clear but include several possibilities. In the Mbita study, the trial was conducted before nets were widely

57

available. Although an added benefit was observed in the current study, it is likely that reducing vector populations by one intervention masks the effects of the other. Another possible difference is that the Mbita study area was bounded on two sides by Lake Victoria and was therefore less subject to mosquitoes flying in from outside the study area. The other study conducted in Asembo Bay showed a significant reduction in larval mosquitoes but no reduction in adults. One obvious difference between the previous and the current studies was that more larval control personnel were used and more emphasis was placed on monitoring. The previous study relied on, at most, two persons to cover four km^2 in four days and had no monitoring. In the current study, two persons covered four km^2 in four days but were followed up on the second day with monitors who treated any habitats with late stage larvae missed by the larval control personnel. These monitors were then followed up by spot checking to verify the accuracy in terms of coverage and habitat status on the same or second day.

The impact of the intervention on the larval population was much greater than that on the adult population. This has also been observed in other studies (Fillinger et al., 2008). It is not clear why there was such a large discrepancy. It is possible that adult mosquitoes fly into the intervention zone from larval habitats located outside the intervention zone (Gillies and DeMeillon, 1968; Killeen et al., 2003). A buffer of 0.5 km was created around the evaluation zone to avoid this, but An. gambiae has been recorded to fly up to 2 km from its breeding sites (Gillies and DeMeillon, 1968; Service, 1997; Killeen et al., 2003). Studies of mosquito populations in a large scale bednet study suggested that there were significant effects on the mosquito populations in control villages located next to

intervention villages. The effects were observed up to 600m away from the intervention villages suggesting the average flight range for *An. gambiae* in this area was less than 600 m (Gimnig *et al.*, 2003b). However, this study can not discount the possibility that mosquitoes flew into the intervention zone from breeding sites outside the intervention zone. A further trial using a larger intervention zone would be required to test this hypothesis.

Molecular species identification demonstrated that *An. arabiensis* was the predominant malaria vector, accounting for 90% of all *An. gambiae* complex caught during the study period. This result is consistent with early study conducted in the same area whereby *An. arabiensis* were the dominant species (CDC unpublished data). In western Kenya, *An. arabiensis* feeds predominantly on cattle and, while frequently caught indoors, spends much of its time outdoors and is therefore less affected by vector control measures targeting the indoor environment (e.g. ITNs and IRS). However, *An. arabiensis* is still capable of transmitting malaria, although less efficiently than *An. gambiae*. Therefore, strategies that better target this mosquito, including larval control, are necessary to further reduce malaria transmission in this region. The control of nuisance mosquitoes was poor, similar to observations in other urban centres in East Africa, where anti-larval measures for malaria control were implemented (Lindsay *et al.*, 2004). Culicine mosquitoes often breed in pit latrines which were not our target habitats. However, the experience of others has shown that control of culicine mosquitoes, accountable for the majority of nuisance biting, would be necessary to attain community recognition and support in larval control activities (Fillinger *et al.*, 2008).

The monitoring system proved to be an effective tool for ensuring all habitats were visited and treated. The monitors carried *Bti* CG which was used when they encountered untreated habitats with late stages. In follow up visits, <10% were found to harbour immature stages of *An. gambiae* and only one habitat was observed to harbour late stage larvae. Independent monitoring of the habitats by a team who did quantitative collections of larvae showed that there was a 97% reduction in late stage (L3, L4 instar and pupae) immature *An. gambiae*.

One advantage of larval control is that many more insecticides and formulations are available for application. Most are safe to non-target organisms and easy to apply. *Bacillus thuringiensis israelensis* was chosen because of rapid killing effect on the larvae, facilitating monitoring. One disadvantage of the *Bti* is its short duration of efficacy. Semi-field studies in western Kenya have shown *Bti* is effective against anopheline mosquitoes for only 1-2 days (CDC unpublished data).

Other larvicides, particularly insect growth regulators, may be available in more persistent formulations, in some cases lasting up to six months (Yapabandara and Curtis, 2002). However, these larvicides may require substantial changes to the monitoring system as they do not cause immediate mortality of the immatures. Furthermore, while a persistent formulation would allow less frequent visits to a given area, in most areas of sub-Saharan Africa where *An. gambiae* breeds in temporary habitats that often appear and disappear with the rains and/or human activity, frequent visits to monitor newly formed habitats may be required regardless of the insecticide used.

The cost of the intervention was similar to that reported for IRS programs (Guyatt *et al.*, 2002). The labour costs for 7 months of treatment were estimated at US$ 2028.56 while the larvicide costs were estimated at US$ 974.4 The monthly cost was therefore US$ 429 (US$ 3002.96/7) and the annual costs were US$ 5148 (US$ 429*12). The annual cost to cover one km^2 of area was US$ 107.25 (US$ 429/4). The population density of Asembo is approximately 275 people per square kilometre. Therefore, the annual cost per person protected is approximately US$ 0.39 (US$ 107.25 /275). At an exchange rate of 75 Kenya Shillings to the US Dollar, the cost was estimated at Kshs. 29.25 (US$ 0.39 *75). Note that these costs do not include the cost of the overall supervisor or some transport costs. However, these were minimal in the current study and would likely add no more than US$ 0.5-1 per person protected per year.

The evaluation of a larval control intervention is a challenge as it is an environmental intervention which requires coverage of a large area for an adequate period of time. Given these challenges, there were several limitations to this study. The biggest limitation was failure to do a true randomization of the larval control intervention as the study area covered only four km^2. The significant pre- or post-intervention collections were not performed to ensure that the intervention and non-intervention zones were similar. Another limitation is failure to include true parasitological outcome indicators e.g. disease incidence. This would be necessary to evaluate the actual impact of malaria disease itself. Based on the data observed here, there was evidence for a pre-existing difference between the two zones independent of the larval control program. Thus, these results should be interpreted with care as this would suggest the true impact of larval

control is less than what was observed here. Conversely, there is increasing evidence that the scale of any vector control intervention, both in terms of geographic and temporal scales is important for maximizing the impact of that intervention. This is likely to be particularly true for larval control which relies on area wide coverage and which may be undermined by in-migration of adult mosquitoes from outside the coverage areas. This study was conducted for seven months and only covered an area of four km^2. While a buffer zone was included to limit in-migration, it is possible that the impact on the adult population would have been greater had the study area been larger.

The findings of this study shows that major malaria vectors in rural western Kenya can be significantly reduced by intensive larviciding with monitoring and the use of bed nets. This finding supports early studies conducted in Mbita, Suba district, western Kenya (Fillinger and Lindsay, 2006) as well as Dar es Salaam (Fillinger *et al.*, 2008). However, further work is encouraged to determine the effectiveness of this program on a larger scale and to better refine the management system that was set in place.

4.1. Conclusions and recommendations

The seven months implementation of intensive larviciding demonstrated significant reduction in the densities and emergence rate of immature stages of malaria vectors in endemic rural settings. However, the impact on immature mosquito was not fully reflected to adult density. Monitoring system proved to be an effective tool of ensuring all habitats were visited and treated.

62

Recommendations:

1. There is need to expand the intervention area with a buffer not less then 2 km to limit flying mosquitoes into intervention zone from breeding site out side the intervention zone.

2. The stability of the effect of larviciding should be monitored for a long period of time to guarantee achievement of maximum possible results on adult densities.

3. Larval control should not be considered as a stand-alone intervention, but should be implemented as part of an integrated malaria vector control program.

4. The cost of intervention in this area could be reduced with larvicides that provide a great residual effect unlike *Bti* which is applied weekly.

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Appendix 1. PCR reagents for identification of the members of *An. gambiae s.l.*

Master mix reagents	X1(μl)
PCR water	13.9
PCR buffer (10x)	2.0
MgCl$_2$ (25 mM)	1.5
Taq DNA polymerase (5 U/μl)	0.1
dNTPs (2.5 mM each)	0.3
GA (10 μM)	0.4
AR (10 μM)	0.4
UN (10 μM)	0.4
DNA template	1.0