RESISTANCE MECHANISMS AND SUSCEPTIBILITY TO ORGANOPHOSPHATES, CARBAMATES AND PYRETHROIDS IN *ANOPHELES GAMBIAE* S.L. GILES (DIPTERA: CULICIDAE) IN HOHOE DISTRICT, GHANA.

BY

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A thesis submitted in partial fulfillment of the requirements for the award of Master of Philosophy degree in Entomology of the University of Ghana, Legon

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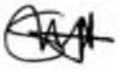
DECLARATION

I certify that all material in this thesis which is not my own work has been duly acknowledged and that no material has previously been submitted and approved for the award of a degree by this or any other university.



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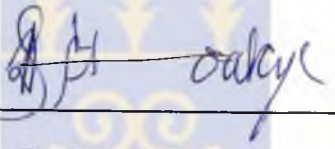

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DEDICATION

To my entire Family and Friends,

Your concern, love and support

Kept me focussed.

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God Bless you all

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LIST OF ABBREVIATIONS

bp	base pairs
ddw	distilled de-ionised water
dCPT	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide phosphate
dTTP	deoxythymidine triaphosphate
EDTA	disodium ethylene diamine tetracetate.$2H_2O$
EtBr	ethidium bromide
EtOH	ethanol
H_2O	water
M	molar (moles per liter)
mM	millimolar
μM	micromolar
ml	milliliter
μl	microliter
Mw	molecular weight
NaOH	sodium hydroxide
KAc	potassium acetate
pH	$-\log_{10}[H^+]$
rpm	revolution per minute
RNase	ribonuclease
s.l.	*sensu lato*
s.s.	*senso stricto*
T_M	melting temperature
g	gram
mg	milligram
μg	microgram

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ABSTRACT

Malaria is a threat to more than 40% of the world's population accounting for more than 300 million acute cases and between 1.1 and 2.7 million deaths annually. Over 90% of the malaria cases are in sub-Saharan Africa, constituting 10% of the total disease burden. Currently activities support the extensive use of insecticide treated materials for malaria control. However these efforts are threatened by the evolution of resistance in the main malaria vectors, *Anopheles gambiae* s.l. towards the commonly used insecticides for mosquito control. The study was done to determine the susceptibility status and the underlying mechanisms of resistance in *An. gambiae* s.l against commonly used insecticides for control in Hohoe District, Volta region of Ghana. Mosquitoes were sampled in four villages; Adabraka, Atabu Newtown, Kledzo and Likpe-Bakwa. Adult mosquitoes were tested against the WHO diagnostic concentrations of 0.75% permethrin, 0.05% deltamethrin, 4% DDT, 5% malathion and 0.1% propoxur. The susceptible Kisumu strain of *An. gambiae* s.l was used as the reference.

Results obtained revealed high levels of resistance to DDT; 6-51% mortality rates in the four villages sampled. Susceptibility to Permethrin was considerably low with 32-82% mortality rates. Mortalities were very high with 0.05% Deltamethrin; 91-97%. All field mosquitoes tested were fully susceptible to Malathion with 100% mortality rates across the 4 villages. Susceptibility to Propoxur was similarly higher; 94-95% mortality rates.

Median Knockdown times in field populations variously increased compared with the susceptible Kisumu strain; 4-6, 1.5-2 and 3 fold with Permethrin, Deltamethrin and

DDT respectively. PCR identification revealed that all the mosquitoes tested were *An. gambiae* sensu stricto with 64% 'Savanna' and 36% 'Mopti' forms. The *kdr* mutation occurred at a frequency of 23.7%, 21.7%, 22.7% and 31% in *An. gambiae* populations of Adabraka, Atabu Newtown, Kledzo and Likpe respectively. Over 70% of the *kdr* mutation occurred in the 'Savanna' form. Biochemical mechanisms of resistance were investigated by the CDC microplate assays protocol. There was a significant elevated activity of mixed function oxidases in *An. gambiae* populations in Likpe as compared to the susceptible Kisumu strain. Activity of Acetylcholine esterase enzyme was significantly elevated in Adabraka population while Glutathione S-transferases showed no significant increase in activity in all the four wild population. There was a significant elevated activity of both α and β-nonspecific esterases in Adabraka and Likpe populations. The high frequency of *kdr* and elevated activity of several detoxification enzymes indicate the occurrence of multi resistance in *An. gambiae* populations in Hohoe district.

Insecticide resistance has been a problem in all insect groups that are vectors of diseases. Regular testing is therefore vital for mosquito control operations because resistant populations of mosquitoes reduce the effectiveness of control procedures. The main defence against resistance is close surveillance of the susceptibility of vector populations so as to detect changes in their susceptibility status at an early stage and to implement resistance management strategies.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Malaria is a life-threatening parasitic disease affecting more than 40% of the world's population and out of the more than 300 million acute cases each year, between 1.1 and 2.7 million people die (RBM, 2002; WHO, 2000). Over 90% of malaria cases are in sub-Saharan Africa, where malaria accounts for 10% of the total disease burden. Children under five and pregnant women are most at risk (TDR/WHO, 2002; RBM/WHO, 2000). Malaria constitutes nearly 25% of all childhood mortality in Africa (WHO, 2000).

Malaria is caused by protozoan parasites of the genus *Plasmodium* and is transmitted amongst humans by mosquitoes of the genus *Anopheles*. There are 120 *Plasmodium* species, of which four; *P. falciparum, P. vivax*, *P. malariae* and *P. ovale* are known to infect humans. All the four species share a common basic life cycle although there are considerable differences in their pathogenecity, epidemiology, appearance, development, and host-parasite relationships. It is *P. falciparum*, however, that causes nearly all of the mortality in cases of malaria infection. Mosquitoes of the genus *Anopheles* are always the vectors and out of the more than 380 species of anopheline mosquitoes, only 60 can transmit malaria. Only female mosquitoes are involved in transmission as the males do not require a blood meal.

The World Health Organization suggests that there are three essential elements of malaria control (WHO, 1995). First is the selective application of vector control by reduction of the numbers of vector mosquitoes and reducing human-mosquito contact. Secondly, the early diagnosis followed by effective and prompt treatment of malaria cases in all areas where people are at risk, and the third element is early detection or forecasting of epidemics and rapid application of control measure.

Since malaria is transmitted by the female *Anopheles* mosquito, a major strategy of control is to attack the vector with insecticides. Extended use, however, has led and continues to lead to the emergence of insecticide resistance in mosquitoes (Hemingway and Ranson, 2000; Philips, 2001). There has recently been an increase in antimalarial activities with the Roll Back Malaria initiative and Global Fund for Health, which support extensive use of pyrethroid-impregnated bed nets for mosquito control campaigns in Africa and other malaria-endemic regions (Curtis *et al*, 1998; Akogbeto and Yakoubou, 1999). However these activities are greatly threatened by the evolution of resistance among malaria vectors towards the commonly used insecticides to control them (Chandre *et al.*, 1999a,b; Hougard *et al.*, 2003; Corbel *et al.*, 2003; Curtis *et al.*, 2003). Furthermore, insecticide resistance is assumed to increase the likelihood of mosquito-borne disease transmission by increasing the vector population size and allowing mosquitoes to live longer in the presence of insecticide (McCarroll and Hemingway 2002).

Mosquitoes can develop resistance through several different mechanisms (Hemingway and Ranson, 2000). Physiological resistance is one way mosquitoes can become immune to insecticides. For example, reduced penetration through the cuticle has been noted in several mosquito species. This gives the detoxification mechanisms in the mosquito more time to deal with uptake of the toxicant hence the greater its chances for survival (Hemingway and Ranson, 2000). Insecticide resistance can also result from reduced sensitivity of the target sites that normally bind to the insecticides. For example, mutations in sodium channels (the target of DDT and pyrethroids) and in acetylcholinesterase (the target of organophosphates and carbamates) have been well documented in many insect species including mosquitoes (Hemingway *et al.*, 2004). Metabolic resistance occurs when detoxification enzymes are used to break down the insecticide into compounds such as amino acids and sugars, which can be metabolized by the mosquito.

Insecticide resistance has been a problem in all insect groups that serve as vectors of emerging diseases (Hemingway and Ranson, 2000). Although mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance foci. Several strategies and recommendations have been proposed for the management of insecticide resistance in field populations. The most important aspect of the management of resistance is to either avoid or delay the onset of resistance by using the available insecticides judiciously, for example as mixtures, in rotation or in mosaics. The practice of using an insecticide until resistance becomes a limiting factor is rapidly eroding the number of suitable insecticides for vector control. Thus, the main defense against resistance is close surveillance of the susceptibility of vector populations.

1.2 Rationale and Objectives

Regular resistance testing is vital for mosquito control operations because resistant populations of mosquitoes reduce the effectiveness of control strategies. The resistant phenotype is relatively easy to monitor with direct insecticide bioassays. However, in many cases the actual molecular and biochemical mechanisms responsible for the resistant phenotypes are still unknown. Currently, the mechanisms that regulate insecticide resistance are poorly understood. *Anopheles gambiae* s.l. has multiple resistance mechanisms that have been field-selected in both East and West Africa through exposure to DDT and pyrethroids (Hemingway *et al.*, 2002). It is not clear how much the current large-scale pyrethroid resistance of mosquitoes in West Africa will affect the extensive use of pyrethroid impregnated bed nets for mosquito control campaigns in Africa, and what will replace the pyrethroid-treated nets if selection of multi-resistance mechanisms results in widespread failure of this strategy (Curtis *et al.*, 1998).

In Ghana several studies on susceptibility/resistance on *An. gambiae* complex to insecticides have been conducted, however they have been focused mainly in the Greater Accra Region and its environs (Adasi *et al.*, 2000; Adeniran, 2002; Otieno, 2004) and in the Western Region (Kristan *et al.*, 2003). It is therefore imperative that more studies on susceptibility/resistance in malaria vectors towards the commonly used insecticides be carried out in different ecological zones of Ghana in order to obtain broader baseline information so as to enable the development malaria vector control programs on national scale and to implement resistance management strategies. Furthermore due to the complex interplay of factors conferring resistance in mosquitoes, there is need for an in-depth study on the underlying mechanisms of resistance especially the biochemical mechanisms in *An.*

gambiae complex, in addition to the knockdown resistance mechanism of which was the focuss of most of the earlier studies in the country.

The present study was therefore carried out to gather information on susceptibility profiles and the underlying mechanisms of resistance against the commonly used insecticides in *An. gambiae* s.l. populations from Hohoe district in the Volta Region, an area in Ghana where no such information exist.

1.2.1 General objective

The general objective of the study was to determine the susceptibility status and the mechanisms of resistance in *Anopheles gambiae* s.l Giles to the commonly used insecticides in the Hohoe area.

1.2.2 Specific objectives

1. To determine the susceptibility status of adult *Anopheles gambiae* s.l to Permethrin, Deltamethrin, DDT, Malathion and Propoxur using WHO adult bioassay methods.
2. To identify *Anopheles gambiae* s.l mosquitoes to species and also the molecular forms of *Anopheles gambiae* s.s.
3. To characterize the *kdr* alleles in populations of *Anopheles gambiae* s.l using a PCR based method.
4. To determine the operative biochemical mechanisms of resistance by conducting enzyme assays for Oxidase, Acetylcholinesterase, Non-specific esterases and Glutathione S-transferase.
5. To relate data on bioassays with those of *kdr* and enzyme assays.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria: Disease and Symptoms

The term malaria is Roman in origin, although the disease was not known by its present name until the mid-eighteenth century. Before then it was referred to variously as ague, intermittent fever, swamp fever, Roman fever, and death fever. Previously, it was thought that "miasma" (bad air or gas from swamps - "mal air ia") caused the disease. Malaria has been known since time immemorial, but it was centuries before the true causes were understood. Hippocrates was the first to describe the manifestations of the disease, and relate them to the time of year and to where the patients lived before this, supernatural were blamed.

There are four species of malaria parasites in humans; *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* (Phillips, 2001). Out of the four, two are most common; *Plasmodium falciparum*, which is found globally but is commonest in Africa, is the most aggressive species, often killing by coma or anaemia (Miller *et al.*, 2002). *Plasmodium vivax*, which ranges widely throughout Asia, Africa, the Middle East, Oceania and the Americas, can cause recurring and debilitating infection, but rarely kills.

The different malaria parasites produce fevers of different frequency, depending on how long it takes to complete shizogony in erythrocytes. Generally the patient will complain of headache, fever and aches and pains all over the body. However, fever is not always present and rigours may or may not be present. At its peak, a person's fever can soar to 41°C (106°F). Several hours later, the fever drops and chills set in. Two to four days later, the cycle repeats.

Diarrhoea and abdominal pain are sometimes present. Spleen and liver are often palpable on clinical examination. This may be misdiagnosed as influenza in non-endemic areas and unless treated promptly, the clinical picture can deteriorate rapidly. A patient with severe and complicated malaria will often present with impaired consciousness, weakness, and jaundice.

Other complications are cerebral malaria, generalised convulsions, normocytic anaemia, renal failure, hypoglycaemia, fluid, electrolyte and acid-base disturbances, pulmonary oedema, circulatory collapse, shock, disseminated intravascular coagulation, hyperpyrexia, hyperparasitaemia, and malarial haemoglobulinurea. These features may occur singly or in combinations. Severe and complicated malaria is usually caused by delay in treating an uncomplicated attack of *P. falciparum*. Cerebral malaria is the most dreaded form of disease and is unique to *P. falciparum*. Red blood cells infected by the parasite are sticky and can gum up the capillaries of the brain (Marsh, 1992; Miller *et al.*, 2002). The victim enters a coma and even if death does not occur, brain damage can be the result. Death can strike in as little as 24 hours from first symptoms. Anemia is another threat due to the parasite's cyclical attacks and rupture of red blood cells.

2.2 Life cycle and transmission of malaria parasites

The *Plasmodium* parasites have a life cycle which is split between a vertebrate host and an insect vector (Figure 1). Briefly, a biting female *Anopheles* mosquito transfers about 10% of its sporozoite load on each occasion, in her saliva, into the circulating blood of the host and within 30 to 45 minutes have entered hepatocytes. Growth and division in the liver for the human malaria parasites take from approximately 6 to 15 days depending on the species,

approximately 6, 10 and 15 days for *P falciparum*, *P vivax*, and *P ovale* and *P. malariae*, respectively. At the end of the pre-erythrocytic cycle, thousands of merozoites are released into the blood flowing through the sinusoids and, within 15 to 20 seconds, attach to and invade erythrocytes.

Recognition and attachment are via a receptor-ligand interaction. In *P. vivax* and *P. ovale*, some of the sporozoites appear to develop for about 24 hours before becoming dormant as a hypnozoite stage. This form can remain as such for months and even years until reactivated to complete the liver cycle, releasing merozoites into the blood to precipitate a relapse infection.

The asexual erythrocytic cycle produces more merozoites that are released with the destruction of the red blood cells after 48 or 72 hours for the human malaria parasites, depending on the species, and which then immediately invade additional erythrocytes. Consequently, they complete schizogony together at the end of the asexual cycle, releasing pyrogenic materials which induce the characteristic fever spike and clinical symptoms. The morbidity and mortality associated with malaria are derived solely from the erythrocytic stages (Miller *et al.*, 2002). The asexual cycle usually continues until controlled by the immune response or chemotherapy or until the patient dies (in the case of *P. falciparum*).

After invading red blood cells, eventually some merozoites differentiate into sexual forms (gametocytes) which have no further activity within the human host and, following ingestion by another female mosquito, will mature to male and female gametes in the blood meal.

After fertilization, the resulting zygote matures within 24 hours to the motile ookinete, which burrows through the midgut wall to encyst on the basal lamina, the extracellular matrix layer separating the haemocoel from the midgut. Within the developing oocysts, there are many mitotic divisions resulting in oocysts full of sporozoites. Rupture of the oocysts releases the sporozoites, which migrate through the haemocoel to the salivary glands to complete the cycle approximately 7 to 18 days after gametocyte ingestion, depending on host-parasite combination and external environmental conditions.

Not all *Anopheles* mosquitoes are vectors for *Plasmodium* parasites and these refractory mosquitoes posses substances toxic to *Plasmodium* within their cells (Beier, 1998). A higher trypsin-like activity has also been found in the midgut of resistant mosquito species, possibly inhibiting ookinete development. Sporogony within the mosquito is governed by environmental temperature as anopheline mosquitoes are poikilotherms. The female anopheline mosquitoes' ability or competence to transmit malaria is governed by a complex interaction of environment, behavioural and biological features, including vector density, blood meal preference, feeding and resting habits, flight range, longevity, humidity and temperature.

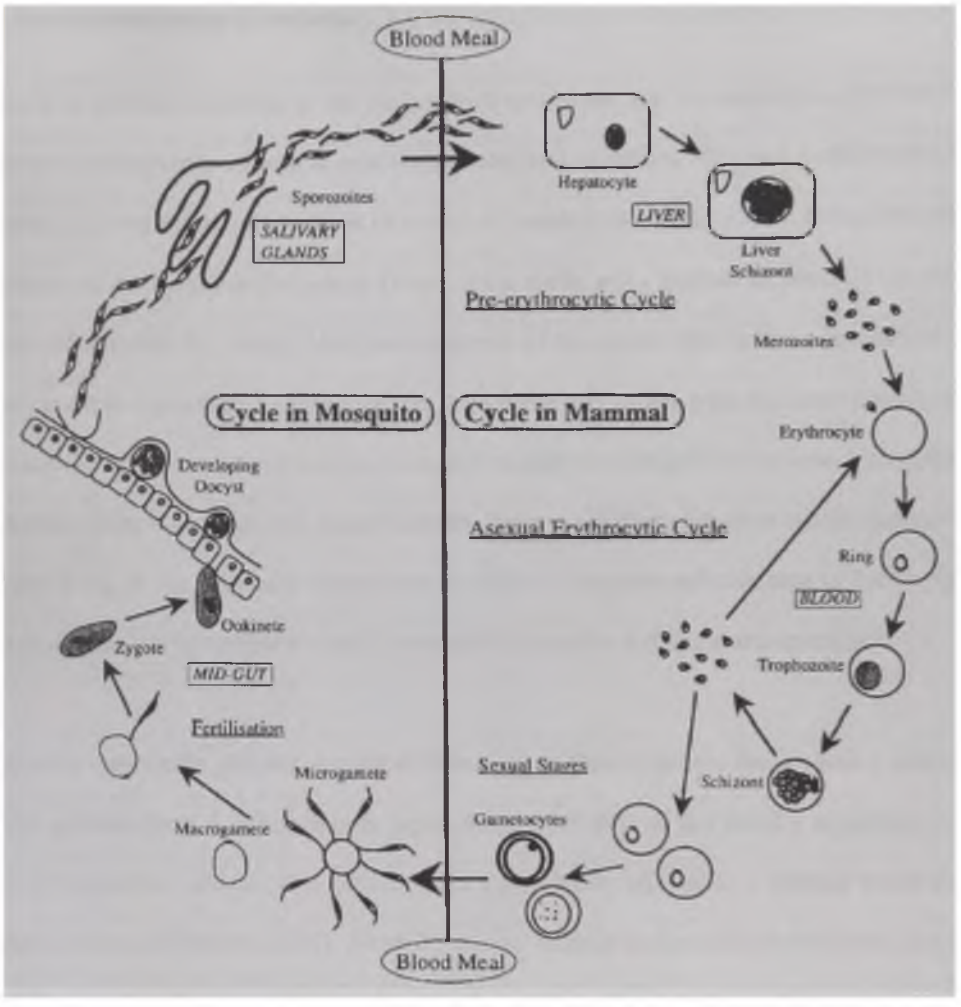

Figure 2.1: The Life cycle stages of malaria parasites (Phillips, 2001).

2.3 Global distribution of Malaria.

Malaria is generally endemic in the tropics, with extensions into the subtropics. Previously extremely widespread, malaria is now mainly confined to Africa, Asia and Latin America (Figure 2.2). Malaria affects the lives of almost all people living in the area of Africa defined by the southern fringes of the Sahara Desert in the north, and a latitude of about 28° in the south (WHO/UNICEF, 2003). Most people at risk of the disease live in areas of relatively stable malaria transmission where infection is common and occurs with sufficient frequency that some level of immunity develops. A smaller proportion of people live in areas where risk of malaria is more seasonal and less predictable, because of either altitude or rainfall patterns. People living in the peripheral areas north or south of the main endemic area or bordering highland areas are vulnerable to highly seasonal transmission and to malaria epidemics.

Malaria is responsible globally, for 500 million cases of clinical disease and presents a public health problem for 2.4 billion people, representing over 40% of the world's population in over 90 countries. Almost 10% of the world's population will suffer a clinical attack of malaria each year (Phillips, 2001). About 90% of all malaria deaths in the world today occur in Africa south of the Sahara. This is because the majority of infections in Africa are caused by *P. falciparum*, the most dangerous of the four human malaria parasites. It is also because the most effective malaria vector, *An. gambiae*, is the most widespread in Africa and the most difficult to control (Greenwood and Mutabingwa, 2002).

Figure 2.2: Global distribution of malaria and drug resistance (UNICEF, 2000).

2.4 Socio-economic burden and risk of malaria

"Where malaria prospers most, human societies have prospered least" (Sachs and Malaney, 2002). Malaria's cost to human and social well-being is enormous. It is a major cause of poverty and poverty exacerbates the malaria situation (UNICEF, 2000). So too is the economic loss, which in Africa alone is estimated at more than $2 billion annually (WHO, 2000). The disease has slowed economic growth in African countries by 1.3% per year, the compounded effects of which are a gross domestic product level now up to 32% lower than it would have been had disease been eradicated from Africa in 1960 (RBM, 2002). Malaria is most intractable for countries in the poorest continent, Africa (Gallup and Sachs, 2001) and poor countries predominate in the same regions as malaria (Miller *et al.*, 2002). Almost all of the rich countries are outside the bounds of intensive malaria.

In all malaria-endemic countries in Africa, 25–40% of all outpatient clinic visits are for malaria (with most diagnosis made clinically). In these same countries, between 20% and 50% of all hospital admissions are a consequence of malaria. With high case-fatality rates due to late presentation, inadequate management, and unavailability or stock-outs of effective drugs, malaria is also a major contributor to deaths among hospital inpatients. Malaria is responsible for a high proportion of public health expenditure on curative treatment, and substantial reductions in malaria incidence would free up available health resources and facilities and health workers' time, to tackle other health problems (WHO/UNICEF, 2003).

In areas of stable malaria transmission, children and pregnant women are the population groups at highest risk of morbidity and mortality. Most children experience their first malaria

infections during the first year or two of life, when they have not yet acquired adequate clinical immunity, which makes these early years particularly dangerous (Greenwood and Mutabingwa, 2002). Ninety percent of all malaria deaths in Africa occur in children aged below 5 years. At least 20% of all deaths in children in this age group is due to the disease. Children who survive malaria may suffer long-term consequences of the infection. Repeated episodes of fever and illness reduce appetite and restrict play, social interaction and educational opportunities, thereby contributing to poor development. An estimated 2% of children who recover from malaria infections affecting the brain (cerebral malaria) suffer from learning impairments and disabilities due to brain damage including epilepsy and spasticity.

Adult women in areas of stable transmission have a high level of immunity, but the normal weakening of the immune system especially during the first pregnancy makes infection more likely and the anaemia associated with pregnancy gives the parasite an advantage. Pregnant women are four times as likely to get the disease, and half as likely to survive cerebral malaria. If they do, their foetus may not as the extreme fevers often cause spontaneous abortion and stillbirths.

2.5 Biology and Life Cycle of the Vectors; the Genus *Anopheles*

Anopheles mosquitoes belong to the order Diptera, Suborder Nematocera, Family Culicidae and Subfamily Anophelinae. They have a worldwide distribution, occurring in both tropical and temperate regions. Of the over 500 known species of *Anopheles*, only about 60 are able to transmit malaria. Important malaria vectors include *An. culicifacies* in South West Asia, *An. darlingi* in North America and *An. albimanus* in Central America (Service, 1993). In Africa, they include *An. pharoencis* in Egypt, *An. gambiae* complex and *An. funestis* in West and East Africa (Nchinda, 1998).

Like all mosquitoes, anophelines go through four stages in their life cycle: egg, larva, pupa, and adult (Figure 2.3). The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. Eggs are generally laid directly on water or damp soil, often in tiny bodies of water such as those formed by flooded hoof prints or tyre tracks. The female anopheline deposits eggs singly on the water surface and the boat-shaped eggs have some grooves on each side to keep them afloat. Eggs hatch in approximately 2 to 3 days after oviposition, although some larvae can remain quiescent in unhatched eggs on damp soil for up to 2 weeks, thus the population can survive periods of erratic rains (Robert and Collins, 1996).

Larva hangs suspended by surface tension, breathing through air tubes and feeding on micro-organisms by filtering the water. Larval development is rapid and can be completed in less than one week in very warm conditions and ample food. Larvae develop through 4 stages, or instars, after which they metamorphose into pupae. At the end of each instar, the larvae

moult, shedding their exoskeleton to allow for further growth. Pupation typically takes place in full sunlight. The coma-shaped pupa is active and does not feed but has to come to the water surface to breathe. Pupal stage is short, approximately 1 to 2 days and adult emerges typically at sunset.

Both male and female adult require at least 24 hours to reach sexual maturity and mating is associated with a swarming behaviour. A swarm consists mainly of males, and females fly into it to mate, which typically takes place after sundown. Mated females seek blood meals only at night. Egg development takes about 2 days during the warm season, but it can be longer in cooler months. Oviposition like blood-feeding also occurs at night. Consequently, the gravid female generally lays her eggs the second night after she has blood fed and after oviposition searches for another blood meal. This repeated feeding and oviposition has major implications for transmission of malaria parasites which have a required developmental cycle in the mosquito (Robert and Collins, 1996).

Since anopheline mosquitoes typically breed in stagnant, unpolluted surface waters they are mainly associated with rural settings. *Anopheles* mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscis, and by the presence of discrete blocks of black and white scales on the wings. Adult *Anopheles* can also be identified by their typical resting position as males and females rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting (figure 2.4).

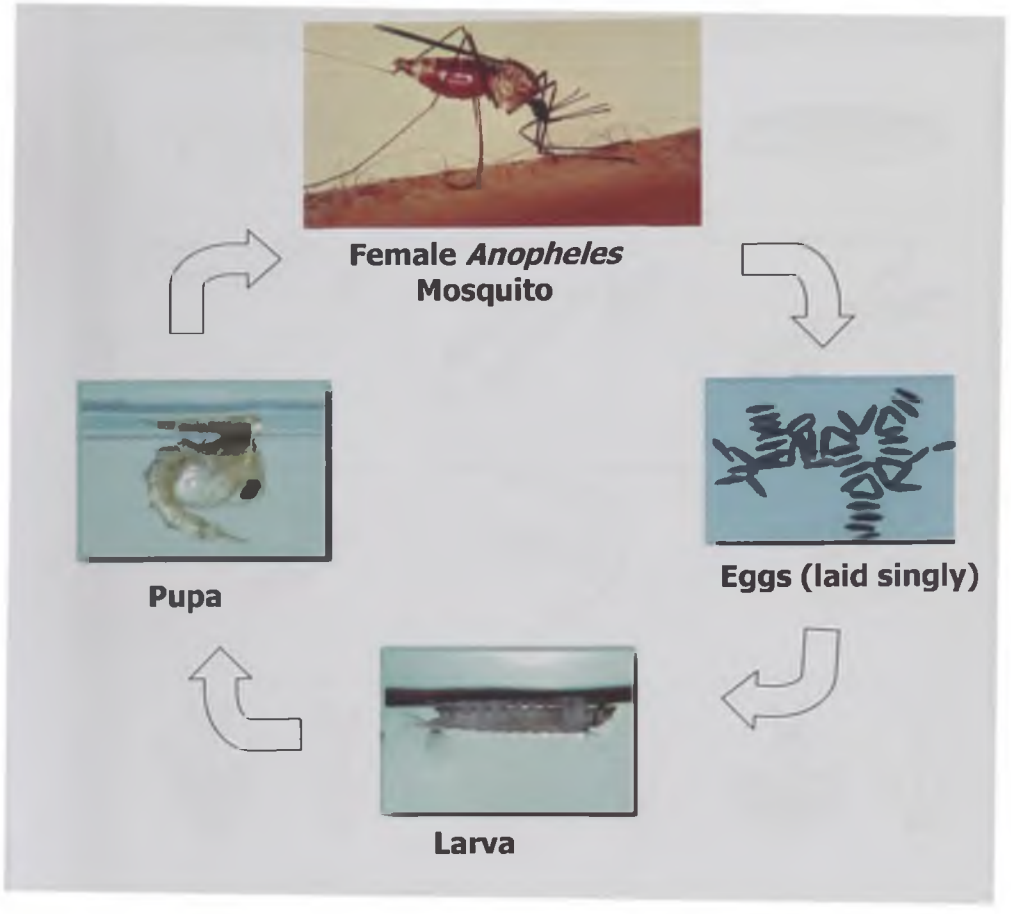

Figure 2.3: Life cycle stages of *Anopheles* mosquitoes

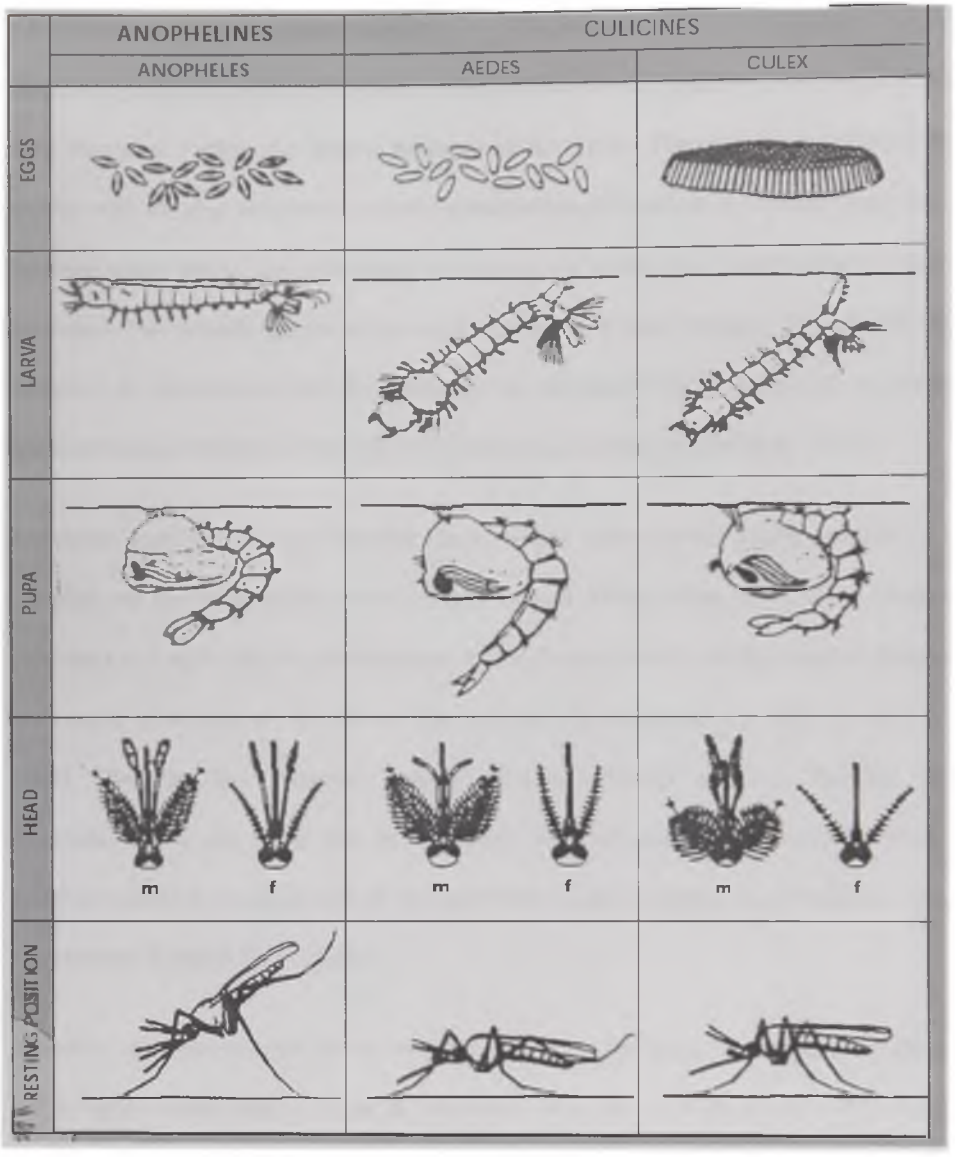

Figure 2.4: Differences between the anopheline mosquito and other common mosquitoes (UNICEF, 2000).

2.5.1 *Anopheles gambiae* Giles complex

Anopheles gambiae complex, the major malaria vector in sub-Saharan Africa, is probably the most important vector of a human pathogen in the world. The complex consists of seven species with varying abilities in malaria transmission (Coluzzi *et al.,* 2002). These are *An. gambiae* sensu stricto, *An. arabiensis*, *An. merus*, *An. melas*, *An. quadriannulatus* and *An. bwambae*. The seventh species is *An. quadriannulatus* B from Ethiopia. Two species of the complex; *An. gambiae* s.s and *An. arabiensis* are the most widely distributed and the most efficient malaria vectors in West Africa (Coetzee *et al.*, 2000; Coluzzi *et al.,* 2002).

Anopheles gambiae s.s, the nominal taxon, is the most anthropophilic member of the complex and the main malaria vector in sub-Saharan Africa (Pates *et al.*, 2001). *Anopheles gambiae* s.s, is split into five chromosomal forms characterized by the presence or absence of paracentric inversions on the second chromosome (Lehmann *et al.*, 2003; Gentile *et al.*, 2002). These are the 'Savanna', 'Mopti', 'Forest', 'Bissau' and the 'Bamako' forms. *Anopheles merus*, *An. melas* and *An. bwambae* are very minor malaria vectors while *An. quadriannulatus* is zoophilic and of no importance as far as malaria transmission in humans is concerned (Coetzee *et al.*, 2000).

Anopheles gambiae complex has an extreme preference for living around human habitations and is highly anthropophilic. Typical oviposition sites are small temporary bodies of water exposed to full sunlight, such as small puddles produced by rain. The number of available larval habitats increases during rainy seasons which makes the annual abundance of this mosquito correlates highly with rainfall.

2.6.0 Malaria control

The control of malaria today is mainly focussed on the mosquito vector and on the parasite.

2.6.1 Historical background of malaria control

Although people were unaware of the origin of malaria and the mode of transmission, protective measures against the mosquito have been used for many hundreds of years. The association with stagnant waters (breeding grounds for *Anopheles*) led the Romans to begin drainage programs, the first intervention against malaria. The inhabitants of swampy regions in Egypt were recorded as sleeping in tower-like structures out of the reach of mosquitoes, whereas others slept under nets as early as 450 B.C. Ways of dealing with malaria followed its proliferation, from administering the herb *qinghaosu* as a treatment in China more than 2000 years ago to employing the bark of the "fever bark tree" (Cinchona) in the seventeenth century and possibly much earlier, in South America, to the use of bednets as a preventive method, going back several millennia. The first recorded treatment dates back to 1600, where the bitter bark of the cinchona tree in Peru was used by the native Peruvian Indians.

Systematic control of malaria started after the discovery of malaria parasite by Laveran in 1889 (for which he received the Nobel Prize for medicine in 1907), and the demonstration by Ross in 1897 that the mosquito was the vector of malaria. These discoveries and the invention of DDT during the World War II, made the idea of global eradication of malaria seem possible. Subsequently, widespread systematic control measures such as spraying with DDT, coating marshes with paraffin (to block Anopheles mosquito larvae spiracles), draining stagnant water, and the widespread use of nets and cheap, effective drugs such as chloroquine were implemented with impressive results.

Despite initial success, there was a complete failure to eradicate malaria in many countries due to a number of factors (Shiff, 2002). Although technical difficulties such as vector and parasite drug resistance have played a part, the main failure to reduce the disease is probably due to social and political factors preventing efficient application of control measures. The hope of global eradication of malaria was finally abandoned in 1969 when it was recognised that this was unlikely ever to be achieved.

2.6.2. Mortality control

The major impact of malaria in any community is that of the death of individuals and to prevent people dying from the disease, appropriate treatment is necessary (Shiff, 2002). This strategy involves detecting presumptive cases, determining which cases are parasite positive and administering effective treatment. Mortality control is the main thrust of the current "Global Malaria Control Strategy". The main problem is that chemotherapy alone is not a means of controlling malaria and is not sustainable in the long term.

Malaria is diagnosed by the clinical symptoms and microscopic examination of the blood. It can normally be cured by antimalarial drugs. Antimalarial drugs form an important element in control programs in treating cases to remove a source of infection for feeding mosquitoes (Shiff, 2002). Current drugs to treat malaria such as doxycycline, proguanil and primaquine attack the liver stage, thus preventing the release of parasites into the bloodstream. Others, such as chloroquine, quinine, sulfadoxine pyrimethamine (Fansidar) and mefloquine, kill the parasite within the red blood cells.

Antimalarials are also used prophylactically (Shiff, 2002). Chemoprophylaxis for malaria is recommended to non-immune persons entering areas endemic for malaria to reduce but not totally eliminate the risk of infection. Chemoprophylaxis is also recommended for high-risk groups, such as young children and pregnant women and recent immigrants from malaria-free areas (Collins *et al*, 2000).

The problem of malaria has been exacerbated in recent years by the development and rapid spread of resistance in *P. falciparum* to the more commonly used and affordable antimalarial drugs (Collins *et al.*, 2000; UNICEF 2000). Drug resistance in malaria, has emerged as one of the greatest challenges facing malaria control today (Boland, 2002). It leads to greater parasite longevity in the host, which, in turn, prolongs the period of infectiveness. Chloroquine resistance, which first appeared in East Africa in the late 1970s, has now spread throughout most of sub-Saharan Africa, and resistance to pyrimethamine–sulfadoxine (Fansidar) has followed rapidly (Figure 2.2). Mefloquine resistance has emerged in South-east Asia. One of the major implications of the diversity of resistance is to make it more critical that public health measures to control malaria be region-specific.

2.6.3 Transmission control

This strategy recognizes that malaria is an important cause of morbidity as well as mortality and it involves all efforts aimed at controlling the vector of the disease (Shiff, 2002). This approach is effective in most epidemiological conditions and is an effective control strategy for a sustained attack on the malaria problem. It is adaptable to the use of insecticide-treated mosquito nets as well as indoor spraying of insecticides. Effective transmission control will

reduce the incidence of infection and re-infection in the community. When Grassi and his Italian colleagues demonstrated that anopheline mosquitoes were the vectors of malaria to humans, the concept of malaria control became synonymous with mosquito control and mosquitoes became the main target of control efforts (Robert and Collins, 1996; Shiff, 2002). In the absence of methods to kill adult mosquitoes, the strategy was to reduce breeding sites.

2.6.3.1 Environmental management

Environmental management is one of the earliest malaria control measures practiced even before mosquitoes were implicated in malaria transmission. The value of environmental management is well recognized as a form of source reduction and involves measures to reduce breeding sites and overall populations of vector species. These include the filling of ditches, covering water containers, flushing irrigation channels, clearing ponds of weed growth, which allows the introduction into ponds of fish which eat mosquito eggs and larvae. There have been some situations where source reduction was effective. In the United States, major modifications of mosquito habitat through the Tennessee Valley Authority malaria control program, habitat degradation, deforestation, flooding, and other effects of development restricted the habitat of the malaria mosquito *Anopheles quadrimaculatus* and led to the local decline of malaria (Shiff, 2002).

However several major environmental changes due to human activities in the quest of development run counter to the source reduction efforts. Mining, logging and land clearance for Agriculture are three such operations which can have a rapid impact on the tropical environment. Extensive borrow pits which hold water are dug alongside new roads

constructed for access to these kinds of developments and the new roads often obstruct existing drainage, causing water to accumulate and thus new mosquito breeding sites emerge (Phillips, 2001).

Moreover, on the whole, anopheline mosquitoes are opportunistic breeders that favour open sunlit pools or small streams and rivulets. In most cases it is impractical to suggest source reduction as an effective control effort for anophelines. Since anophelines are opportunists, their populations expand during rainy spells and they breed in such a variety of situations that any attempts to limit the extent of suitable habitat will not be very successful. Importantly, it is not the number of mosquitoes that is critical in the cycle but, rather, the length of mosquito survival which contributes to the efficient transmission of malaria (Roberts and Collins, 1996; Shiff, 2002).

2.6.3.2 Intradomicile application of residual insecticides

Intradomicile application of residual insecticides, also referred to as indoor spraying, has been the mainstay of malaria control operations since the early parts of the last century. The rationale, in short, is indoor spraying with a persistent insecticide that remains active on the sprayed surface for weeks or even months to kill or at least repel the adult female mosquito (Shiff, 2002). The motivation for this method is based on the feeding and resting habits of most malaria vectors (Curtis and Towson, 1998). The majority of important malaria vectors feed late at night, with peak biting activities between the hours of 20:00 and 05:00 nightly. They are also highly anthropophilic and endophagic. The term endophily refers to the

preference of a female mosquito to rest indoors during the period between the end of feeding and the onset of the search for an oviposition site.

Residual house spraying is likely to be effective only if the mosquito species concerned is endophilic or at least partially endophilic, because the mosquito needs to rest on the insecticide-treated walls for a sufficient time if it is to pick up a lethal dose. Naturally endophilic species include *An. gambiae* s.s. and *An. funestus* in Africa, *An. culicifacies* in India, and *An. minimus* in East and Southeast Asia, (Pates and Curtis, 2005). Endophilic behavior varies among species and is affected by insecticidal irritancy. The spraying of the walls and ceilings of houses with residual insecticides such as DDT reduces the survival prospects of indoor resting *Anopheles* mosquitoes sufficiently to greatly reduce the chance of malaria transmission.

However, behavioral resistance in vectors in some countries has arisen in response to prolonged spraying programs (Pates and Curtis, 2005). Exophilic behavior has evolved in certain populations exposed to prolonged spraying programs. This can have an impact on a control effort and may result from an immediate response to the irritant insecticides (DDT or pyrethroids), or it may be a genetic trait evolved under selection from the presence of insecticides in houses. Insecticide irritancy can be demonstrated by a strong stimulation to take off and fly, a high proportion of mosquitoes exiting from a treated house, or both (Pates and Curtis, 2005).

2.6.3.3 Insecticide treated materials

The use of ITNs is a new and somewhat revolutionary tool for effective vector control. Bednets are an effective method to reduce malaria transmission as they stop more humans being infected or infectious humans from transmitting the parasite. The use of bednets is especially important if the room does not have ceiling and insect screens which cover doors and windows to stop mosquitoes from getting in. Before the development of insecticide treated nets (ITNs) as a new technology in the mid-1980s, people in many countries were already using nets, mainly to protect themselves against biting insects and for cultural reasons.

It was only recently appreciated that a net treated with insecticide offers much greater protection against malaria. Insecticide treated bednets locate a deposit of a quick-acting insecticide of low human toxicity between a sleeper and host-seeking mosquitoes. Thus a chemical barrier is added to the often incomplete physical barrier provided by the net (Miller *et al.*, 1991; Hodjati *et al.*, 2003). Not only does the net act as a barrier to prevent mosquitoes biting, but also the insecticide repels, inhibits or kills any mosquitoes attracted to feed (Mbogo *et al.*, 1996; Mathenge *et al.*, 2001). Thus ITNs provide protection both to individuals sleeping under them and to other community members. The effect is so significant that use of ITNs is considered to be one of the most effective prevention measures for malaria (WHO/UNICEF, 2003). The use of insecticide treated bed nets (ITNs) for both individual and collective protection against malaria has shown potential, reducing childhood malaria morbidity by 50% and global mortality by 20–30% in The Gambia, Ghana, and Kenya (Alonso *et al.*, 1991; Choi *et al.*, 1995; Binka *et al.*, 1996).

The insecticides of choice for bed net impregnation are pyrethroids because of their high efficacy, rapid rate of knockdown, strong mosquito excito-repellent properties and low mammalian toxicity (Hougard *et al.*, 2003; Corbel *et al.*, 2002; Curtis *et al.*, 2003; Zaim *et al.*, 2000). However, the increasing resistance of malaria vectors to pyrethroids threatens to reduce the potency of this important method of vector control (Curtis *et al.*, 2003; Corbel *et al.*, 2002). Furthermore pyrethroid-treated nets have been reported to be involved in the selection for the *kdr* resistance allele (Fanello *et al.*, 1999).

The World Health Organization (WHO) recommends the large-scale use of ITNs to control malaria transmission because they offer a good cost-efficiency ratio based on active community involvement (Diabate *et al.*, 2002). To be an effective control intervention for the malaria vectors, a high coverage is required for ITN to act, through a 'mass effect'. It is therefore easier to expand ITN coverage in areas where there is already a culture of mosquito-net usage and the most suitable areas to be targeted will be those where at least 20% of households already have at least one net each (Manga, 2002). Once such areas have been identified, a local plan for improving coverage and for ensuring that at least 80% of the nets in use are (re)impregnated with insecticide is to be developed.

2.6.3.4 Personal protection

The number of bites can also be reduced by wearing long sleeved shirts and trousers to reduce the amount of exposed skin, the use of insect repellents on clothes and exposed skin (especially those containing DEET) and spraying bedrooms with aerosols to kill any mosquitoes before sleeping.

2.6.3.5 Transgenic mosquitoes

Consideration of the potential use of genetically modified organisms (GMOs) is driven by the realization of the enormous human cost of diseases like malaria and of the inadequacy of present control measures (Alphey *et al.,* 2002). GMOs could be used in either of two ways for malaria control. The idea is to generate transgenic mosquitoes that express antiparasitic genes in their midgut epithelium, thus rendering them inefficient vectors for the disease. Because mosquitoes are obligatory vectors for malaria transmission, the spread of malaria could be curtailed by rendering them incapable of transmitting parasites (Beier, 1998; Alphey *et al.,* 2002; Ito *et al.*, 2002). These strategies target the malaria parasite, rather than the mosquito itself, for reduction. An alternative use of genetic engineering for malaria control takes a more traditional approach. This involves targeting the mosquito population per se for reduction through sterile male release.

However, the release of large numbers of insects presents other specific challenges: for example, the need to release only male mosquitoes so as not to increase the number or nature of mosquito bites per person per night. *Plasmodium*-refractory mosquitoes are being rapidly developed for malaria control but will only succeed if they can successfully compete for mates when released into the wild (Okanda *et al.*, 2002). Precopulatory behavioural traits maintain genetic population structure in wild mosquito populations and mating barriers have foiled previous attempts to control malaria vectors through sterile male release.

2.6.4 Multilateral malaria research and control programs

Multilateral programs are those activities involving two or more nations that are channeled through an international or regional agency (Alilio *et al.*, 2004). Examples of these programs include the Multilateral Initiative on Malaria (MIM), the Roll Back Malaria (RBM) Partnership, the Global Fund for HIV, Tuberculosis and Malaria (Global Fund), the Medicines for Malaria Venture (MMV), and the Malaria Vaccine Initiative (MVI). These programs have gained prominence due to their great potential for facilitating important discoveries and coordination of large-scale control actions, which cannot be achieved by a single African country working alone (Shiff, 2000).

2.7 Insecticide Resistance

Resistance is defined as the acquired ability of an insect population to tolerate doses of insecticide which will kill the majority of individuals in a normal population of the same species (WHO, 1992). Several years of intensive use of organic insecticides to control arthropod pests and disease vectors has led to the selection of pesticide resistance in some species. Resistance to insecticides among mosquitoes that act as vectors of disease emerged more than 25 years ago in Africa, America and Europe (Weill *et al.,* 2003).

Factors that induce resistance are numerous and the mechanism adopted by organism depends on the prevailing pressure and on the mode of action of the insecticide in use. Intoxication of arthropod by a pesticide encompasses different levels of pharmacokinetic interaction: penetration of barrier tissue, distribution, storage, metabolism in internal tissue, and molecular interaction with the ultimate target site. Many chemicals are being used against arthropods and there are hundreds of examples of resistance, and a number of resistance mechanisms have been identified.

2.7.1 Mechanisms of insecticide resistance

Three main mechanisms of resistance to insecticides occur: reduction of insecticide penetration, increased degradation and modification of the insecticide target (Hemingway *et al.*, 2004).

2.7.1.1 Reduced penetration

Here the composition of the insect's exoskeleton becomes modified in ways that inhibit insecticide penetration (Matsumura, 1983). Decreased penetration of insecticides would allow ample time for detoxifying enzymes to metabolize the chemical and therefore would be less effective. Reduced cuticular penetration alone usually confers only a low level of resistance, however in combination with other mechanisms, it can potentially result in large non-additive increase in resistance (Oppernoorth, 1985). Plapp and Hoyer (1968) reported reduced penetration of dieldrin and DDT in a strain of housefly.

2.7.1.2 Metabolic resistance

Enzyme detoxification, by modifying or increasing endogenous enzymes within the insect, is major mechanism of resistance (Chareonviriyaphap *et al.*, 2000). In metabolic resistance the metabolic pathways of the insect become modified in ways that detoxify the insecticide, or disallow metabolism of the applied compound into its toxic forms. Metabolic resistance to insecticides is mediated by qualitative and quantitative changes in proteins that can often be difficult to define precisely at the biochemical level. Three broad enzyme classes are involved in insecticide detoxification, the mixed function oxidases (MFO), esterases and glutathione S-transferases. Their involvement in resistance is commonly identified by increases in the characteristic metabolites they produce. All three classes exist in multiple forms within each species and it is often not known whether increased activity arises from qualitative or quantitative changes in these enzyme complex. Increased synthesis of these enzymes seem to result from gene amplification.

2.7.1.2.1 Glutathione S-transferases

Glutathione S-transferases (GSTs) are soluble dimeric proteins that are ubiquitous in nature. They are involved in the metabolism, detoxification and excretion of a large number of endogenous and exogenous compounds from the cell (Ortelli *et al.*, 2003). GSTs catalyses the conjugation of glutathione with compounds having a reactive electrophilic centre, leading to the formulation of a water-soluble, less reactive product. Although there are many examples of increased metabolism of insecticide or model substrates by glutathione S-transferases of resistant insects, few are characterized at the molecular level.

GSTs have no direct role in the metabolism of pyrethroid insecticides but they play a very important role in conferring resistance to this insecticide class by detoxifying lipid peroxidation products induced by pyrethroids (Hemingway *et al.*, 2004). GSTs may also protect against pyrethroid toxicity in insects by sequestering the insecticide. Insect GSTs are of particular interest because of their potential to cause resistance to all the major families of insecticide. Metabolism mediated by these enzymes has been implicated in DDT and organophosphate resistance. Increased levels of DDT-dehydrochlorinase have been reported in different species resistant to DDT. Biochemical studies on partially purified GST fractions from DDT-resistant and susceptible *A. gambiae* have indicated that resistance was associated with both qualitative and quantitative changes in multiple GST enzymes (Ortelli *et al.*, 2003)

2.7.1.2.2 Mixed function oxidases (MFO)

MFO enzymes are of a great significant both in mammals and arthropods in giving protection to a variety of insecticides, particularly to some chlorinated hydrocarbons, to many

organophosphates and carbamates and to some pyrethroids. Monooxygenases are a chain of enzymes, with the rate limiting enzyme usually being cytochrome P450. Alterations in this rate-limiting enzyme can dictate levels of resistance to pyrethroids, organophosphates, and carbamate insecticides using this metabolic mechanism (Chareonviriyaphap *et al.*, 2003)

An increase in MFO activity is one of the most versatile mechanisms of resistance in insects. Insect P-450 enzymes also activate certain types of insecticides, for instance the conversion of phosphorothioates (P=S) to phosphate (P=O). This can result in an increase in potency for inhibition of acetylcholinesterase by 3 or 4 orders of magnitude. Monooxygenases can contribute to Malathion resistance in two ways, by either increasing the rate of metabolism to non-toxic products, or decreasing the rate at which the insecticidal malaoxon is produced from the malathion parent compound (Karunaratne and Hemingway, 2001).

Elevated monooxygenase activity is associated with pyrethroid resistance in *An. stephensi*, *An. subpictus, An. gambiae* and *C. quinquefasciatus* (Hemingway and Ranson, 2000). Elevated levels of mixed function oxidases were found to be responsible for the detoxification of pyrethroids in resistant *Anopheles funestus* Giles from northern Kwazulu/Natal in South Africa and the Beluluane region of southern Mozambique (Brooke *et al.*, 2001) and were further implicated to be conferring cross-resistance to the carbamate insecticide propoxur. Similarly, monooxygenases have been responsible for degradation of pyrethroids in *Anopheles pseudopunctipennis* (Ocampo *et al.*, 2000).

2.7.1.2.3. Esterases and Hydrolases

Esterases are the most significant enzymes for insecticide detoxification in insects. Organophosphate, carbamate and pyrethroids contain carboxylester and phosphotriester bonds that are subject to attack by esterase enzymes. These esterases can often be separated into isozymes with different substrate specificities (Chareonviriyaphap *et al.*, 2000). Polymorphism is a notable characteristic of insect esterases. Esterases are widely distributed in many insect tissues such as gut, cuticle and fat body and multiple forms of esterases are present in the soluble, cytosolic fraction of insect. Few of the multiple forms of esterase isozymes that exist in insects participate in insecticide metabolism, where each isozyme probably has a certain range of substrates. Unlike the monooxygenase reaction, esterases do not utilize high energy co-factors.

Elevated esterase activity has been linked to pyrethroid, organophosphate and carbamate resistance patterns in a variety of insects. Pyrethroids are insecticidal esters derived from primary alcohols and are thus susceptible to hydrolysis by esterases. Chareonviriyaphap *et al.*, (2000), have reported highly elevated esterase levels in *Anopheles albimanus* resistant to deltamethrin in Guatemala and they further suggest this may limit pyrethroid use against *An. albimanus* population in parts of Central America.

Different types of esterases (A1, B1, A2, B2) have been recognized in organophospates insecticide resistant populations of *Culex pipiens* complex throughout the world and overproduction of nonspecific esterases is a common mechanism of resistance. For esterase B1, resistance to OP insecticides has been shown to be due to sequestration of insecticide and

overproduction of all esterase B is due to gene amplification. Enzymatic assays suggested that sequestration rather than metabolism is the primary mode of operation of these esterases on malathion.

The basis of malathion resistance in the adults of *An. arabiansis* from Sudan was a carboxylesterase. Malathion resistance due to an increase in degradation at the carboxylester linkage is a common detoxification pathway that has been implicated in *An. culicifacies*; *An.stephensi* Liston. Esterase dependent cross- resistance between Organophosphates, carbamate and pyrethroids has been detected in several insect species. *An. gambiae* from Kenya has been reported to have demonstrated elevated oxidase and esterase levels in permethrin-resistant (Vulule *et al.*, 1999). Similarly, Brogdon *et al.* (1999a, b) have reported oxidase-based and esterase-based resistance mechanisms alone and in combination in permethrin-resistant *An. albimanus* from Guatemala.

2.7.1.3 Target-site mechanisms

Alterations of amino acids responsible for insecticide binding at its site of action cause the insecticide to be less effective or even ineffective. Non-silent point mutations within structural genes are the most common cause of target-site resistance (Hemingway and Ranson, 2000). For selection of the mutations to occur, the resultant amino acid change must reduce the binding of the insecticide without causing a loss of primary function of the target site. Therefore the number of possible amino acid substitutions is very limited. Hence, identical resistance-associated mutations are commonly found across highly diverged taxa. The degree to which function is impaired by the resistance mutation is reflected in the fitness

of resistant individuals in the absence of insecticide selection. This fitness cost has important implications for the persistence of resistance in the field.

2.7.1.3.1 Knock-down resistance

The target of organochlorines especially DDT and synthetic pyrethroids are the sodium channels of the nerve sheath (Hemingway *et al.*, 2004). In insects, an important mechanism of pyrethroid resistance is due to a modification of the voltage-gated sodium channel protein recently shown to be associated with mutations of the para-type sodium channel gene, (Martinez-Torres *et al.*, 1988). A reduction in the sensitivity of the insect's voltage-gated sodium channels to the binding of insecticides causes the resistance phenotype known as knockdown resistance (kdr) (Hemingway and Ranson, 2000). DDT-pyrethroid cross-resistance may be produced by single amino acid changes in the axonal sodium channel insecticide-binding site.

Pyrethroid insecticides have a rapid 'knock-down' effect. However, the intensive use of DDT and pyrethroids has led to the development of *kdr* in many insect species including *Anopheles* mosquitoes (Hemingway *et al.*, 2004). The use of both DDT and pyrethroids in the control of rice and cotton pests is likely to have contributed significantly to the development of resistance in *An. gambiae* from West Africa (Hemingway *et al.*, 2004; Martinez-Torres *et al.*, 1998). Already Pyrethroid resistance has been noted in *An. albimanus*, *An. stephensi*, and *An. gambiae* among the malaria vectors.

The development of pyrethroid resistance in *An. gambiae* is particularly important given the recent emphasis by the WHO and other organizations on the use of pyrethroid-impregnated bednets for malaria control. Two cases of pyrethroid resistance in *An. gambiae,* from the Ivory Coast (Chandre *et al.*, 1999) and Kenya (Vulule *et al.*, 1999) are well documented. However, in Cameroun, Senegal and Botswana, *An. gambiae* populations have been reported to be fully susceptible to pyrethroids (Chandre *et al.*, 1999a). The West African focus appears to be larger and has higher levels of resistance than that in East Africa (Hemingway and Ranson, 2000). Resistance due to *kdr* can develop in a short period of introduction of pyrethroids for mosquito control. Approximately one year after bed nets impregnated with permethrin were introduced as a malaria control measure in the northern part of Thailand, evidence of physiological resistance was reported (Chareonviriyaphap *et al.*, 2002).

Several studies have reported that, the *kdr* mutation has been found widely distributed in the Savanna form of *An. gambiae* s.s., but never in wild populations of the Mopti form or *An. arabiensis*, even in areas where both occur in sympatry with resistant Savanna populations (Chandre *et al.*, 1999; Awolola *et al.*, 2003; Berzosa *et al* 2002). As a result it was suggested that, the absence of the kdr mutation in the M form involves an additional pyrethroid resistance mechanism in *An. gambiae* s.s. (Awolola *et al.*, 2003). However, already low levels of the kdr mutation has recently been detected in *An. arabiensis* during an extensive survey of pyrethroid resistance in *An. gambiae* s.l. in Burkina Faso (Diabate *et al.*, 2004), Ethiopia (Balkew *et al.*, 2003) and Western Kenya (Stump *et al.*, 2004). Similarly, Weill *et al.*, (2000) has reported kdr mutation in the Mopti form of *An.gambiae* s.s. The detection of this mutation in both *An. arabiensis* and the M form of *An. gambiae* s.s., is important at both epidemiologic and fundamental levels. Most susceptibility/resistance studies in Ghana have

reported insensitivity of the sodium channels in *An. gambiae* populations, as characterized by increased knockdown and high frequency of the kdr mutation allele (Adasi *et al.*, 2000: Adeniran, 2002: Otieno, 2004).

2.7.1.3.2 Insensitive acetylcholinesterase

The target of organophosphate and carbamate insecticides is acetylcholinesterase (AcChE) in nerve synapses. AcChE is a key enzyme in the cholinergic synapses where it rapidly terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. Organophosphates and carbamates are substrates of AcChE and their hydrolysis results in the phosphorylation or carbamylation of the active serine followed by dephosphorylation or decarbamylation. The deacetylation of the acetylated enzyme by its natural substrate acetylcholine is a rapid process, 1000 s^{-1} in insects. However, dephosphorylation or decarbamylation is very long and takes several days while synaptic transmission remains blocked, resulting to the death of the insect, (Hemingway *et al.*, 2004: Shi *et al.*, 2004).

This resistance is frequently due to a loss of sensitivity of the insect's acetylcholinesterase enzyme to organophosphates and carbamates. This insensitivity results from a single amino-acid substitution in the enzyme (Weill *et al.*, 2003). At least five point mutations in the acetylcholinesterase insecticide-binding site have been identified that singly or in concert cause varying degrees of reduced sensitivity to organophosphates and carbamate insecticides. Already resistance to carbosulfan, a carbamate insecticide, due to insensitive acetylcholinesterase has been detected in field populations of *Anopheles gambiae* in Ivory Coast (N'Guessan *et al.*, 2003; Corbel *et al.*, 2003).

2.7.1.3.3 GABA receptor changes

The γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both insects and vertebrates (Ffrench-Constant *et al.*, 2000). The GABA receptors belong to a superfamily of neurotransmitter receptors that also includes the nicotinic acetylcholine receptors (Hemingway and Ranson, 2000). These receptors are formed by the oligomerization of five subunits around a central transmitter-gated ion channel. An alanine-to-serine substitution in the putative channel-lining domain of the GABA receptor confers resistance to cyclodienes such as dieldrin (Hemingway and Ranson, 2000). Resistance to dieldrin was recorded in the 1950s, but the involvement of the GABA receptors in this resistance was not elucidated until the 1990s.

Although cyclodiene resistance is historically very widespread and in the past accounted for over 60% of reported cases of resistance, cyclodienes themselves have been largely with-drawn from use, and therefore, in relative terms, the overall frequency of resistance cases is declining (Ffrench-Constant *et al.*, 2000). However, resistance seems to be able to persist in the absence of extensive insecticide selection, representing a threat for novel insecticides interacting with the cyclodiene binding site, such as the fipronils. Further, cyclodiene type insecticides, such as endosulfan, are still used to control multiple resistant pests including the whitefly.

2.8 Management of Insecticide Resistance

Insecticide resistance has been a problem in all insect groups that serve as vectors of emerging diseases. Although mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance foci. The main defense against resistance is close surveillance of the susceptibility of vector populations.

In the present situation of insecticide resistance status in malaria vectors, the future of vector control mainly relies on the strategies for the management of insecticide resistance. So far the approach has been the replacement of insecticide by an effective and preferably by a new group of insecticides. Subsequent replacement of insecticides has led to the development of multiple-resistant malaria vectors. It may be mentioned that subsequent change of insecticides has burdened the programme with increased costs. Not many new insecticide molecules are available for vector control in the immediate future. What is needed for the present day vector control programme is an approach for the management of existing resistance in malaria vectors and to limit its further spread. The strategy for this approach is to use the available insecticides rationally.

The most important aspect of the management of resistance is to either avoid or delay the onset of resistance by effectively manipulating or influencing the factors responsible for the development of resistance (Hemingway *et al.*, 2004). Various methods emphasize on the strategic use of available insecticides to delay the onset of resistance. The methods include avoidance of use of insecticides that induce broad-spectrum resistance mechanisms and

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confer cross-resistance to chemically related and unrelated insecticides. The sequential use of insecticides in rotation is preferred. The need of the hour is intensive research on management tactics and integration of such tested strategies in the ongoing vector control programmes. In malaria endemic areas, there is a need for comparative studies on susceptible and refractory populations for as many known vectors as possible (Chareonviriyaphap *et al.*, 2002).

Among the strategies proposed for resistance management is the use a pyrethroid and a non-pyrethroid insecticide in combination on the same mosquito net, either separately or as a mixture (Darriet *et al.*, 2003; Corbel *et al.*, 2003). Mixtures are particularly promising if there is potentiation between the two insecticides as this would make it possible to lower the dosage of each hence an advantage in terms of lower cost and toxicity (Hougard *et al.*, 2003). The possible use of non-pyrethroid insecticides, such as carbamates, on nets is a promising alternative solution because these insecticides are effective against susceptible and pyrethroid-resistant populations of *Anopheles* and *Culex* mosquitoes (Corbel *et al.*, 2003).

confer cross-resistance to chemically related and unrelated insecticides. The sequential use of insecticides in rotation is preferred. The need of the hour is intensive research on management tactics and integration of such tested strategies in the ongoing vector control programmes. In malaria endemic areas, there is a need for comparative studies on susceptible and refractory populations for as many known vectors as possible (Chareonviriyaphap *et al.*, 2002).

Among the strategies proposed for resistance management is the use a pyrethroid and a non-pyrethroid insecticide in combination on the same mosquito net, either separately or as a mixture (Darriet *et al.*, 2003; Corbel *et al.*, 2003). Mixtures are particularly promising if there is potentiation between the two insecticides as this would make it possible to lower the dosage of each hence an advantage in terms of lower cost and toxicity (Hougard *et al.*, 2003). The possible use of non-pyrethroid insecticides, such as carbamates, on nets is a promising alternative solution because these insecticides are effective against susceptible and pyrethroid-resistant populations of *Anopheles* and *Culex* mosquitoes (Corbel *et al.*, 2003).

CHAPTER THREE

MATERIALS AND METHODS

3.0 Standard solutions

All solutions and reagents used were prepared according to standard procedures as shown in Appendix I.

3.1 Study area

The studies were carried out in three suburb villages of Hohoe town; Adabraka (N 07° 09.881' E 000° 28.514'), Atabu Newtown (N 07° 08.153' E 000° 28.815') and Kledzo (N 07° 07.353' E 000° 27.523') and Likpe (N 07° 09.799' E 000° 35.313'), a rural village lying on the foot of mountain ranges bordering Ghana and Togo.

Hohoe District is one of the twelve administrative districts of the Volta-Region and has a land surface area of 1,172km^2. It is located within longitude 0°15E and 0° 45E and latitude 6° 45'N and 7° 15'N and lies almost at the heart of the Volta-Region. It shares borders with the Republic of Togo on the east, on the southeast with Ho District, on the southwest with Kpando District and on north with Jasikan District. The District capital Hohoe is 220km from Accra, the national capital.

The District houses part of the Akwapim-Togo ranges extending beyond the country's eastern boundary all the way to Western Nigeria. Within these ranges is the Afadjato – the highest elevation in Ghana (880.3m). Some of the low-lying areas have swamps which are

used for rice cultivation. Annual rainfall is between 1016mm-1210mm. There is 4-5 month dry season between November and April. Hohoe District falls within the forest-savanah transitional ecological zone of Ghana with the forest part at its southern and eastern sector and tapering into the middle of the district. The eastern highlands are clothed with high forest. The soils generally tend to be sandy overlying iron pans. Bottom lands carry heavy silts and cracking clays and as a consequence, drainage is very poor, subjecting the area to extreme variations in soil moisture. Both savannah and forest crops do well in the district. Some of these are cocoa, coffee, oil palm, bananas and plantains, rice, cassava, yams, maize, millet and groundnuts. The year 2000 population figure for Hohoe district (based on DSDA II headcount) is 144,511 with the gender breakdown established as 70,754 males and 73,547 females.

3.2 Field collection of mosquitoes

Mosquitoes were collected as adults and larvae in the above mentioned sites between August 2004 and April 2005.

3.2.1 Adult mosquito collection

In each village, houses (Figure 3.1 and 3.2) were randomly selected for adult mosquito collection in rooms where people sleep. The rooms of selected houses were thoroughly searched during the day for indoor resting *Anopheles* mosquitoes with the aid of a torch light. Adult anophelines were identified by their characteristic resting position, with the body resting at an angle of 45^0 to the surface. Only bloodfed and gravid adult females were collected using plastic aspirators and carefully transferred into paper cups by gently blowing them out of the aspirator. They were then transported to the laboratory where they were maintained until they were ready to lay eggs.

3.2.2 Larval mosquito collection

Where the number of adults was very low, mosquitoes were collected as larvae from randomly selected larval sites around the sampled houses. The larval sites comprised mainly of abandoned fish ponds, tyre tracks, shallow water wells and an open concrete water tank (Figure 3.3 and 3.4). The *Anopheles* larvae were identified by their characteristic resting position, with the body lying parallel to the surface and just below the surface film. Larvae were collected using the dipping method with the aid of copper ladles, transferred into plastic jars which had perforated covers for ventilation and transported to the laboratory for rearing.

Figure 3.1: Typical adult mosquito collection house at Adabraka village.

Figure 3.2: Typical adult mosquito collection house at Atabu Newtown village.

Figure 3.4: Typical larval mosquito collection site at Atabu Newtown village. A shallowly-dug water well.

Figure 3.3: Typical larval mosquito collection site at Adabraka village. An abandoned fish pond filled with rain water.

3.2.3 Laboratory rearing of mosquitoes

In the laboratory gravid females were carefully transferred into 1 foot square cages and provided with 10% sugar solution soaked in cotton wool. Petri dishes lined with moist filter paper were placed at the base of the cages for them to lay eggs on. The eggs when laid were then carefully transferred into plastic trays containing water for them to hatch. Larvae were later reared in plastic containers of 5cm x 27cm x 36cm that contained water to the depth of not more than 2cm (Figure 3.5 a). Field collected larvae were also transferred into similar trays and in cases where the water from their natural habitats was unsuitable, tap water was used. Larvae were fed on a mixture of two parts finely ground gold fish food (Nutrifin™, Warden Corporation U.S.A) and one part brewer's yeast (Saf-instant®, France) in water. Pupae were collected each day using Pasteur pipettes and transferred into small beakers and placed in labelled cages (Figure 3.5 b) for adult emergence. The emerging adults were similarly fed on 10% sugar solution soaked in cotton wool. Only 2-5 days old females were picked from the cages and used for bioassays.

The temperature and relative humidity during the rearing period was within the range 25-30°C and 55-78% respectively and a natural photoperiod was maintained. The cages were mounted on oil moats to prevent ants from entering the cages. Dead mosquitoes were also picked up daily to prevent attraction of ants and mould formation. Other precautions such as overcrowding of larvae in the tray and not using much feed to avoid scum formation were taken during rearing.

a)

b)

Figure 3.5: Mosquito rearing system; a) Plastic pans for rearing larvae b) Wooden cages for rearing adults.

3.2.4 Morphological identification of *Anopheles gambiae* s.l. mosquitoes

The *Anopheles* mosquitoes were identified using the keys by Giles and de Mellion (1968) and Hervy *et al.* (1998). The adults usually rest with the body at an angle to the surface and most of them have spotted wings. The number, length and arrangement of the spots differ considerably in different species. The females have non-plumose antennae and palps as long as the proboscis and usually lie closely alongside (Service, 1980) while the males have plumose antennae. Apart from the morphological identification of *Anopheles* from other mosquitoes, Morphological identifications were carried out to separate *culex* and *Aedes* species and to distinguish *An. funestus* from *An. gambiae* s.l. *Anopheles gambiae* complex species have 5 pale spots on the coastal margin of the wings, anal vein colouration with 3 white spots and a dark apical fringe and white speckle (or spots in the median part) tibia ornamentation. In contrast, *An.funestus* and other *Anopheles* species have 4 pale spots on the costal margin on the wings, entirely dark anal vein colouration and entirely dark tibia ornamentation.

3.2.5 Preservation of mosquito samples

Randomly selected mosquitoes were placed in paper cups and killed by freeing at -20° C for at least 10 minutes. Specimens for identification of species and molecular forms and for Kdr analysis using molecular methods were preserved dry over silica gel in individual tubes. Specimens for biochemical analysis were quickly transferred to tubes and stored at -80^0 C until ready to use.

3.3 Bioassay Experiments

The aim of the bioassay was to measure the time it took for a given insecticide to kill the adult mosquitoes. The susceptibility tests were carried out using the World Health Organization test kits for adult mosquitoes (WHO, 1998b). The kit is basically comprised of insecticide impregnated test papers and non-impregnated papers for control and plastic tubes that are marked red for exposure and marked green for holding (Figure 3.6). The papers were impregnated with the WHO-recommended discriminating dosages of 5% Malathion, 0.1% Propoxur, 0.05% Deltamethrin, 0.75% Permethrin and 4% DDT. For each test, batches of 25 female mosquitoes aged between 2-5 days old were aspirated from the cages and transferred into paper cups where they were held for 1 hour. They were then aspirated into exposure tubes lined with the insecticide impregnated papers for 1 hour; during which the number of mosquitoes knocked down was recorded after 10, 15, 20, 30, 40, 50 and 60 minutes. Where the number knocked down after 60 minutes was less than 80%, number knocked down was recorded for 20 more minutes (i.e after 80minutes) in the holding tube. A mosquito was considered knocked down if it lay on its side on the floor of the exposure tube and was unable to fly (WHO, 1998b).

Mosquitoes were then transferred into holding tubes by gently blowing them through the open space between the exposure and the holding tubes. Cotton soaked in 10% sucrose was placed on top of the holding tube. This was to avoid death by starvation. The mortality was scored after 24 hours post-exposure and each test in each site was replicated four times. None of the impregnated papers was used for more than four times. The resistance or susceptibility status was evaluated based on the WHO criteria i.e. 98-100% mortality indicated

susceptibility; 80-97% mortality required confirmation and less than 80% mortality indicated resistance (WHO, 1981; WHO, 1998b). When the control mortality was between 5% and 20% the mean observed mortality was corrected using Abbott's formula (Abbott, 1925). An experiment was repeated if control mortality was more than 20%. The susceptibility of the wild populations to the tested insecticides was compared with that of the susceptible Kisumu strain.

a)

b)

Figure 3.6: Bioassay experiment set set-up; a) Exposure tubes with insecticide impregnated papers (b) Holding tubes.

3.4 Molecular Studies

Molecular methods were used to identify species of *Anopheles gambiae* complex, molecular forms of *An. gambiae* s.s and the *Kdr* alleles.

3.4.1 DNA Extraction

Genomic DNA extraction was carried out using two methods: the Bender buffer method (a modified protocol of Collins *et al.*, 1987) and homogenised legs/ parts of mosquito. For the Bender buffer extraction, each mosquito was homogenised in 1.5ml Eppendorf tube containing 100µl of the buffer (preheated at 65°C) using a sterile plastic pestle followed by incubation at 65°C for 30 minutes. Then 125 µl of phenol was added to the homogenate, mixed well by vortexing and spun at 14,000 rpm for 10 minutes. The supernatant was transferred into a fresh tube and 250 µl of chloroform added, vortexed briefly and spun as described. The supernatant was again transferred into a fresh tube and 250µl of pre-chilled absolute ethanol and 10 µl of 8M potassium acetate added followed by incubation at -40°C for 1 hour or -20°C overnight. The DNA was pelleted by centrifugation at 10000 rpm for 10 min and the supernatant discarded. Two hundred micro litres of 70% ethanol added to the pellet, the tube gently swirled and the DNA repelleted by centrifugation at 10000 rpm for 5 minutes. The supernatant was discarded and the tube inverted over a tissue paper to dry. The DNA pellet left was re-dissolved in 25µl TE plus RNase (5µg/ml). It was then stored at -20°C until ready for use. This method was used mainly for the *kdr* analysis because the PCR with the DNA extracted from mosquito legs proved unsuccessful.

The ground leg extract involved grinding a single mosquito leg or wing with a plastic pestle in 50 µl of sterile double distilled water (sdd H_2O) in 1.5ml Eppendoff tube. The homogenate was boiled for 10minutes, spun briefly and used directly as a DNA template for PCR.

3.4.2 PCR identification of *Anopheles gambiae* complex

Anopheles gambiae sibling species identification was carried out according to the method of Scott *et al.* (1993). Five sets of primers abbreviated as UN, GA, ME, AR and QD designed from the DNA sequence of the intergenic spacer region of *An. gambiae* complex of ribosomal DNA (rDNA) were used for species identification (Table 1). The sequence details of these primers, expected sizes of the PCR products and their melting temperatures are also given in Table 2. The UN primer anneals to the same position on the rDNA sequences of all five species, GA anneals specifically to *An. gambiae* s.s., ME anneals to both *An. merus* and *An. melas*, AR to *An .arabiensis* and QD to *An. quadriannualatus*.

The PCR reaction mixture of 20 µl contained 1x reaction buffer (Buffer C), 200µM each of the four oligonucleotide triphosphate (dNTPs), 0.25 µM each of oligonucleotide primers and 0.5U of DNA *Taq* polymerase enzyme. Five microliters of mosquito DNA (from single ground leg extraction method) template was used as the template for the amplification reaction. The reaction mixture was made up to 20 µl with sterile double distilled water.

The reaction mixture was spun down briefly and overlaid with mineral oil to avoid evaporation and refluxing during thermo cycling. The PCR thermal cycling was as follows; an initial step of 3 min at 94°C, followed by 35 cycles with denaturation at 94°C for30s, annealing at 50°C for 30s and extension at 72 °C for 60s and ended with a final cycle at 94°C

for 30s, annealing at 50 °C for 30s and extension at 72°C for 10mins using a PCR Express Thermal Cycler (Hybaid Ltd., UK).

The amplified products were analysed by agarose gel electrophoresis. Ten microliters of each PCR product were added to 1μl of 10x Orange G loading dye and electrophoresed in 2% agarose gel stained with 0.5μg/ml of ethidium bromide. The electrophoresis was run in 1X Tris acetate-EDTA (TAE) buffer at 100V for one hour and were visualized and photographed over a UVP dual intensity transilluminator at short wavelength using a Palorid direct screen instant camera fitted with an orange filter, a hood and a Polaroid Type 667 film. The film was processed as recommended by the manufacturer (Polaroid Inc., USA). The amplified PCR product was identified to the sibling species on the basis of the diagnostic band size determined by comparison with the mobility of a standard 100bp DNA ladder (Sigma, USA).

3.4.4 Identification of the molecular forms of *Anopheles gambiae* s.s

The identification of *An. gambiae* s.s to the molecular forms was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The method of Fanello *et al.* (2002) involving a combination of the protocols established by Scott *et al.* (1993) and Favia *et al.* (1997) was used. This method allows for simultaneous identification of *An. gambiae* s.l. as well as the M and S forms within the *An. gambiae* s.s. It is based on the fact that GCG^C restriction site for *Hha*1 enzyme (Favia *et al.*, 1997) lies within the *An. gambiae* specific fragment (Scott *et al.*,1993) which makes it possible to digest this fragment directly in order to differentiate M and S molecular forms.

The PCR reaction is the same as described in Section 5.4.2. After amplification, 1U of *Hha*1 enzyme (Promega, USA) in 10X enzyme buffer (Promega, USA) and nuclease free water were added to 10µl of PCR product to make a 20µl of the reaction mixture. The digestion was carried out at 37°C for 6 hours in a thermal cycler and the products electrophoresed through ethidium bromide-stained 2% agarose gel and visualised under UV light. Different band sizes are obtained between the PCR-amplified fragments and the fragments obtained after digestions are due to the presence of a restriction site for *Hha*I (Fanello *et al.*, 2002) at position 469 in all taxa except *An. merus*, and of a second restriction site at position 475 in *An. quadriannulatus*, *An. melas* and *An. merus*. The *An. gambiae* S-form digestion is characterized by two fragments, 257 and 110bp long, resulting from the presence of the *Hha*I restriction site. The *An. gambiae* M-form does not have this restriction site and thus is characterized by a single 367bp fragment.

3.4.5 PCR detection of the *kdr* alleles in *Anopheles gambiae* complex

The PCR-based method of Martinez-Torres *et al.* (1998) was used to detect *kdr* genes in the mosquitoes. DNA extraction was performed as described in Section 3.5.1. The primers used were Agd1 and Agd2 (Oligos Etc. Inc., USA) and Agd3 and Agd4 (Oswel, UK) [Table 2]. A total of 40 survivors from the bioassay plus 20 individuals of the general population of each of the sites were chosen at random for the *kdr* analysis. *Kdr* genotyping of susceptible and resistant individuals was possible after amplifying the DNA template from mosquitoes following the PCR conditions of 94°C for 3mins (initial denaturation), followed by 45cycles of 94°C for 30sec, 50°C for 30sec and 72°C for 1min. There was a final extension cycle of 94°C for 30sec, 50°C for 30 sec and 72°C for 10min followed by 4°C for cooling.

The products were electrophoresed through ethidium bromide-stained 2% agarose gel and visualized under UV light. *Kdr* genotypes of both the susceptible and resistant individuals were then recorded. Expected sizes for susceptible, resistant and control were 137bp, 195bp and 293bp respectively. The positive controls were laboratory susceptible strains of *An. gambiae* from Kenya (Kisumu).

Table 1: Oligonucleotides primer sequences, melting temperatures and the expected band sizes of the PCR amplified DNA products for identification of *An. gambiae* species complex (Scott *et al.*, 1993).

Primer	Sequence(5"-3")	T_M(°C)	Band size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	56	468
GA	CTC GTT TGG TCG GCA CGT TT	62	390
ME	TGA CCA ACC CAC TCCCTT GA	90	464
AR	AAG TGT CCT TCT CCATCCTA	78	315
QD	CAG ACC AAG ATG GTTAGT AT	54	153

Table 2: Sequence details of the *kdr* primers and their melting temperatures

Primer	Sequence (5"-3")	T_M(°C)
Agd 1	ATA GAT TCC CCG ACC ATG	54
Agd 2	AGA CAA GGA TGA TGA ACC	64
Agd 3	AAT TTG CAT TAC TTA CGA CA	40
Agd 4	CTG TAG TGA TAG GAA ATT TA	52

3.5 Biochemical assays

The purpose of conducting these assays was to determine activity of detoxification enzymes in the field populations as compared to that of a susceptible 'Kisumu' strain. The Centre for Disease Control (CDC, USA) microplates assays protocol with minor modifications was used (CDC, 2002). The enzymes tested were oxidases, glutathione S-transferases, acetylcholinesterase and non-specific esterases.

3.5.1 Sample preparation

Batches of 30 randomly selected frozen mosquitoes were placed individually in 1.5ml Eppendorf tubes using fine forceps and 100µl of phosphate buffer (pH 7.2) added. Mosquitoes were then thoroughly homogenized using either Teflon or plastic pestles and the crude homogenate, diluted to a final volume of 1ml with additional 900µl of the buffer.

For each individual mosquito, 100µl each of the homogenate was loaded into flat-bottomed microplate wells in triplicate (Figure 3.7). For an enzyme assay, homogenates of the first mosquito were loaded into the first three wells across i.e. A 1-3. The homogenate of next mosquito was loaded in the wells directly below the first i.e. B 1-3 and this continued down the plate until the end, then moving to the right and beginning at the top again. Wells A 4-6 contained the ninth mosquito. The last 6 lower wells on the plate were loaded with the positive and negative controls. Fresh pipettor tip was used for each homogenized mosquito sample to avoid cross-contamination.

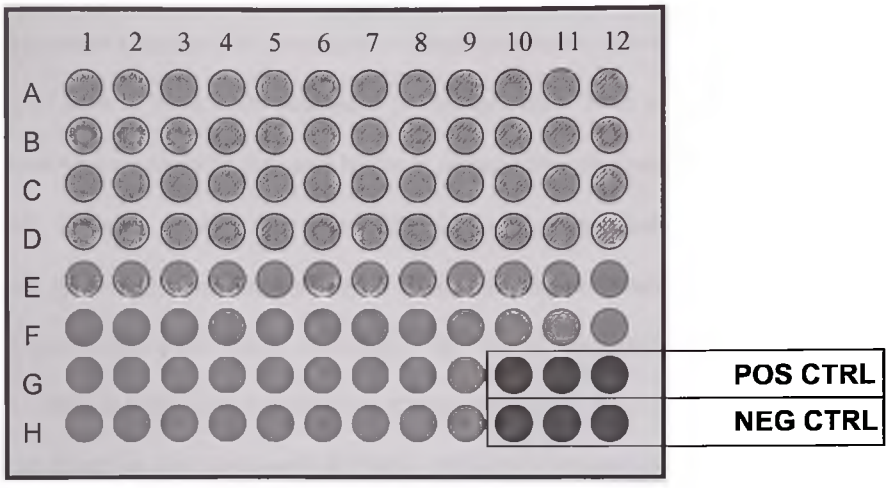

Figure 3.7: An illustration of a flat-bottomed microtiter plate used for biochemical assays. Homogenates were loaded in the following order; Mosquito # 1 went into wells A1, A2, A3, mosquito # 2 went into wells B1, B2, B3, mosquito # 8 went into wells H1, H2, H3 and so on.

3.5.2 Calibration curves

Calibration curves were obtained for protein, oxidases and the non-specific esterases.

3.5.2.1 Protein

Bovine serum albumin (BSA) was used to obtain a calibration curve for protein by dissolving 0.02g of BSA in 10ml 0.1M potassium phosphate buffer. Then serial dilutions of the stock solution were made using the same buffer to obtain concentrations within the range 0.25-2.0 mg/ml. For each dilution 5µl were loaded into wells in triplicates and 250µl of Bradford reagent (protein dye concentrate) added. The plate was incubated and read after 5 minutes using an ELISA plate reader at 620nm. The buffer was used as the negative control. Concentrations were plotted against absorbance to obtain a calibration curve which was then used to determine the corresponding protein content in mosquito samples.

3.5.2.2 Oxidase

Cytochrome-C was used to obtain a calibration curve for oxidase. 80mM solution of cytochrome –C was prepared (0.01g in 100ml 0.25M sodium acetate buffer pH 5) and serial dilutions were made using the same buffer to obtain concentrations within the range 7.467-0.933mM. Hundred microlitres of each dilution were loaded into each well in triplicates followed by the addition of 200µl of 16mM 3, 3', 5, 5'-Tetramethyl-Benzidine Dihydrochloride (TMBZ) solution. Twenty-five microlitres of 3% hydrogen peroxide were then added to each well and the plate incubated for five minutes. Potassium phosphate buffer was used as the negative control. The plates were read as described at 620nm. Concentrations were plotted against absorbance to obtain a calibration curve.

3.5.2.3 Non- specific esterases

Alpha and beta-napthol were used to obtain calibration curves for non-specific esterases. Thirty millimolar (30mM) solutions of α- and β-napthol were prepared (0.0433g in 10ml acetone). Serial dilutions of the two stock solutions were made using 0.1M Potassium phosphate buffer to obtain concentrations within the range 7.5-0.17mM. Five microlitres of each dilution was loaded into wells in triplicates and 100µl of Fast Blue B salt solution added. The same buffer was used as the negative control. The plates were incubated at room temperature and read after 2 minutes as described at 540nm. Concentrations were plotted against absorbance to obtain a calibration curve.

3.5.3 Enzyme activity assays

For each test for activity of oxidases, Acetylcholine esterase and glutathione S-transferase 30 mosquitoes were used, while for the non-specific esterases 20 mosquitoes were used.

3.5.3.1 Protein assay

Total protein content was determined in 2 batches of mosquito samples. Batch one comprised of samples from 4 field populations and the susceptible Kisumu strain which were used for oxidase, glutathione S-transferase and acetylcholinesterase assays. Batch two comprised of samples from 3 field populations and the susceptible Kisumu strain which were used for the elevated non-specific esterase assays. For each mosquito homogenate 20µl was loaded into appropriate wells in the microplate, followed by 80µl of 0.1M potassium phosphate buffer. Two hundred microlitres protein dye (Bradford reagent) were added and the plate read immediately (T_0) using an ELISA plate reader at 620 nm. Potassium phosphate buffer was

used as the negative control. The quantity of protein (mg) per mosquito was estimated from the BSA calibration curve.

3.5.3.2 Oxidase assay

This assay measures the heme peroxidase levels in the test population. To conduct the assay, 100µl of mosquito homogenate were transferred into appropriate wells followed by 100µl Tetramethyl-Benzidine Dihydrochloride (TMBZ) solution. Then 25µl of 3% hydrogen peroxide was added into each well. Cytochrome-C and potassium phosphate buffer were used as positive and negative controls respectively. The plates were read after 5 minutes incubation as described at 620nm. The concentration of oxidase was calculated from a calibration curve obtained for cytochrome-C and the specific activity of oxidase expressed as mmole of product/min/mg protein per mosquito.

3.5.3.3 Acetylcholinesterase assay

This assay measures the amount of acetylcholine esterase (AcChE) present. To conduct the assay, 100µl of mosquito homogenate was transferred into in appropriate wells followed by the addition of 100 µl of 26mM Acetylthiocholine iodide (ATCH) solution into each well. 0.1M potassium phosphate buffer was used as the negative control. Then 100µl of 3.3mM dithio-bis-2-nitrobenzoic acid (DTNB) solution was added into each well and the plate read immediately (T_0) with ELISA microplate reader using 414 nm filter. The plate was incubated and read again after ten minutes (T_{10}) and the difference between the two readings used for statistical analysis. The concentration of AcChE produced was calculated using Beer's law

used as the negative control. The quantity of protein (mg) per mosquito was estimated from the BSA calibration curve.

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(Hemingway, 1998). The specific activity was then expressed as mmole of product/min/mg protein per mosquito.

3.5.3.4 Glutathione S-transferases

This assay measures the level of Glutathione S-Transferase (GSTs) present in each mosquito. To conduct the assay, 100µl mosquito homogenate was transferred into each well in triplicates. Then 100µl a 0.02mM reduced glutathione solution was added into each well followed by the addition of 100 µl of 1mM 1-chloro-2, 4'-dinitrobenzene (cDNB) solution and the plate read immediately (T_0) at 340nm filter. The plate was then incubated and read again after five minutes (T_5) and the difference between the two readings (T_5-T_0) used for statistical analysis. The concentration of GSTs produced was calculated by following the method of Hemingway (1998) using Beer's law. The specific activity was then expressed as mmole of product/min/mg protein per mosquito.

3.5.3.5 Non-specific esterases

This assay measures levels of non-specific ß-esterases present. To conduct the assay, 100 µl mosquito homogenate was pippeted into each well and 100µl of 30mM α and β naphthyl acetate solutions added, and incubated at room temperature for ten minutes. One hundred microlitres of Fast Blue B salt solution was then added to each well and further incubated for two minutes. Potassium phosphate buffer and α/β-naphthol were used as the negative and positive controls respectively. The plates were read as described at 540 nm. The concentration of naphthol produced from the esterase reactions was calculated from standard

curves obtained for α and β naphthol. Results were expressed as mmole of product/min/mg protein per mosquito triturate.

3.6 Data Analysis

Abbott's formula was used to correct the observed mortality in adult susceptibility tests (Abbott, 1925). The KT50 and KT95 values were estimated from the time mortality regression curves using probit analysis (Finney, 1971). Observed differences in resistance between susceptible and wild populations were analyzed by Student's *t*-test. A one-way analysis of variance (ANOVA) was used to compare the protein content and enzyme expression levels between susceptible and wild populations. Chi-square test (X^2) was used to test for relationships between site, molecular forms and *kdr*. All levels of statistical significance were determined at $P<0.05$.

CHAPTER FOUR

RESULTS

4.1 Bioassays

A total of 3,125 adult female *An. gambiae* s.l. were studied. For each site and for each test, 100 test and 25 control mosquitoes were used. For permethrin, deltamethrin, malathion and propoxur 100 test and 25 control susceptible Kisumu strain mosquitoes were used while for DDT 80 test and 20 control were used.

4.1.1 Permethrin (0.75%)

Mortality in the field populations ranged between 24 and 48% across the 4 replicates with a mean of 32% (±10.83) for Adabraka, 60 to 100% with a mean of 78% (±16.49) for Atabu Newtown. For Kledzo mortalities ranged from 72 to 100% with 82% (±13.27) mean mortality while in Likpe, mortality was between 44 and 64% with mean of 57% (2.31). No mortality was scored of the wild mosquito populations used for control tests. There was however 100% mortality in the susceptible Kisumu strain (Figure 4.1).

The median knockdown time (KT_{50}) obtained ranged between 39.1 - 65 minutes for the wild populations. In the Kisumu susceptible strain KDT_{50} was 10 minutes thus indicating a 3.9 to 6.5 fold increase in the wild populations. The KDT_{95} in wild populations ranged from 113.4 to 147.1 minutes while in the Kisumu susceptible strain it was 25 minutes. The details are given in Table 3.

4.1.2 Deltamethrin (0.05%)

Mortalities in the field populations ranged from 96-100% across the 4 replicates with a mean of 97% (±2.00) for Adabraka and Atabu Newtown while Kledzo had 97% (±3.83), thus indicating susceptibility in these populations. However for Likpe mortality ranged between 84-100% with a mean of 91% (±6.83), thus indicating reduced susceptibility (Figure 4.1). No mortality was scored of the wild mosquito control populations but 100% mortality was recorded in the susceptible Kisumu strain.

The median knockdown time in wild populations was in the range of 24.5 to 33.5 minutes while in the susceptible Kisumu strain KDT_{50} was 17 minutes thus indicating a 1.4 to 1.9 fold increase in wild population. The KDT_{95} ranged from 42.8 to 55 minutes in the wild populations while in the Kisumu susceptible strain it was 19 minutes (Table 3).

4.1.3 DDT (4%)

The mortalities observed for Adabraka mosquito population ranged from 8-24% with mean of 16% (±6.53), while in Atabu Newtown mortalities ranged from 40-64% with a mean of 51% (±10.00). The least mortality rate was observed in the Kledzo population which ranged from 0-20% with a mean of 6% (±9.52). For the Likpe population, mortality ranged from 36-56% with a mean of 46% (±8.33). Results from these tests thus indicate very high levels of resistance to 4% DDT in all the field populations while there was full susceptiblity in the susceptible Kisumu strain (Figure 4.1). No mortality was recorded among the control mosquito populations from the villages.

The median knockdown time (KDT_{50}) was recorded in only Atabu Newtown village and the susceptible strain populations. This is because less than 50% had been knocked down at the end of the exposure period for the other three villages. Thus the KDT_{50} was 77.5 and 23.4 minutes for Atabu Newtown and the Kisumu susceptible strain respectively, thus indicating a 3.3 fold increase in the wild population as compared with the susceptible strain. KDT_{95} was 138.3 and 43.1 minutes for the two populations respectively (Table 4).

4.1.4 Malathion (5%)

Mortality in wild populations was 100% (±0.00) in all the 4 replicates tested across the four wild populations as well as with the Kisumu susceptible strain (Figure 4.2). Thus there was full susceptibility in all the test populations. Control mortality was 0% for both field populations and the susceptible strain.

4.1.5 Propoxur (0.1%)

In the wild populations, the mortality rates recorded were between 92-96% with a mean of 95% (±2.00) for Adabraka and Kledzo and 94% (±2.31) for Atabu Newtown and Likpe populations respectively (Figure 4.2). There was 100% mortality for the Kisumu susceptible strain while with the control populations no mortality was recorded.

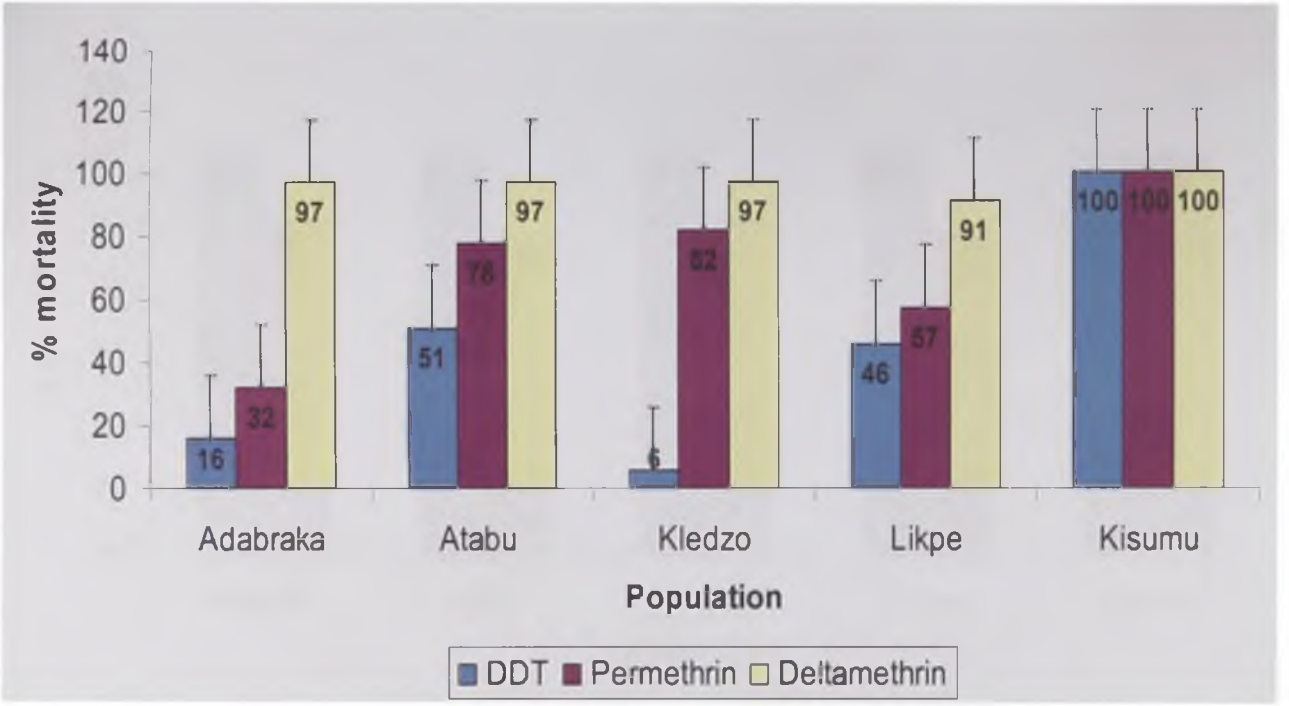

Figure 4.1: Mean percent mortalities recorded for pyrethroids; Permethrin (0.75%), Deltamethrin (0.05%) and DDT (4%) in the four wild populations and the susceptible Kisumu strain.

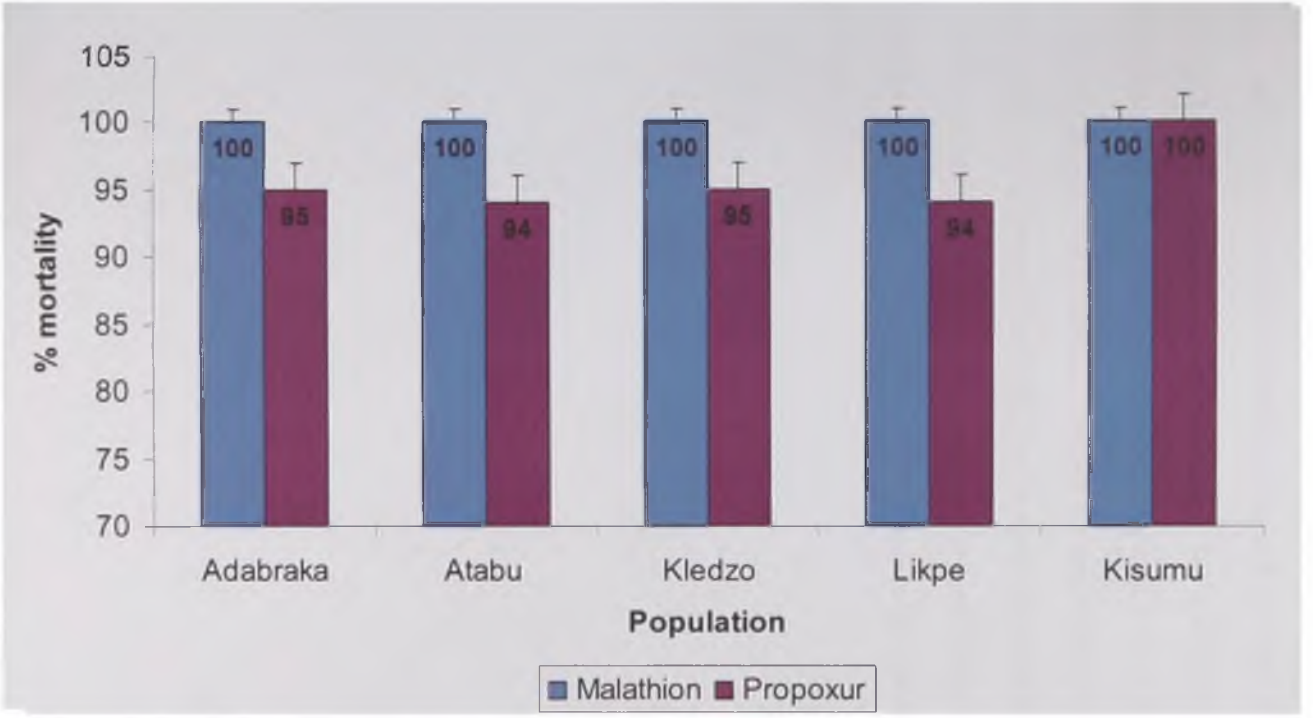

Figure 4.2: Mean percent mortalities recorded for malathion (5%) and propoxur (0.1%) in the four wild populations and the susceptible Kisumu strain.

4.1.6 Resistance ratios

Resistance ratios were determined for the pyrethroids. For permethrin the RR_{50} ranged from 3.9 to 6.5 with the lowest and highest RR_{50} occuring in Kledzo and Adabraka populations respectively (Table 3). Similarly the highest mortality with permethrin was recorded in the Kledzo while the lowest was in Adabraka and therefore there was a similar trend in mortalities and RR_{50} in these populations. However this was not the case for Atabu Newtown in which despite having relatively high RR_{50} like in Adabraka, mortality was instead higher. With deltamethrin RR_{50} was very low when compared with permethrin and was in the range of 1.4 and 1.9 and unlike permethrin, the lowest RR_{50} occurred in Adabraka while the highest was in Kledzo.

Table 3: Knockdown times (in minutes) for Permethrin, Deltamethrin and DDT in the four wild populations and the susceptible Kisumu strain and Resistance ratios.

Population	Permethrin		Deltamethrin		DDT		RR_{50}	
	KDT_{50}	KDT_{95}	KDT_{50}	KDT_{95}	KDT_{50}	KDT_{95}	Permethrin	Deltamethrin
Adabraka	65.00	147.14	24.45	51.05	*	*	6.5	1.4
Atabu newtown	62.95	142.29	25.63	42.82	77.54	138.34	6.3	1.5
Kledzo	39.13	97.17	33.52	55.04	*	*	3.9	1.9
Likpe	49.95	113.37	25.58	51.24	*	*	4.9	1.5
Kisumu	10	25	17	19	23.42	43.14	-	-

* indicates that knockdown time could not be recorded because less than 50% of the population had been knocked down at the end of the holding period.

KDT_{50} = the time it took for 50% of the test mosquitoes to be knocked down
KDT_{95} = the time it took for 95% of the test mosquitoes to be knocked down
RR_{50} was calculated by dividing KDT_{50} of field populations by that of the susceptible Kisumu strain.

4.2 Molecular studies

A total of 200 specimens (50 per site) were processed by polymerase chain reaction (PCR) for species identification, molecular forms of An. *gambiae* s.s. and *kdr* alleles.

4.2.1 Species and molecular forms identification

PCR amplification was successful in 174 (87%) specimens which were all positively identified as *An. gambiae* s.s. In Adabraka, of the 42 identified *An. gambiae* s.s, 40 were successfully identified to molecular forms, with 26 being 'S' and 14 being 'M'. Out of the 46 identified *An. gambiae* s.s in Atabu Newtown, 36 were successfully identified to molecular forms, and they comprised of 7 'S' and 26 'M' forms. In Kledzo 33 *An. gambiae* s.s were successfully identified to molecular forms; 27 were 'S' and 6 were 'M' forms. In Likpe, 38 *An. gambiae* s.s were successfully identified to molecular forms; 35 were 'S' and 3 were 'M' forms (Table 4).

4.2.2 Distribution of the *kdr* allele in *An. gambiae* s.s. population

In the four field populations, 63% of the samples tested were *kdr* positive while 37% were *kdr*-negative. In Adabraka 24 specimens were *kdr* positive while 14 were *kdr* negative. In Atabu Newtown, 15 were *kdr* positive and 18 were negative. In Kledzo, of the 32 specimens 23 possessed the *kdr* allele and 9 were *kdr* negative and in Likpe 32 of the 45 studied were *kdr* positive. Over 70% of the *kdr* mutation was associated with the 'S' form in the wild *An. gambiae* s.s populations.

Chi-square tests revealed no significant relationship between molecular forms and *kdr* (Pearson Chi-square value = 1.019, p = 0.313).

Table 4: Molecular identification of *Anopheles gambiae* s.s samples into 'M' and 'S' forms and distribution of *kdr* alleles in the four field populations.

	Molecular form	
Site	**'S'**	**'M'**
Adabraka	26 (65%)	14 (35%)
Atabu newtown	7 (21.2%)	26 (78.8%)
Kledzo	27 (78.8%)	6 (21.2%)
Likpe	35 (83%)	7 (17%)
Total	**95 (64%)**	**47 (36%)**

Table 5: Distribution of *kdr* alleles into the 'M' and 'S' forms of *Anopheles gambiae* s.s in the four field populations.

	'S' form		'M' form	
Site	***Kdr* +ve**	***Kdr* -ve**	***Kdr* +ve**	***Kdr* -ve**
Adabraka	17 (65%)	9 (35%)	7 (58%)	5 (42%)
Atabu newtown	4 (57%)	3 (53%)	11 (42%)	15 (58%)
Kledzo	18 (67%)	9 (33%)	5 (100%)	0
Likpe	27 (71%)	11 (29%)	2 (50%)	2 (50%)
Total	**66 (67%)**	**32 (33%)**	**27 (57%)**	**20 (43%)**

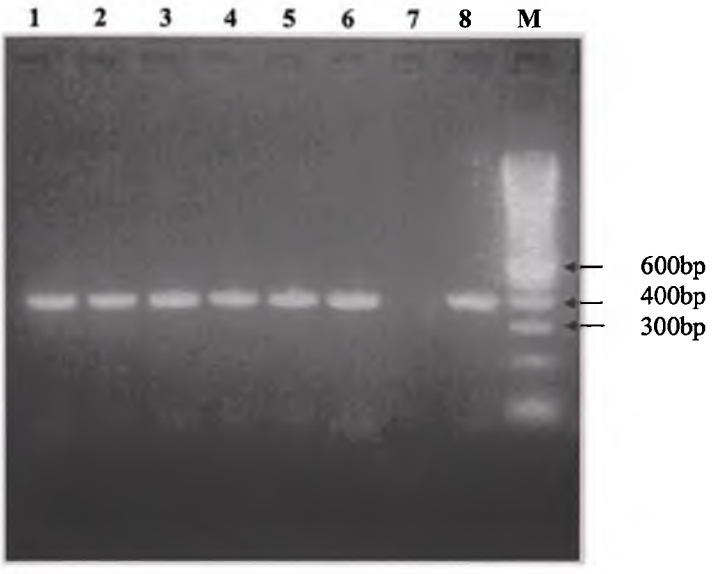

Figure 4.3: An example of electrophoresed ethidium bromide-stained 2.0% agarose gel of PCR products for the detection of species of *An. gambiae* complex. Lanes 1-6 = *An. gambiae s.s.*; lane 7 = negative control; lane 8 = positive control; lane M = 100bp DNA size marker.

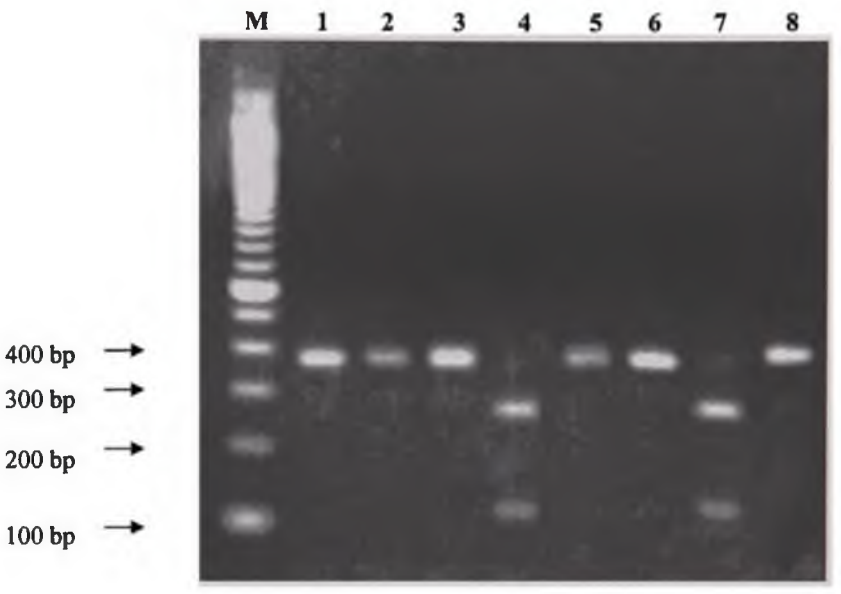

Figure 4.4: An example of electrophoresed ethidium bromide-stained 2.0% agarose gel of *Hha*1 restriction enzyme analysis of *An. gambiae s.s.* PCR products for the detection of M and S molecular forms. Lane M =100bp DNA size marker; lanes 1-3 = M form; lane 4 = S form; lanes 5-6 = M form; lane 7 = S form; lane 8 = undigested *An. gambiae s.s.* PCR product.

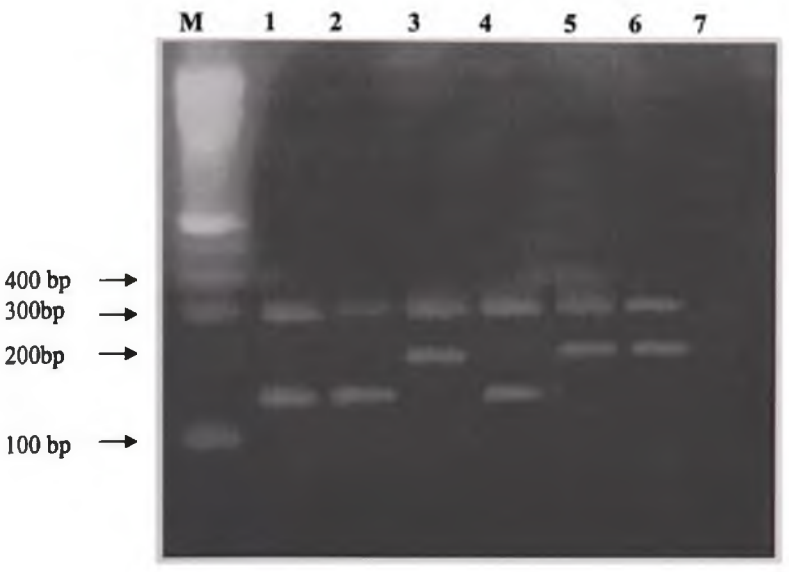

Figure 4.5: An example of electrophoresed ethidium bromide-stained 2.0% agarose gel of *An. gambiae s.s.* PCR products for the detection of *kdr* alleles. Lane M =1 00bp DNA size marker; lanes 1-2 = *kdr* susceptible; lane 3 = *kdr* resistant; lanes 4 = *kdr* susceptible; lane 5-6 = *kdr* resistant; lane 7 = negative control.

4.3 Biochemical assays

A total of 190 wild mosquitoes and 80 from the susceptible Kisumu strain were assayed for the activities of oxidase, acetylcholine, glutathione S-transferase and esterase enzymes. Detailed information on absorbances obtained and enzyme activities of individual mosquitoes are shown in appendix III.

4.3.1 Calibration curves

The results obtained are illustrated in Figure 4.6. All values were in the range of 92 – 99% and therefore all the curves exhibited good linearity hence indicating the reliability of the methods used.

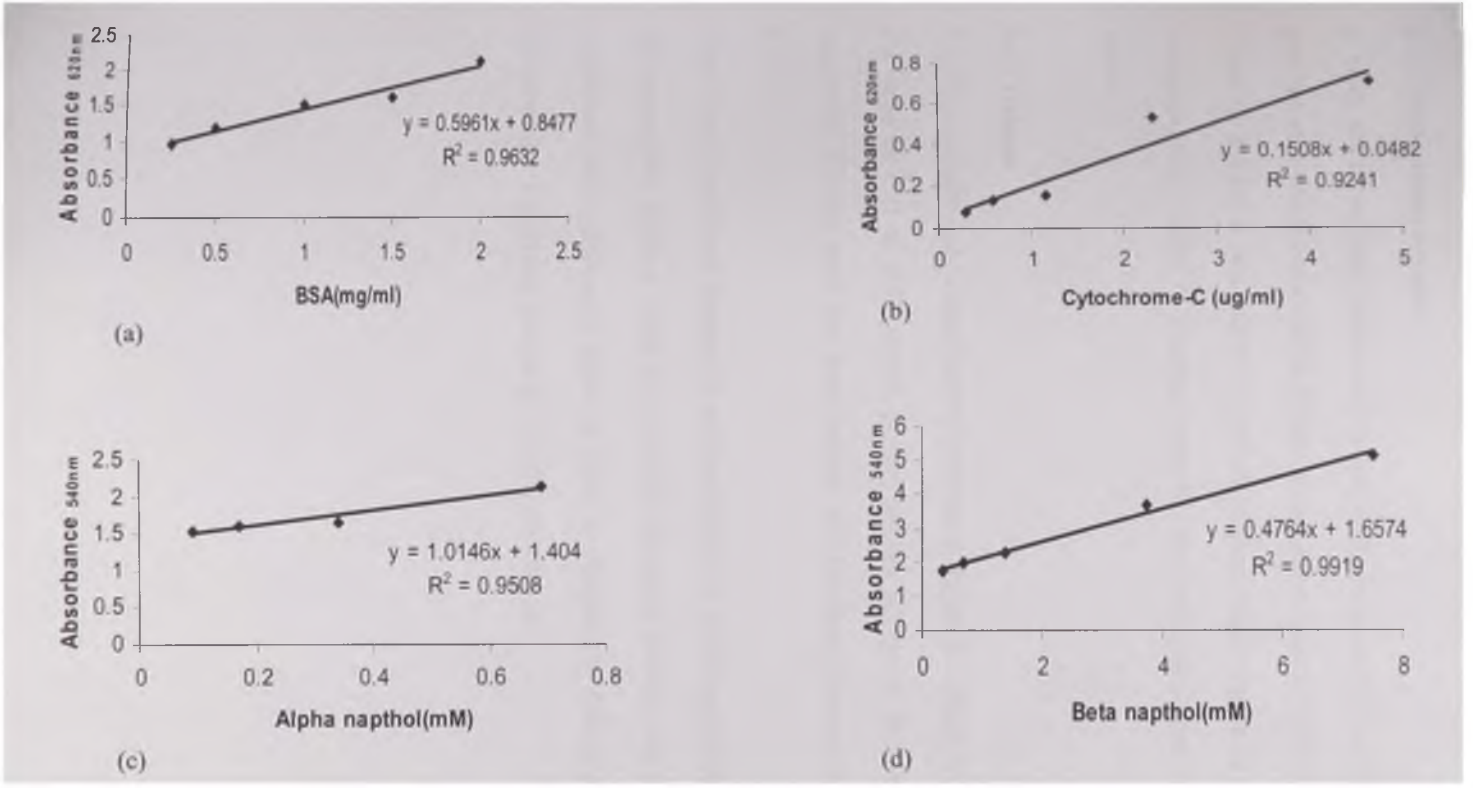

Figure 4.6: Calibration curves for (a) Protein (b) Oxidase (c) Alpha esterase and (c) Beta esterase.

R^2 = Coefficient of regression.

4.3.2 Total protein content

In batch one mean total protein content was in the range of 0.2 to 0.52 mg/ml in the wild populations while the susceptible Kisumu strain had a mean of 0.53 mg/ml protein content (Table 6). For batch two, mean total protein content ranged from 0.22 to 0.35 mg/ml in the wild populations (Table 7). The susceptible Kisumu strain had a mean of 0.47 mg/ml protein content.

4.3.3 Oxidase

Distribution pattern of oxidase activity is shown in Figure 4.7. Mean enzyme activity was in the range of 0.11 to 0.53 mmole product/min/mg protein in the wild populations. The susceptible Kisumu strain had mean activity of 0.10 mmole product/min/mg protein (Table 6).

There was a significant increase in enzyme activity in Likpe population when compared to the susceptible Kisumu strain ($P = 0.005$). Similarly oxidase activity within the field populations was significantly higher in Likpe as compared to Adabraka ($P = 0.005$), Atabu Newtown ($P = 0.005$) and Kledzo ($P = 0.005$) populations.

Table 6: Mean activity of Oxidase, Acetylcholinesterase (AcChE) and Glutathione-S-transferase (GST) in the four wild populations and the susceptible Kisumu strain.

Population		Enzyme activity (mmole product/min/mg protein)		
	Protein	Oxidase	AeChE ($x10^{-6}$)	GST (10^{-3})
Adabraka	0.20±0.07	0.27±0.67 [a]	1.12±2.38 [b]	0.77±1.50 [a]
Atabu Newtown	0.39±0.12	0.11±0.04 [a]	0.22±0.06 [a]	0.33±0.07 [a]
Kledzo	0.52±0.11	0.12±0.04 [a]	0.36±0.09 [a]	0.58±0.07 [a]
Likpe	0.37±0.26	0.53±0.94 [b]	0.50±0.13 [a]	0.31±0.11 [a]
Kisumu	0.53±0.15	0.10±0.03 [a]	0.28±0.10 [a]	0.56±0.16 [a]

Means followed by same letter in a column are not significantly different at $P < 0.05$.

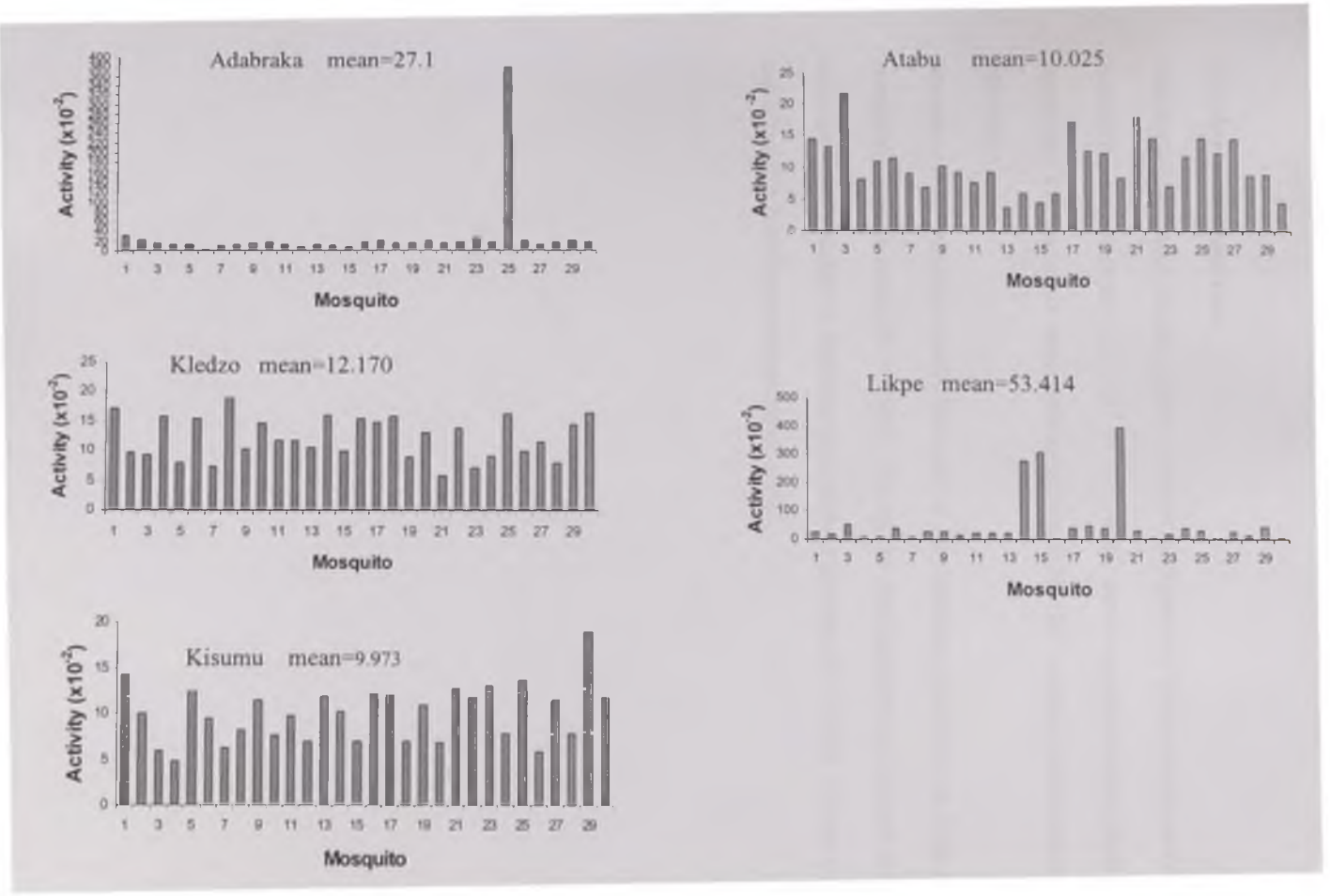

Figure 4.7: Distribution patterns of Oxidase activity in the four wild populations and the susceptible Kisumu strain.

Activity = mmole product/min/mg protein

4.3.4 Acetylcholinesterase

Distribution pattern of AcChE activity is shown in Figure 4.8. Mean enzyme activity in wild populations ranged from 0.22 $x10^{-6}$ to 1.12 $x10^{-6}$ mmole product/min/mg protein. In the susceptible Kisumu strain mean activity of was 0.28 $x10^{-6}$ mmole product/min/mg protein (Table 6).

Enzyme activity was significantly increased in the Adabraka population as compared to the susceptible Kisumu strain (P = 0.009). Among the field populations, enzyme activity was also significantly higher in Adabraka than Atabu Newtown (P = 0.009), Kledzo (P = 0.009) and Likpe (P = 0.009) populations respectively.

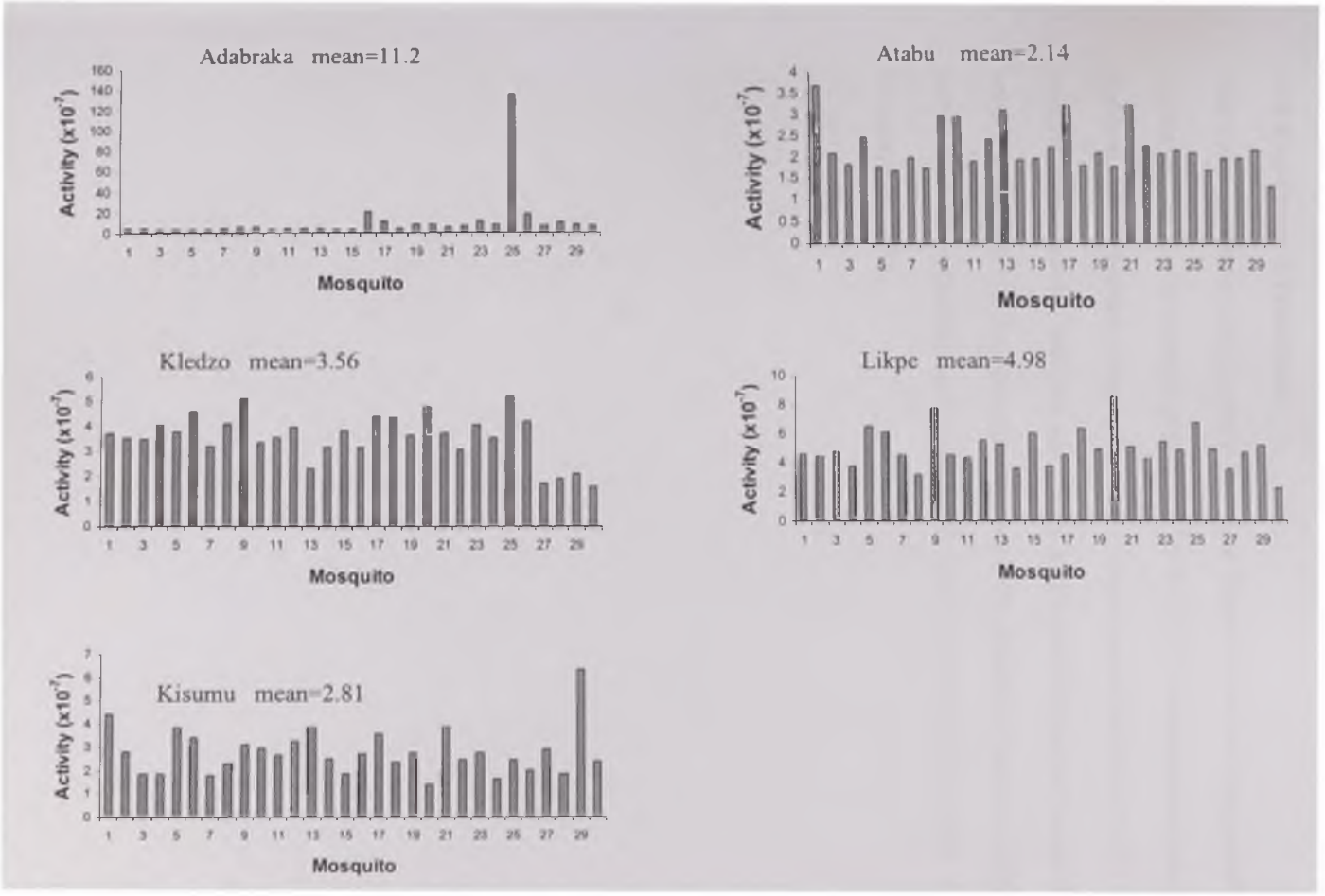

Figure 4.8: Distribution patterns of AcChE activity in the four wild populations and the susceptible Kisumu strain.

Activity = mmole product/min/mg protein

4.3.5 Glutathione S-Transferase

Distribution pattern of GST activity is shown in Figure 4.9. In the wild populations mean enzyme activity was in the range of 0.31 $x10^{-3}$ to 0.77 $x10^{-3}$ mmole product/min/mg protein (Table 6). The susceptible Kisumu strain had a mean enxyme activity of 0.56 $x10^{-3}$ mmole product/min/mg protein. Adabraka and Kledzo populations showed an increase in enzyme activity as compared to the susceptible Kisumu strain although it was not significant (P = 0.056), while Atabu Newtown and Likpe populations had a much lower enzyme activity than the susceptible strain.

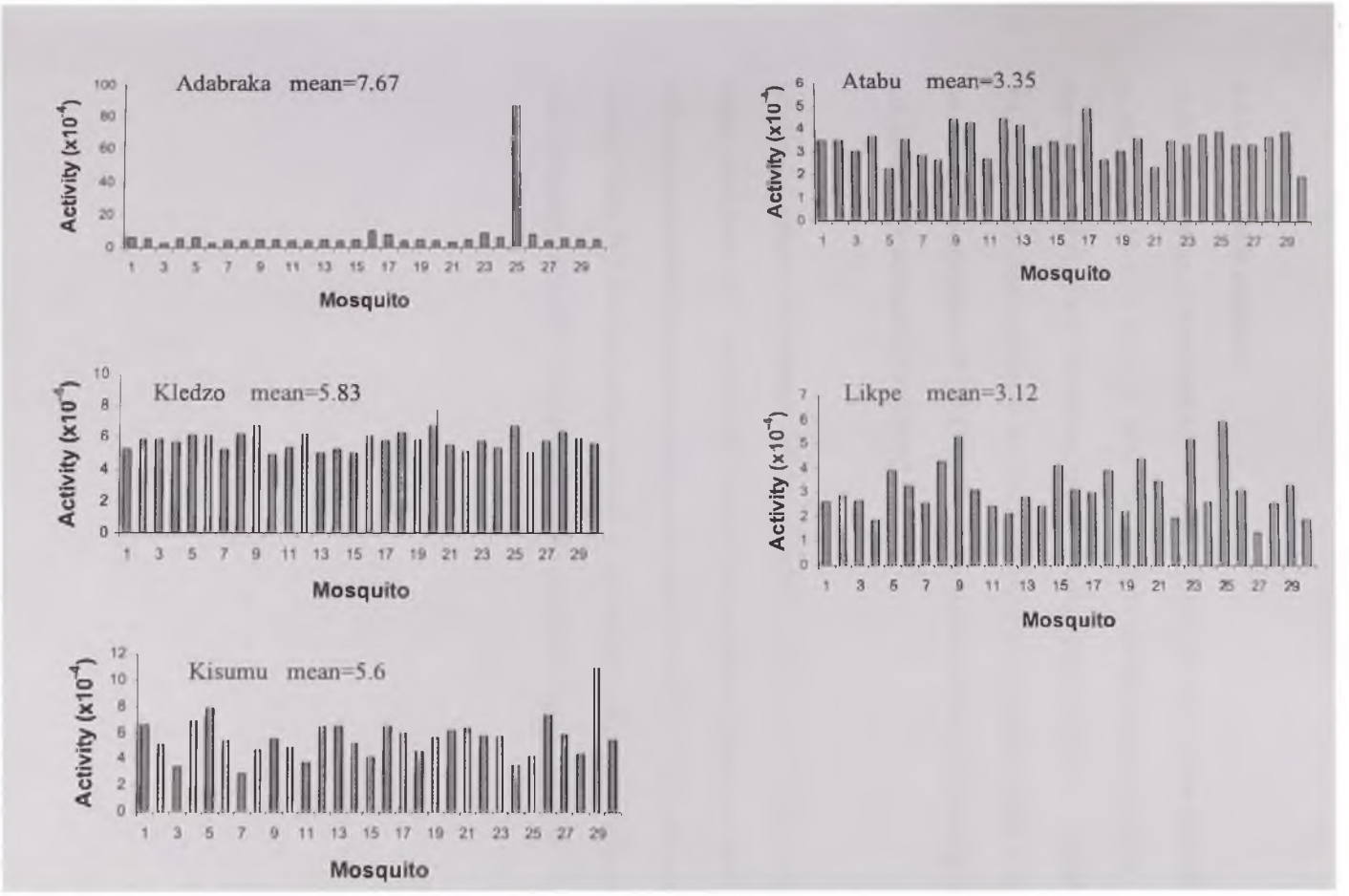

Figure 4.9: Distribution patterns of GST activity in the four wild populations and the susceptible Kisumu strain.

Activity = mmole product/min/mg protein

4.3.6 Non-specific esterases

Distribution pattern of α-esterase activity is shown in Figure 4.10. Mean enzyme activity was in the range of 0.23 $x10^{-1}$ to 0.94 $x10^{-1}$ mmole product/min/mg protein in the wild populations and 0.19 $x10^{-1}$ in the susceptible Kisumu strain (Table 7). Enzyme activity in the field populations of Adabraka and Likpe was significantly higher when compared to the susceptible Kisumu strain (P = 0.000). However enzyme activity in Kledzo population was not significantly increased (P = 0.000)

Distribution pattern of β-esterase activity is shown in Figure 4.11. Mean enzyme activity ranged from 0.90 $x10^{-1}$ to 2.55 $x10^{-1}$ mmole product/min/mg protein in wild populations while the susceptible Kisumu strain had a mean activity of 0.72 $x10^{-1}$ mmole product/min/mg protein (Table 7). Likewise, enzyme activity in the field populations of Adabraka and Likpe was significantly elevated when compared to the susceptible Kisumu strain (P = 0.000).

Table 7: Mean activity of Non-specific esterases in the four wild populations and the susceptible Kisumu strain.

Population		Enzyme activity (m-mole product/min/mg protein)	
	Protein	α-esterase ($x10^{-1}$)	β-esterase ($x10^{-1}$)
Adabraka	0.22±0.06	0.92±0.99 [b]	2.55±0.91 [b]
Kledzo	0.35±0.18	0.23±0.18 [a]	0.90±0.57 [a]
Likpe	0.24±0.14	0.94±0.55 [b]	2.52±1.61 [b]
Kisumu	0.47±0.10	0.19±0.10 [a]	0.72±0.15 [a]

Means followed by same letter in a column are not significantly different at $P < 0.05$.

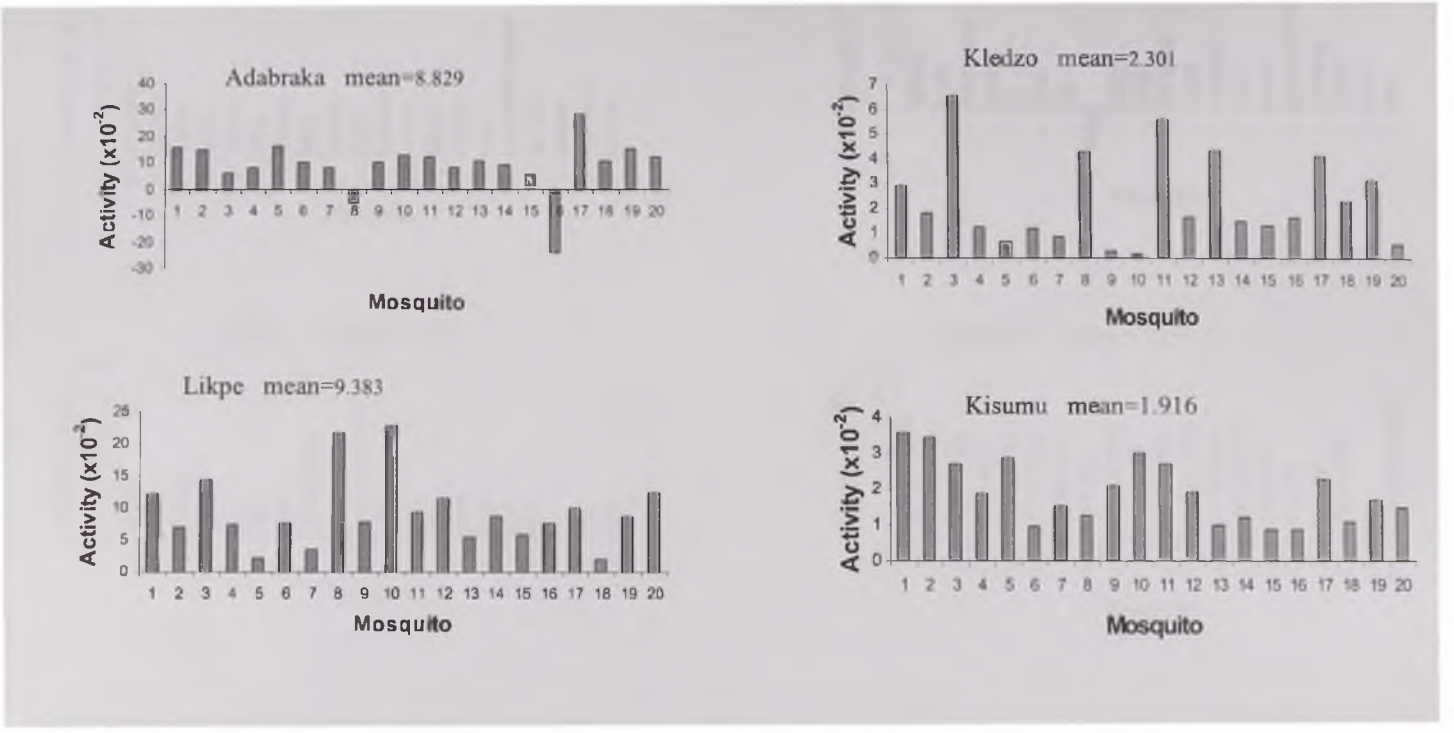

Figure 4.10: Distribution patterns of α-esterase activity in three wild populations and the susceptible Kisumu strain.

Activity = mmole product/min/mg protein

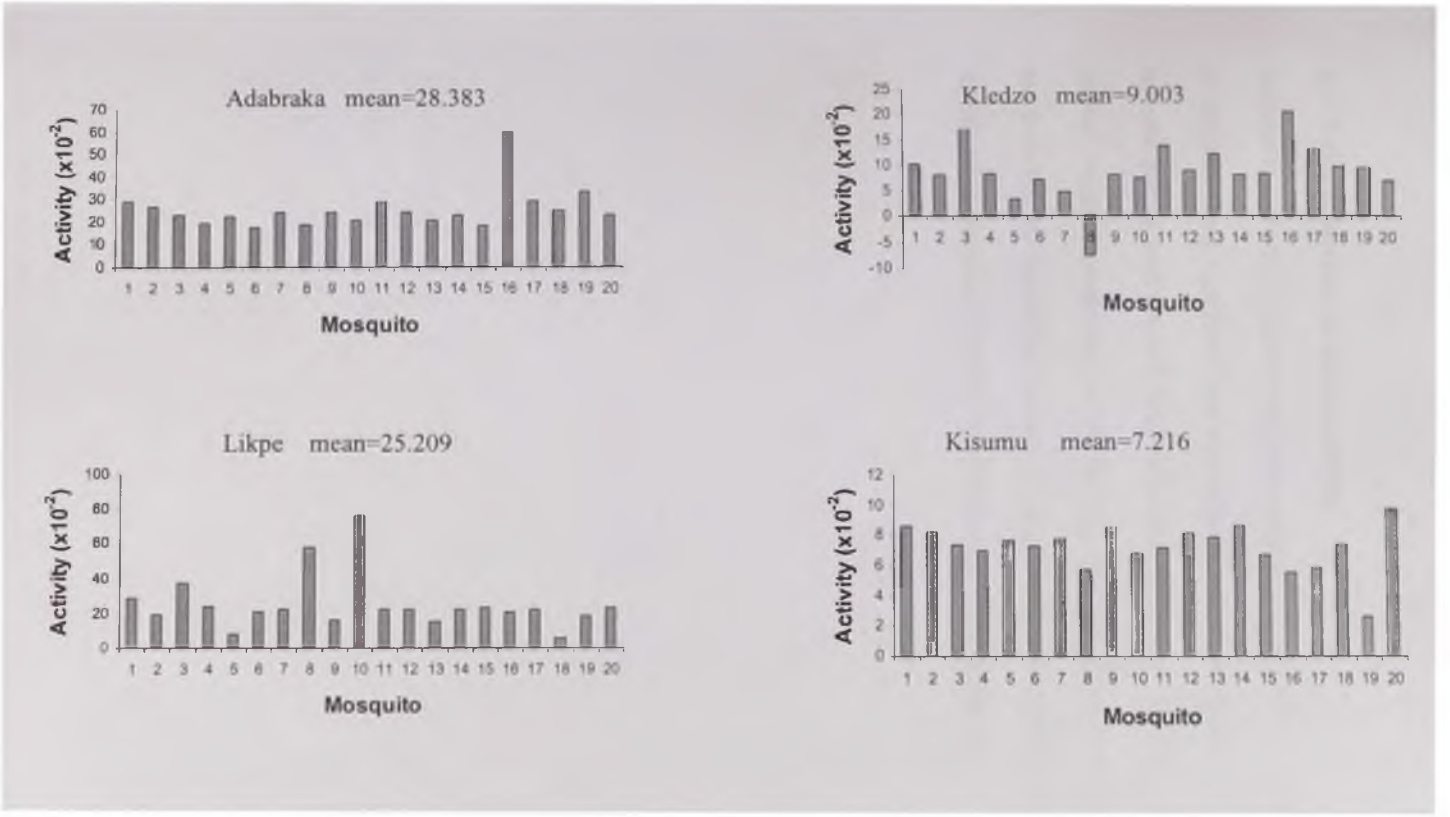

Figure 4.11: Distribution patterns of β-esterase activity in three wild populations and the susceptible Kisumu strain.

Activity = mmole product/min/mg protein

4.3.7 Level of increase in enzyme activity

Increase in activity of oxidase was in the range of 1.1 to 5.4 fold, with only the highest level of increase being significant and occurring in Likpe population. For AcCHE the level of increase ranged from 0.8 to 4 fold and as with oxidase the highest level was significant but instead was in Adabraka population. Activity of GSTs increased by between 0.6 to 1.4 fold, but there was no significant increase among the field populations. The level of increase in activity had a similar pattern for both α- and β-esterases (Table 8).

Table 8: Level of increase in enzyme activity in field populations as compared with the susceptible Kisumu strain.

Population	Level of increase in enzyme activity				
	Oxidase	AcCHE	GST	α-esterase	β-esterase
Adabraka	2.7	4*	1.4	4.8*	3.5*
Atabu Newtown	1.1	0.8	0.6	**	**
Kledzo	1.2	1.3	1	1.2	1.3
Likpe	5.4*	1.8	0.6	4.9*	3.5*

*= Enzyme activity was significantly elevated
**=Enzyme activity was not tested
Level of increase in enzyme activity was calculated by dividing field population value by that of the susceptible Kisumu strain.

CHAPTER FIVE

DISCUSSION

Insecticide resistance has been a problem in all insect groups that are vectors of diseases. Although mechanisms by which insecticides become less effective are similar across all vector taxa, each resistance problem is potentially unique and may involve a complex pattern of resistance foci. Regular testing is therefore vital for mosquito control operations because resistant populations of mosquitoes reduce the effectiveness of control procedures. The main defense against resistance is close surveillance of the susceptibility of vector populations (Brogdon and McAllister, 1998). The initial step in identifying a potential problem is to detect changes in the susceptibility of a population of vectors through bioassays. Biochemical and molecular methods can detect resistance mechanisms in individual insects and therefore resistance can be confirmed with the use of only a small number of insects.

The actual molecular mechanisms that regulate insecticide resistance are poorly understood. *Anopheles gambiae* has multiple resistance mechanisms that have been field-selected in both East and West Africa through exposure to DDT and pyrethroids (Hemingway *et al.*, 2002). Identification of resistance mechanisms helps determine the cross-resistance spectrum, facilitates the choice of alternative insecticides and allows detailed mapping of areas with resistant populations. This study therefore set out to determine the susceptibility status of *An. gambiae* complex towards various commonly used insecticides and to determine the underlying mechanisms of resistance by using molecular and biochemical assays.

In this study, susceptibility tests results generally showed high levels of resistance to permethrin and DDT in filed populations of *An. gambiae* as shown by the very low mortalities. This is strongly supported by the greatly increased median knockdown times in the field populations. Higher KT_{50} values in field populations have been suggested to give an early indication of the involvement of *kdr* mechanism of resistance (Chandre *et al.*, 1999a, b). Deltamethrin and propoxur resulted in slightly reduced susceptibility levels while all tested samples were fully susceptible to Malathion. Deltamethrin, an α-cyano pyrethroid and more toxic than permethrin, despite giving very high mortality, still had a resistance ratio ranging from 1.4 to 2, thus implying low levels of resistance. These results are therefore consistent with those of other studies conducted in Accra, where *An. gambiae* s.l. has been reported to be resistant to permethrin and DDT (Adasi *et al.*, 2000: Adeniran, 2002: Achonduh, 2005). However the results obtained are in contrast with those of Kristan *et al.* (2003) who reported full susceptibility of *An. gambiae* s.l. field populations to permethrin and DDT in the Western Region of Ghana.

Morphological identification revealed *An. gambiae* s.s. as the dominant *Anopheles* mosquito species at all the study sites. These results are consistent with that of other studies carried out in the country (Adasi *et al.*, 2000; Adeniran, 2002; Midega, 2002; Kristan *et al.*, 2003 and Otieno, 2004). This is explained by the fact that the study area is comprised of a high altitude humid forest zone with higher rainfall making it is more favourable to *An. gambiae* s.s while *An. arabiensis* prefers more of dry savanna areas (Colluzi *et al.*, 2000). In Ghana *An. arabiensis* has been found to predominate in the arid savanna of the northern region (Appawu *et al.*, 1994).

Both the 'M' and 'S' molecular forms of *An. gambiae* s.s were present and occurred in sympatry at all the study sites. Generally the 'S' form occurred at higher frequencies than the 'M' form in the study area with an exception of one site, Atabu Newtown where the 'M' form occurred at a very high frequency than the 'S' form. This is a low lying swampy area that is normally flooded during the rainy season. The 'M' form has generally been associated with urban environments and flooded/irrigated areas while the 'S' form has been associated with rural areas. The fact that the 'S' form occurred at a very high frequency has serious implications in malaria control efforts since it has been strongly associated with the *kdr* mechanism of resistance (Chandre *et al.*, 1999a,b). In this study the resistant phenotype of *kdr* was 70% in the 'S' form. Nevertheless it is very remarkable that 30% of resistant phenotype occurred in the 'M' form.

PCR identification of the *kdr* allele revealed both the resistant and susceptible phenotypes across all the collection sites. The resistance phenotype generally occurred at a higher frequency and was mainly associated with the 'S' form of *An. gambiae* s.s. Atabu Newtown however had more of the susceptible phenotype and this is mainly due the fact that the 'M' form which is less associated with the *kdr* occurred at a very high frequency. The observed high frequency of the resistant phenotype of *kdr* is supported by the high increase in knockdown times in field populations as compared with the susceptible Kisumu strain. This high *kdr* frequency by no means accounts for the very low mortalities observed in field populations with permethrin and DDT.

Biochemical assays showed elevated levels of activity of detoxification enzymes in some field populations. Mean enzyme activity of oxidase was significantly higher in Likpe population as compared with the susceptible Kisumu strain. Likewise this population exhibited very high levels of permethrin and DDT resistance and thus strongly suggesting the implication of oxidases in addition to *kdr* in the observed resistance in this population. Mixed function oxidases may be involved in the detoxification of almost all insecticides but are most notably associated with pyrethroid resistance (Hemingway & Ranson, 2000; Brooke *et al.*, 2001; Chareonviriyaphap *et al.*, 2003). In *An. gambiae*, metabolic resistance to pyrethroids has been shown to involve increased levels of cytochrome P_{450} (Ranson *et al.*, 2000). Work by Vulule *et al.* (1999) demonstrated the involvement of P_{450} enzymes in pyrethroid resistance in a Kenyan population of *An. gambiae*, where treatment with P_{450} inhibitor piperonyl butoxide (PBO) partially reversed permethrin resistance in this strain.

Activity of both α and β esterase was significantly increased in Adabraka and Likpe populations. Bioassays show that the Adabraka population had the highest levels of resistance to both permethrin and DDT. This strongly suggests the involvement of esterases as well as *kdr* and oxidases in the observed high resistance to permethrin and DDT in this population. Pyrethroids are insecticidal esters derived from primary alcohols and are thus susceptible to hydrolysis by esterases. Elevated esterase activity has been linked to pyrethroid resistance patterns in a variety of insects. *Anopheles albimanus* resistant to deltamethrin demonstrated elevated esterase levels in Guatemala (Brogdon *et al.*, 1998). Chareonviriyaphap *et al.* (2000) found extremely high elevated esterase levels in the

pyrethroid resistant *An. albimanus* colony in Guatemala, and they concluded pyrethroid use against them in Central America may be limited.

GST enzyme activity in field populations was not significantly elevated when compared with the susceptible Kisumu strain. Several studies have reported similar findings. Chareonviriyaphap *et al.* (2003) did not find any association between insecticide resistance in *An. minimus* with activity of GSTs. The mean level of GST activity across test populations of *An. funestus* in South Africa was not significantly higher than that of the corresponding susceptible reference strain (Brooke *et al.*, 2001). There are currently no published records implicating GSTs in pyrethroid resistance in mosquitoes and the mixed function oxidase synergist PBO has not been implicated in GSTs activity inhibition (Brooke *et al.* 2001).

However GSTs have been implicated in resistance to DDT (Penilla *et al.*, 1998; Ranson *et al.*, 2000; Prapanthadara *et al.*, 2002). GST activity was implicated in the observed high resistance to DDT and permethrin field populations of *An. gambaiae* in the Greater Accra area (Achonduh, 2005). The high level of DDT resistance detected in field populations of *An. albimanus* in southern Mexico was attributed to elevated levels of GST activity leading to increased rates of metabolism of DDT to DDE (Penilla *et al.*, 1998). The GSTs of *Anopheles gambiae* have been studied extensively because of their involvement in DDT resistance. GSTs from a DDT-resistant strain of *An. gambiae* had an altered GST profile compared with susceptible insects (Prapanthadara *et al.*, 1993). Ortelli *et al.* (2003) have indicated that DDT resistance in *An. gambiae* is associated with both qualitative and quantitative changes in multiple GST enzymes. Vontas *et al.* (2001) have implicated elevated GSTs with a

predominant peroxidase activity to pyrethroid resistance in a laboratory selected colony of the brown planthopper *Nilaparvata lugens*.

There was a significantly elevated level of non-specific acetylcholinestrease in the Adabraka population as compared to the susceptible Kisumu strain. This does not seem to be consistent with the bioassay results which showed that *Anopheles gambiae* was fully susceptible to Malathion, an organophosphate while susceptibility to propoxur, which is a carbamate, was only slightly reduced in this population. However this slightly reduced susceptibility to propoxur could still be attributed to the activity of this enzyme. Studies by Penilla *et al.* (1998) attributed the carbamate resistance in *An. albimanus* population in Mexico to an altered acetylcholinesterase based resistance mechanism. Carbamate resistance as a result of insensitive acetylcholinesterase has recently been detected in *Anopheles gambiae* s.s. populations from Côte d'Ivoire (Corbel *et al.*, 2003). In Accra *Anopheles gambiae* was reported to be fully susceptible to propoxur (Adeniran, 2002).

Elevated esterase occurs in a number of mosquitoes that are resistant to organophosphate and carbamate insecticides (Hemingway *et al.*, 1998). Beach *et al.* (1989) implicated elevated esterases for organophosphate and pyrethroid resistance in *An. albimanus* from the coastal areas of Guatemala where organophosphate and carbamate insecticides have been used for agriculture. Malathion-specific carboxylesterase mechanisms were found in *A. culicifacies* and *A. subpictus*, both giving high rates of insecticide metabolism in Sri Lanka (Karunaratne and Hemingway, 2001). Thus the slight reductions in susceptibility to propoxur could be due to the activity of both esterases and acetylcholinesterase.

Resistance is not evolving through unique new mechanisms but instead existing mechanisms are being enhanced, and cross-resistance is occurring. Multi-resistance (two or more resistance mechanisms in the same insect) is becoming widespread as control programs make sequential use of one chemical class after another (Brogdon *et al.*, 1998; Brogdon *et al.*, 1999b). Vulule *et al.* (1999) suggested that use of impregnated nets selects for higher oxidase and esterase levels in *An. gambiae* to metabolize permethrin acquired from the nets. It has been suggested that deltamethrin selection appears to select initially a monooxygenase-based mechanism and when this mechanism is blocked by treatment with PBO, selection of a *kdr*-type mechanism is accelerated, as is evident from increased cross-resistance to DDT in the adults selected with deltamethrin–PBO (Kumar *et al.*, 2004).

Similarly, P450-dependent oxidases were implicated in permethrin resistance in *Culex quinquefasciatus* Say from, Côte d'Ivoire and Burkina Faso in which *kdr* associated with DDT cross resistance and a dramatic loss of permethrin knockdown effect on adults was also involved (Chandre *et al.*, 1998). Studies by Brogdon *et al.* (1999a) identified an oxidase resistance mechanism producing DDT–pyrethroid cross-resistance in Guatemalan *An. albimanus* Wiedemann. Interestingly, only adult female mosquitoes express the oxidase mechanism which coexists with an elevated esterase mechanism. Brooke *et al.* (2001) found the evidence for mixed function oxidase based cross-resistance between pyrethroids and the carbamate, propoxur in *An. funestus* rather unusual.

The findings of the current study are consistent with most of the other studies elsewhere as there is evidence of cross and multi-resistance. Results of bioassays, molecular and biochemical assays show an interesting trend. For example the occurrence of high levels of

permethrin and DDT resistance is associated with the occurrence of significant levels of esterases, oxidases and a high frequency of the resistant *kdr* phenotype. In Adabraka and Likpe there was a significant increase in esterase activity as well as a high frequency of the resistant *kdr* phenotype. Furthermore activity of oxidases was significantly higher in the Likpe population. Thus there is a strong indication that both oxidase and esterase enzyme systems and *kdr* mechanisms are working in concert to confer the observed high levels of permethrin and DDT resistance in these *An. gambiae* populations.

The lack of a significant increase in enzyme activity in the other two field populations of Atabu Newtown and Kledzo is rather surprising as they both had high resistance to DDT and significantly reduced susceptibility to permethrin. Although esterase assay was not conducted in Atabu Newtown population none of the enzymes tested was significantly high either. However it is worth noting that occurrence of *kdr* was equally high in both populations and that it is probably the only mechanism conferring resistance. Another point worth considering is the fact that since it is the means which were used to compare the enzyme activity, there are higher chances that some individual mosquitoes in these populations might have enzyme activity high enough to metabolize some of these insecticides as is shown by the distribution patterns of enzyme activity.

Currently, the most advocated worldwide malaria vector control strategy appears to be the use of pyrethroid-impregnated bed nets. This is because they are less expensive than spraying walls with residual insecticides, are effective in reducing child deaths and can be better administered through a horizontal community-based program. The evolution of the *kdr* and biochemical mechanisms of resistance by no means poses a threat to this strategy. Initially

there was an early optimism that because of their rapid toxicological action pyrethroids would not produce resistance.

In this study, resistance was generally high against permethrin, which is commonly used for impregnating bednets and curtains as well as indoor spraying against disease causing and biting nuisance mosquitoes. This could have serious implications in malaria transmission as well as compliance to ITN programmes. It has already been pointed out that any failure in nuisance control due to resistance is likely to demotivate people in using impregnated materials (Chandre *et al.*, 1998). The high mortalities with deltamethrin gives hope that other pyrethroids are still effective in controlling *An. gambiae* populations. However the slight reduction in susceptibility as indicated by the slight increase in KT_{50} should be closely monitored. The same case applies to propoxur and malathion, since already the carbamates and organophosphates have been suggested as alternatives, should the pyrethroids fail to be effective in ITN programmes due to high levels of resistance (Darriet *et al.*, 2003; Corbel *et al.*, 2003; Hougard *et al.*, 2003).

To compromise vector control with insecticides, the level of resistance must be high enough to adversely affect disease transmission and in many cases, vector control may not be affected by the observed levels of resistance in the vectors (Brogdon and McAllister, 1998). Even in areas of high pyrethroid resistance and high *kdr* gene frequency in *An. gambiae*, deltamethrin impregnated bednets remain effective in vector control and should still be considered as a practical means of personal protection against malaria (Darriet *et al.*, 2000). If for example, the level of resistance is lower than 10%, resistance will not affect disease control efforts (Brogdon and McAllister, 1998). Thus in this situation, increasing surveillance

and monitoring level and frequency of resistance would be sufficient without the need for change in control methods.

Insecticide susceptibility has been described as a resource and resistance surveillance as an essential step in resistance management (Brogdon and McAllister, 1998).. Resistance surveillance has three objectives; first to provide baseline data for program planning and pesticide selection before the start of control operations. Secondly, to detect resistance at an early stage so that timely management can be implemented. Even detection of resistance at a late stage can be important in elucidating the causes of failure of disease control. However, in such cases, any management other than replacement of the pesticide may not be possible. Thirdly, resistance surveillance is essential to continuously monitor the effect of control strategies on resistance.

In conclusion, the current study forms a baseline survey in the study area and since no earlier study has been conducted, resistance has been inferred by comparison with the susceptible Kisumu strain. Since it is the first study in Hohoe and although resistance levels in *An. gambaie* populations in agree with those of other areas, there is need for regular surveillance to find out if resistance is rapidly evolving or it has stabilized. For example, in western Kenya, pyrethroid resistance appeared soon after bed nets were introduced. After 2 years, the resistance level had not changed significantly, possibly because of the continual massive introduction of susceptible genes (Vulule *et al.*, 1996). This study has demonstrated the existence of several resistance mechanisms operating together in *An. gambaie* populations. It is therefore recommended that investigations on multi-resistance mechanisms in *An. gambaie*

populations be conducted in different ecological zones of the country as most previous studies have been focusing only on the *kdr* mechanism.

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APPENDIX I

Preparation of standard solutions

The following standard solutions were prepared using sterile double distilled water (sddw).Where appropriate, the solutions were autoclaved at 1211b/sq in. for 15minutes in an Eyela Autoclave (Rikikkaki, Tokyo)

1. Solutions for DNA extraction

a) Bender buffer

0.1M NaCl, 0.2 M sucrose, 0.1M Tris-HCl pH 7.5, 0.05M EDTA pH 9.1, 0.5% SDS. Stored at 4°C.

b) 0.5M EDTA (pH 8.0)

186.1 g/l in water, pH adjusted with NaOH pellets and stored room temperature.

c) KAc(8M)

60ml of 5M KAc and 11.5ml glacial acetic acid in 28.5ml distilled water.

d) RNase

10mg/ml. Sterilised by filtration and stored at-20°C

e) TE (pH 8.0)

10mM Tris-HCl (pH 8.0), 1mM EDTA (pH8.0). Stored at room temperature.

f) TE + RNnase (5µg/ml)

5µl of RNase (10mg/ml) solution, 995µl of TE (pH8.0). Stored at -20°C.

2. Solutions for Electrophoresis

i) Agarose gels

10X TAE buffer

242g Tris base, 57.1ml glacial acetic acid, 100ml of 0.5 m EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000ml with ddw.

EtBr (10mg/ml)

1g of EtBr was completely dissolved in 100ml ddw and stored in the dark at room temperature.

ii) Gel loading buffer

Orange G

20% (w/v) Ficoll, 25 mM EDTA, 2.5% (w/v) orange G. Stored at 4°C.

3. Solutions for Biochemical Assays

a) Buffers

i. 0.1M potassium phosphate buffer(pH7.2)

1.7g monobasic potassium phosphate in 454.4ml distilled water plus 6.6g dibasic potassium phosphate in 545.5ml distilled water. Adjust pH to 7.2 Stored at room temperature

ii. 0.25M Sodium acetate (NaAc) buffer (pH 5.0).

Mix 83ml of 3M sodium acetate solution with 900ml of distilled water. Adjusted to pH5 using glacial acetic acid and the final volume made to 1 litre with distilled water. Stored at room temperature

b) Solutions for enzyme determination assays

a) β-naphthyl acetate: 56 mg β-naphthyl acetate was dissolved in 20 ml acetone followed by addition of 80 ml of 0.1M K_3PO_4 buffer and stored at 4°C.

b) α-naphthyl acetate: 56 mg α-naphthyl acetate was dissolved in 20 ml acetone followed by addition of 80 ml of 0.1M K_3PO_4 buffer and stored at 4°C.

c) Fast Blue solution: 150mg fast blue B salt was dissolved 15ml distilled water followed by the addition of 35ml of 5% sodium lauryl sulfate. This was stored at in the dark and used within 2 hours after preparation

d) TMBZ: 50mg 3,3',5,5'-Tetramethyl-Benzidine Dihydrochloride (TMBZ) was dissolved in 25ml methanol and 75ml 0.25M NaAc buffer added. This was at stored for a few days at 4°C.

e)cytochrome –C: 0.01g cytochrome–C was dissolved in 100ml 0.25M NaAc buffer and used immmediately

f) Bradford reagent (protein dye concentrate): 20 ml Protein dye concentrate was mixed with 80 ml distilled H_2O and stored indefinitely at 4°C in a light proof bottle

g) Reduced glutathione: 61 mg reduced glutathione was dissolved in 100 ml K_3PO_4 buffer and stored for 3-4 days at 4°C.

h) cDNB: 20 mg 1-chloro-2,4'-dinitrobenzene(cDNB) was dissolved in 10 ml acetone followed by the addition of 90 ml KPO_4 buffer . This was stored for 3-4 days at 4°C.

i) ATCH: 75 mg acetylthiocholine iodide (ATCH) was dissolved in10 ml acetone and made up to 1ml with 0.1M K_3PO_4 buffer. This was stored for 3-4 days at 4°C

j) DTNB: 13 mg dithio-bis-2-nitrobenzoic acid (DTNB) was dissolved in 100ml K_3PO_4 buffer and stored for 3-4 days at 4°C.

APPENDIX II

W.H.O Susceptibility Tests Results

i) 0.75% Permethrin

a) Adabraka village

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	1	2	0	3
15	3	2	2	4
20	5	2	4	6
30	6	7	9	8
40	9	12	11	12
50	10	12	11	12
60	10	12	11	13
80	10	12	13	16
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	6	7	7	12
Observed mortality (%)	24	28	28	48
Control mortality (%)	0	0	0	0
Corrected mortality (%)	24	28	28	48

b) Atabu Newtown

No. knocked down after exposure for minutes	**Replicates**			
	1	**2**	**3**	**4**
10	1	0	1	0
15	3	2	3	3
20	5	6	5	6
30	10	10	6	7
40	13	14	7	9
50	13	15	7	9
60	13	17	8	12
80	13	18	9	12
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	15	19	19	25
Observed mortality (%)	60	76	76	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	60	76	76	100

c) Kledzo village

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	1	1	1	1
15	3	2	7	4
20	6	3	11	15
30	11	11	16	19
40	15	14	16	20
50	15	14	16	20
60	17	16	18	21
80	19	18	19	21
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	21	18	18	25
Observed mortality (%)	84	72	72	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	84	72	72	100

d) Likpe village

No. knocked down after exposure for minutes	**Replicates**			
	1	**2**	**3**	**4**
10	0	0	0	1
15	4	4	2	5
20	7	5	4	8
30	11	12	9	13
40	13	14	10	13
50	13	15	12	13
60	14	15	15	14
80	16	20	19	15
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	16	11	15	15
Observed mortality (%)	64	44	60	60
Control mortality (%)	0	0	0	0
Corrected mortality (%)	64	44	60	60

e) Susceptible Kisumu strain

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	7	14	15	16
15	14	17	18	21
20	20	20	22	23
30	23	22	24	25
40	25	25	25	25
50	25	25	25	25
60	25	25	25	25
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	24	24	23	24
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	4	0	4
Corrected mortality (%)	100	100	100	100

ii) 0.05% Deltamethrin

a) Adabraka

No. knocked down after exposure for minutes	**Replicates**			
	1	**2**	**3**	**4**
10	5	4	0	6
15	6	7	3	7
20	11	13	11	9
30	15	21	20	16
40	20	23	22	19
50	22	22	24	23
60	25	25	25	23
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	24	24	25	24
Observed mortality (%)	96	96	100	96
Control mortality (%)	0	0	0	0
Corrected mortality (%)	96	96	100	96

b) Atabu Newtown

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	1	0	1	1
15	4	3	2	3
20	11	7	5	10
30	15	23	18	17
40	24	24	24	23
50	24	25	25	24
60	24	25	25	24
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	24	24	25	24
Observed mortality (%)	96	96	100	96
Control mortality (%)	0	0	0	0
Corrected mortality (%)	96	96	100	96

c) Kledzo

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	0	1	0
15	0	0	2	2
20	6	3	5	7
30	11	8	8	14
40	17	21	13	22
50	20	23	22	25
60	21	25	24	25
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	23	24	25	25
Observed mortality (%)	92	96	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	92	96	100	100

d) Likpe

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	3	0	4
15	4	7	3	8
20	8	13	6	12
30	15	20	22	20
40	22	20	22	24
50	24	21	22	24
60	24	23	24	24
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	25	21	23	22
Observed mortality (%)	100	84	92	88
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	84	92	88

e) Susceptible Kisumu strain

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	6	5	6	8
15	13	7	10	14
20	20	14	15	16
30	24	24	20	25
40	25	25	23	25
50	25	25	25	25
60	25	25	25	25
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	4	4	0	0
Corrected mortality (%)	100	100	100	100

iii) 4% DDT

a) Adabraka

No. knocked down after exposure for minutes	**Replicates**			
	1	**2**	**3**	**4**
10	0	0	0	0
15	0	0	0	0
20	0	0	0	0
30	0	0	0	0
40	0	0	0	0
50	2	0	1	0
60	2	0	1	0
80	2	0	1	0
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality at the end of holding period	6	4	2	4
Observed mortality (%)	24	16	8	16
Control mortality (%)	0	0	0	0
Corrected mortality (%)	24	16	8	16

b) Atabu Newtown

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	0	0	0
15	0	1	0	0
20	1	1	0	0
30	2	5	2	2
40	3	7	10	7
50	5	8	11	8
60	6	9	11	10
80	7	10	11	11
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality at the end of holding period	13	16	10	12
Observed mortality (%)	52	64	40	48
Control mortality (%)	0	0	0	0
Corrected mortality (%)	52	64	40	48

c) Kledzo

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	0	0	0
15	0	0	0	0
20	0	0	0	0
30	0	0	0	0
40	0	0	0	0
50	2	0	0	0
60	0	0	0	0
80	2	0	0	0
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality at the end of holding period	5	0	0	1
Observed mortality (%)	20	0	0	4
Control mortality (%)	0	0	0	0
Corrected mortality (%)	20	0	0	4

d) Likpe

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	0	0	0
15	0	0	0	0
20	0	0	0	1
30	0	0	0	3
40	1	0	0	5
50	2	2	1	6
60	2	2	1	6
80	4	5	2	9
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality at the end of holding period	11	9	12	14
Observed mortality (%)	44	36	48	56
Control mortality (%)	0	0	0	0
Corrected mortality (%)	44	36	48	56

e) Susceptible Kisumu strain

No. knocked down after exposure for minutes	Replicates			
	1	2	3	4
10	0	2	4	2
15	1	2	7	4
20	15	13	19	19
30	16	16	20	20
40	20	18	20	20
50	20	18	20	20
60	20	18	20	20
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality at the end of holding period	20	20	20	20
Observed mortality (%)	100	100	100	100
Control mortality (%)	4	0	0	4
Corrected mortality (%)	100	100	100	100

iv) 5% Malathion

a) Adabraka

	Replicates			
	1	**2**	**3**	**4**
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	23	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

b) Atabu Newtown

	Replicates			
	1	**2**	**3**	**4**
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	24	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

c) Kledzo

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	23	25	23
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

d) Likpe

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	24	24	25	24
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	4	0	0	0
Corrected mortality (%)	100	100	100	100

e) Susceptible Kisumu strain

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	25	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

v) 0.1% Propoxur

a) Adabraka

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	25	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

b) Atabu Newtown

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	23	21	24	23
Mortality a the end of holding period	24	23	23	24
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	4
Corrected mortality (%)	96	92	92	96

c) Kledzo

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	21	21	18	25
Mortality a the end of holding period	24	23	24	24
Observed mortality (%)	96	92	96	96
Control mortality (%)	0	0	0	0
Corrected mortality (%)	96	92	96	96

d) Likpe

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	25	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

e) Susceptible Kisumu strain

	Replicates			
	1	2	3	4
Total exposed	25	25	25	25
Total control	25	25	25	25
Mortality a the end of exposure period	25	25	25	25
Mortality a the end of holding period	25	25	25	25
Observed mortality (%)	100	100	100	100
Control mortality (%)	0	0	0	0
Corrected mortality (%)	100	100	100	100

APPENDIX III

Biochemical Assays Results

a) Glutathione S-Transferase (340nm)

Mosq. #	Adabraka		Atabu		Kledzo		Likpe		Kisumu	
	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity
1	0.076	0.000645	0.044	0.000345	0.176	0.000527	0.087	0.000261	0.174	0.000664
2	0.065	0.000513	0.088	0.000346	0.16	0.000589	0.077	0.000283	0.137	0.000515
3	0.035	0.000249	0.089	0.000297	0.161	0.000587	0.072	0.000262	0.141	0.000344
4	0.062	0.000497	0.061	0.000363	0.176	0.000571	0.057	0.000185	0.225	0.000691
5	0.057	0.000598	0.058	0.000222	0.14	0.000608	0.089	0.000387	0.174	0.00079
6	0.044	0.000259	0.147	0.000351	0.174	0.000612	0.092	0.000324	0.189	0.000539
7	0.059	0.000409	0.099	0.000282	0.187	0.000522	0.09	0.000251	0.169	0.00029
8	0.053	0.000367	0.083	0.000262	0.193	0.000619	0.132	0.000423	0.192	0.000467
9	0.072	0.000492	0.063	0.000436	0.168	0.000781	0.114	0.00053	0.146	0.000557
10	0.071	0.000446	0.062	0.000424	0.177	0.000491	0.111	0.000308	0.142	0.000484
11	0.051	0.000379	0.083	0.000264	0.213	0.000533	0.097	0.000243	0.143	0.000375
12	0.052	0.000348	0.07	0.00044	0.181	0.000621	0.061	0.000209	0.141	0.000653
13	0.061	0.000471	0.11	0.000413	0.174	0.000497	0.098	0.00028	0.149	0.00065
14	0.069	0.000416	0.061	0.00032	0.239	0.000525	0.111	0.000244	0.189	0.000523
15	0.074	0.000421	0.078	0.000341	0.139	0.000497	0.114	0.000407	0.164	0.000416
16	0.036	0.001018	0.073	0.000326	0.18	0.000616	0.09	0.000308	0.177	0.000645
17	0.052	0.000746	0.054	0.000482	0.161	0.000576	0.083	0.000297	0.151	0.000594
18	0.054	0.000401	0.066	0.000257	0.164	0.000626	0.101	0.000386	0.152	0.000453
19	0.043	0.000486	0.06	0.000297	0.15	0.000587	0.057	0.000223	0.164	0.000562
20	0.045	0.000417	0.082	0.000354	0.137	0.000779	0.077	0.000438	0.119	0.000611
21	0.045	0.00032	0.034	0.000225	0.138	0.00055	0.086	0.000343	0.137	0.000642
22	0.038	0.000461	0.064	0.000341	0.19	0.000514	0.072	0.000195	0.157	0.000579
23	0.041	0.000852	0.072	0.000323	0.14	0.000575	0.126	0.000518	0.172	0.000574
24	0.068	0.000597	0.081	0.000365	0.178	0.000538	0.086	0.00026	0.156	0.000351
25	0.051	0.008655	0.091	0.000377	0.161	0.000669	0.142	0.00059	0.155	0.000428
26	0.041	0.000745	0.062	0.000324	0.135	0.000511	0.081	0.000307	0.203	0.00073
27	0.056	0.000352	0.074	0.000326	0.224	0.000576	0.052	0.000134	0.176	0.000588
28	0.038	0.000516	0.066	0.000356	0.206	0.000639	0.082	0.000254	0.174	0.000435
29	0.052	0.000477	0.096	0.000379	0.178	0.000595	0.099	0.000331	0.154	0.001097
30	0.048	0.00047	0.051	0.000191	0.232	0.000561	0.077	0.000186	0.173	0.000545
Mean		**0.000767**		**0.000335**		**0.000583**		**0.000312**		**0.00056**

b) AcCHE (414nm)

Mosq. #	Adabraka		Atabu		Kledzo		Likpe		Kisumu	
	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity
1	0.362	5.03E-07	0.288	3.7E-07	0.756	3.71E-07	0.931	4.57E-07	0.709	4.43E-07
2	0.354	4.58E-07	0.324	2.09E-07	0.587	3.54E-07	0.737	4.44E-07	0.454	2.79E-07
3	0.303	3.53E-07	0.335	1.83E-07	0.583	3.48E-07	0.793	4.74E-07	0.459	1.83E-07
4	0.292	3.83E-07	0.251	2.45E-07	0.763	4.06E-07	0.712	3.79E-07	0.36	1.81E-07
5	0.191	3.28E-07	0.283	1.77E-07	0.531	3.78E-07	0.917	6.53E-07	0.598	4.45E-07
6	0.301	2.9E-07	0.429	1.68E-07	0.791	4.56E-07	1.054	6.08E-07	0.731	3.42E-07
7	0.405	4.6E-07	0.425	1.98E-07	0.599	3.2E-07	0.978	4.47E-07	0.636	1.79E-07
8	0.506	5.74E-07	0.335	1.73E-07	0.781	4.1E-07	0.611	3.21E-07	0.573	2.28E-07
9	0.504	5.64E-07	0.262	2.97E-07	0.671	5.11E-07	1.017	7.75E-07	0.495	3.09E-07
10	0.373	3.84E-07	0.261	2.92E-07	0.739	3.36E-07	0.995	4.52E-07	0.526	2.93E-07
11	0.377	4.59E-07	0.365	1.9E-07	0.861	3.53E-07	1.053	4.32E-07	0.615	2.64E-07
12	0.415	4.55E-07	0.232	2.39E-07	0.708	3.98E-07	0.976	5.48E-07	0.428	3.24E-07
13	0.398	5.03E-07	0.503	3.1E-07	0.485	2.27E-07	1.13	5.28E-07	0.543	3.88E-07
14	0.386	3.81E-07	0.222	1.91E-07	0.875	3.15E-07	0.986	3.55E-07	0.549	2.49E-07
15	0.4	3.73E-07	0.272	1.95E-07	0.653	3.82E-07	1.029	6.02E-07	0.442	1.84E-07
16	0.462	2.14E-06	0.302	2.21E-07	0.56	3.14E-07	0.666	3.73E-07	0.454	2.71E-07
17	0.489	1.15E-06	0.217	3.17E-07	0.747	4.38E-07	0.765	4.48E-07	0.553	3.56E-07
18	0.405	4.93E-07	0.282	1.8E-07	0.695	4.35E-07	1.016	6.35E-07	0.48	2.35E-07
19	0.464	8.6E-07	0.256	2.07E-07	0.565	3.62E-07	0.771	4.94E-07	0.489	2.74E-07
20	0.522	7.91E-07	0.251	1.77E-07	0.513	4.78E-07	0.909	8.47E-07	0.164	1.38E-07
21	0.51	5.95E-07	0.293	3.17E-07	0.566	3.7E-07	0.78	5.1E-07	0.5	3.84E-07
22	0.34	6.75E-07	0.255	2.23E-07	0.683	3.02E-07	0.956	4.23E-07	0.405	2.44E-07
23	0.335	1.14E-06	0.279	2.05E-07	0.599	4.03E-07	0.799	5.38E-07	0.503	2.75E-07
24	0.549	7.89E-07	0.289	2.13E-07	0.71	3.52E-07	0.979	4.85E-07	0.443	1.63E-07
25	0.487	1.35E-05	0.306	2.07E-07	0.764	5.2E-07	0.988	6.73E-07	0.542	2.45E-07
26	0.625	1.86E-06	0.194	1.66E-07	0.673	4.17E-07	0.795	4.93E-07	0.334	1.97E-07
27	0.622	6.4E-07	0.27	1.95E-07	0.41	1.73E-07	0.839	3.54E-07	0.528	2.89E-07
28	0.468	1.04E-06	0.22	1.94E-07	0.379	1.93E-07	0.913	4.64E-07	0.45	1.84E-07
29	0.525	7.89E-07	0.329	2.13E-07	0.386	2.11E-07	0.951	5.21E-07	0.542	6.32E-07
30	0.466	7.47E-07	0.21	1.29E-07	0.401	1.59E-07	0.567	2.25E-07	0.465	2.4E-07
Mean		**1.12E-06**		**2.14E-07**		**3.56E-07**	0.931	**4.98E-07**		**2.81E-07**

c) Oxidase (620nm)

Mosq. #	Adabraka		Atabu		Kledzo		Likpe		Kisumu	
	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity
1	0.202	0.303921	0.127667	0.145736	0.2937	0.171138	0.1723	0.262127	0.2087	0.142536
2	0.162	0.209284	0.193	0.132583	0.1603	0.096115	0.1697	0.174886	0.162	0.099622
3	0.131667	0.139112	0.327	0.216633	0.1573	0.092677	0.2317	0.500047	0.1517	0.058794
4	0.113	0.121208	0.107667	0.08171	0.256	0.157025	0.1483	0.089635	0.115	0.047835
5	0.101333	0.129397	0.170333	0.108617	0.1253	0.078132	0.217	0.099559	0.1747	0.133773
6	0.086667	0.053387	0.253	0.113696	0.2367	0.15447	0.268	0.366906	0.1903	0.094397
7	0.107333	0.095051	0.184	0.089966	0.1587	0.071884	0.2653	0.102512	0.204	0.062332
8	0.116333	0.10955	0.140333	0.067454	0.3077	0.193587	0.1917	0.257812	0.1917	0.081278
9	0.14	0.146225	0.111	0.101495	0.1423	0.101974	0.275	0.245136	0.1773	0.114685
10	0.156667	0.159343	0.106333	0.092185	0.2753	0.146554	0.2547	0.128144	0.1433	0.075482
11	0.113	0.11236	0.151333	0.076227	0.2473	0.115967	0.2997	0.224935	0.207	0.096973
12	0.099	0.07946	0.099667	0.092097	0.1927	0.115381	0.2097	0.205882	0.1117	0.06857
13	0.116333	0.121999	0.089667	0.037577	0.205	0.104236	0.1943	0.201436	0.164	0.117751
14	0.117	0.09669	0.096	0.058595	0.3593	0.159125	0.253	2.742844	0.205	0.101118
15	0.108333	0.079381	0.091667	0.044722	0.167	0.098883	0.2403	3.036048	0.1637	0.06833
16	0.074667	0.177616	0.104667	0.059205	0.2407	0.153293	0.1743	0.052018	0.1913	0.121505
17	0.104	0.186789	0.13	0.170345	0.2263	0.148315	0.161	0.375327	0.1797	0.120422
18	0.135	0.15039	0.184667	0.124308	0.2243	0.156549	0.2467	0.486622	0.1467	0.068486
19	0.099333	0.134199	0.153	0.12071	0.1443	0.087614	0.209	0.393074	0.185	0.109131
20	0.133	0.182998	0.131	0.083289	0.146	0.129656	0.209	3.930743	0.1047	0.067714
21	0.13	0.1358	0.164	0.178385	0.11	0.057512	0.2237	0.299349	0.1647	0.127184
22	0.106333	0.163519	0.165	0.145068	0.2677	0.13812	0.2207	0.090086	0.184	0.116568
23	0.101	0.256154	0.115	0.069899	0.1213	0.070081	0.2203	0.18055	0.215	0.129542
24	0.129	0.165367	0.159	0.116315	0.177	0.090733	0.2883	0.381934	0.1947	0.076862
25	0.144	3.789139	0.198333	0.144403	0.216	0.162392	0.1977	0.279812	0.2587	0.135336
26	0.094667	0.198761	0.147667	0.121447	0.16	0.098602	0.206	0.080642	0.1167	0.057429
27	0.127333	0.115487	0.188	0.143528	0.2403	0.115089	0.2717	0.242235	0.1947	0.113907
28	0.099	0.161038	0.117333	0.086459	0.1573	0.078844	0.2207	0.109356	0.1817	0.077739
29	0.142	0.200551	0.145	0.089037	0.233	0.143787	0.3477	0.438119	0.1623	0.189291
30	0.118667	0.161676	0.098	0.043533	0.3383	0.1633	0.1213	0.046664	0.2083	0.117531
Mean		**0.271195**		**0.100257**		**0.121701**		**0.534148**		**0.099737**

d) Protein (620nm)

Mosq. #	Adabraka		Atabu		Kledzo		Likpe		Kisumu	
	Absorb	Conc.	Absorb	Conc.	Absorb	Conc.	Absorb	Conc.	Absorb	Conc.
1	0.967667	0.201342	0.977667	0.218121	1.188	0.5705	0.9603	0.1884	1.115	0.448
2	0.977	0.216443	1.106667	0.434564	1.1247	0.4643	1.0128	0.2765	1.119	0.4547
3	0.990667	0.239933	1.145333	0.511745	1.1273	0.4686	0.935	0.146	1.2657	0.7008
4	0.974667	0.213087	1.019	0.286913	1.1617	0.5263	1.113	0.4446	1.1797	0.5565
5	0.945	0.162752	1.114333	0.446309	1.0823	0.3931	1.25	0.6745	1.0723	0.3763
6	1.021667	0.290268	1.275333	0.716443	1.1373	0.4854	0.99	0.2383	1.205	0.599
7	0.995	0.246644	1.206333	0.600671	1.2127	0.6119	1.35	0.8423	1.4407	0.9945
8	0.955	0.246644	1.171333	0.541946	1.1657	0.5331	0.98	0.2215	1.2667	0.7025
9	0.997	0.25	0.995333	0.246644	1.067	0.3674	1.0673	0.368	1.115	0.448
10	1.010333	0.271812	0.997	0.25	1.2153	0.6163	1.23	0.6409	1.147	0.5017
11	0.985333	0.229866	1.168	0.536913	1.255	0.6829	1.113	0.4446	1.2363	0.6515
12	1.002333	0.255034	1.010333	0.271812	1.145	0.4983	1.034	0.3121	1.068	0.3691
13	0.98	0.221477	1.119667	0.454698	1.2047	0.5985	1.02	0.2886	1.0813	0.3914
14	1.017	0.283557	1.042	0.325503	1.3113	0.7773	0.8657	0.0297	1.2157	0.6169
15	1.026667	0.300336	1.081	0.39094	1.133	0.4782	0.863	0.0252	1.249	0.6728
16	0.884	0.060403	1.076333	0.38255	1.1457	0.4995	1.423	0.9648	1.1273	0.4686
17	0.918667	0.119128	0.962	0.191275	1.1327	0.4777	0.9193	0.1196	1.107	0.4346
18	0.984667	0.229866	1.109	0.437919	1.1147	0.4475	0.9447	0.1622	1.1893	0.5727
19	0.938	0.151007	1.053667	0.345638	1.1083	0.4367	0.945	0.1628	1.1453	0.4988
20	0.958	0.184564	1.084333	0.395973	1.027	0.3003	0.8577	0.0163	1.0463	0.3327
21	0.991	0.239933	1.002333	0.258389	1.1033	0.4284	0.987	0.2332	1.0653	0.3646
22	0.932	0.14094	1.038667	0.32047	1.2247	0.632	1.302	0.7617	1.1243	0.4636
23	0.897	0.082215	1.075	0.380872	1.0957	0.4156	1.074	0.3792	1.1533	0.5122
24	0.964333	0.194631	1.074333	0.379195	1.1847	0.5649	0.997	0.25	1.3	0.7584
25	0.854	0.010067	1.093667	0.412752	1.093	0.4111	0.9747	0.2126	1.2167	0.6186
26	0.903667	0.09396	1.043	0.327181	1.117	0.4513	1.312	0.7785	1.1313	0.4753
27	1.006333	0.271812	1.079333	0.387584	1.2437	0.6639	1.0667	0.3669	1.153	0.5117
28	0.923	0.125839	1.036667	0.317114	1.1763	0.5508	1.222	0.6275	1.2553	0.6834
29	0.959	0.186242	1.106	0.432886	1.1527	0.5112	1.01	0.2718	0.991	0.2399
30	0.951667	0.174497	1.120333	0.456376	1.269	0.7064	1.22	0.6242	1.171	0.5419
Mean		**0.177768**		**0.370302**		**0.518988**		**0.369083**		**0.532036**

e) α-esterase (540nm)

Mosq. #	Protein		Adabraka		Kledzo		Likpe		Kisumu	
	Absorb	Conc.	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity
1	1.110667	0.440716	2.499	0.15749	1.449667	0.028787	1.727333	0.122532	1.75433	0.035845
2	1.150333	0.50727	2.509	0.14749	1.332	0.018022	1.791	0.069108	1.831	0.034372
3	1.181667	0.559844	1.706	0.061538	1.796	0.065435	2.238333	0.14289	1.717667	0.026819
4	1.170667	0.541388	1.801	0.078817	1.192	0.012385	1.597	0.073384	1.491667	0.018813
5	1.129	0.471477	2.24	0.160829	1.237667	0.006666	1.464667	0.022741	1.645667	0.028582
6	1.083333	0.394854	2.402	0.102101	1.212667	0.012181	1.880333	0.075971	1.191	0.009524
7	1.11033	0.440151	1.929	0.079183	1.275333	0.008404	1.213333	0.034075	1.331	0.015341
8	1.193	0.578859	0.376	-0.05536	0.727333	0.042648	1.935	0.216967	1.351667	0.012427
9	1.064	0.362416	2.204	0.101624	1.056	0.003071	2.111667	0.078047	1.366	0.020694
10	1.218667	0.621924	2.647	0.128294	1.038	0.001927	1.518333	0.228776	1.88833	0.030005
11	1.219333	0.623042	2.319	0.121215	1.954667	0.055679	1.996	0.092541	1.797667	0.026842
12	1.08233	0.393171	1.975	0.080432	1.33	0.016785	2.253	0.114163	1.369667	0.019275
13	1.079	0.387584	2.11	0.105643	1.790667	0.043344	1.651333	0.055099	1.19333	0.009831
14	1.018	0.285235	2.254	0.093365	1.265	0.014538	1.98	0.087158	1.179	0.012286
15	1.09	0.40604	1.79	0.055138	1.239667	0.013086	1.403333	0.058132	1.179	0.00863
16	1.167667	0.536354	0.346	-0.23666	1.106333	0.016322	1.717667	0.075233	1.232667	0.008672
17	1.162667	0.527965	2.595	0.2834	1.685333	0.04122	1.949667	0.100801	1.573	0.022583
18	1.129667	0.472596	2.182	0.10848	1.387333	0.022667	1.660333	0.019327	1.25667	0.010927
19	1.137667	0.486018	2.086	0.151547	1.739667	0.031468	2.255	0.086292	1.398667	0.016868
20	1.041	0.323826	2.055	0.120404	1.355	0.005645	2.799	0.123475	1.242	0.014979
Mean		**0.468036**		**0.088296**		**0.023014**		**0.093836**		**0.019166**

f) β-esterase (540nm)

Mosq. #	Protein		Adabraka		Kledzo		Likpe		Kisumu	
	Absorb	Conc.	Absorb	Activity	Absorb	Activity	Absorb	Activity	Absorb	Activity
1	1.110667	0.440716	3.716	0.292221	2.297	0.10255	2.408333	0.282331	2.564667	0.086003
2	1.150333	0.50727	3.65	0.266289	2.169333	0.081708	2.997667	0.190811	2.774333	0.082235
3	1.181667	0.559844	3.542	0.232035	3.218	0.195197	4.25	0.374799	2.73667	0.073289
4	1.170667	0.541388	2.771	0.195481	1.882	0.083476	2.706	0.238829	2.549333	0.069496
5	1.129	0.471477	2.477	0.223095	1.833667	0.034442	2.359667	0.0808	2.466333	0.0766
6	1.083333	0.394854	3.278	0.175261	1.839	0.071143	3.215333	0.204396	2.059667	0.072739
7	1.11033	0.440151	3.76	0.241791	2.178	0.046701	1.983333	0.220095	2.345667	0.077067
8	1.193	0.578859	3.038	0.188567	1.069	-0.07422	3.369	0.579745	2.308667	0.057438
9	1.064	0.362416	3.761	0.238618	1.752	0.081081	3.078667	0.157339	2.169333	0.084751
10	1.218667	0.621924	3.575	0.207028	1.549	0.076211	2.456667	0.765125	2.812	0.068175
11	1.219333	0.623042	4.036	0.281271	3.189667	0.136538	3.280333	0.224754	2.937667	0.07172
12	1.08233	0.393171	3.871	0.241751	2.414	0.087688	3.952	0.222626	2.24733	0.081728
13	1.079	0.387584	3.009	0.207615	3.028	0.121101	2.565667	0.153773	2.147	0.0782
14	1.018	0.285235	4.038	0.228141	2.113667	0.080852	3.361667	0.221492	1.83866	0.086605
15	1.09	0.40604	3.506	0.183189	2.14	0.082515	2.293333	0.231133	1.96966	0.066705
16	1.167667	0.536354	2.47	0.59901	1.814	0.203602	2.721	0.20407	2.11667	0.055481
17	1.162667	0.527965	2.382	0.290292	2.947667	0.129199	2.903333	0.222513	2.16033	0.057866
18	1.129667	0.472596	3.577	0.244965	2.325	0.095661	2.504	0.051607	2.396333	0.073726
19	1.137667	0.486018	3.243	0.332676	3.060333	0.095419	3.623667	0.186172	1.174	0.025962
20	1.041	0.323826	2.809	0.229435	2.244333	0.069764	4.377333	0.229507	2.2176	0.097561
Mean		**0.468036**		**0.283835**		**0.090032**		**0.252096**		**0.072167**