

Malaria Vector Status and Insecticide Susceptibility Studies in Zimbabwe

By

Givemore Munhenga

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Department of Biological Sciences

Faculty of Science

University of Zimbabwe

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Abstract

The major problem that has evolved from malaria control programmes that utilize insecticides for vector control is the development of insecticide resistance. In Zimbabwe, despite decades of pesticide usage there is limited information on susceptibility status of major malaria vectors and possible mechanisms responsible for resistance. Susceptibility status of vectors provides useful information for making rational decisions on control strategies.

The malaria vector status and their susceptibility to insecticides were studied in Gokwe, Gwave village in the Midlands province of Zimbabwe. Standard WHO bioassays, using 0.75% permethrin, 4% DDT, 5% malathion, 0.1% bendiocarb and 4% dieldrin were done on wild-collected adult anopheline mosquitoes. Susceptibility tests were done on the F₁ generation of *Anopheles arabiensis* reared from wild-caught females using 0.75% permethrin and 4% DDT. Molecular and biochemical assays were carried out to identify *kdr* mutations in individual mosquitoes and to determine expression levels of non-specific esterases, monooxygenases, glutathione-S-transferases and altered acetylcholinesterase (AChE).

A total of 648 anophelines were collected, with the majority (98%) being members of the *An. gambiae* complex. Species in the *An. gambiae* complex were identified by polymerase chain reaction (PCR) and *An. arabiensis* (72.8%) predominated all the other sibling species. Among the *An. arabiensis* females, 0.5% was positive for *Plasmodium falciparum*. WHO diagnostic tests showed that 53% of the *An. arabiensis* were resistant to permethrin and 32% to DDT. Insecticide susceptibility tests on F₁ *An. arabiensis* families showed an average mortality of 85.9% (n=567) after exposure to 4% DDT and 69.8% (n=372) after exposure to 0.75% permethrin. Six families showed cross resistance to both DDT and permethrin. Biochemical assays of F₁ *An. arabiensis* families revealed comparatively high levels of monooxygenase (48%, n = 33, p<0.05), glutathione S-transferase (26%, n = 31, p<0.05) and general esterases activity compared to the reference colony. Insensitive acetylcholinesterase was detected in 23.5% (n = 33). The *kdr* analysis by PCR revealed presence of both East and West Africa mutation, but was not confirmed by sequencing.

The significant elevation of various enzyme systems in the F₁ progeny and detection of families showing cross resistance to permethrin and DDT is suggestive of existence of multiple resistances in *An. arabiensis* population from Gwave. This has serious implication on malaria control. Continued use of these insecticides is likely to further select resistant vectors. Use of mosaic insecticides or rotational use of insecticides is recommended.

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LIST OF ABBREVIATIONS

Bp	base pair
°C	degrees Celsius
DDT	diethyl diphenyl trichloroethane
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleic acid
<i>et al.</i>	and others
hr	hour(s)
kb	kilo base
M	Molar
mg	Milligram
MgCl ₂	magnesium chloride
min	minute(s)
ml	Millilitre
mm	Millimetre
mM	Millimole
ng	Nanogram
nm	Nanomole
%	Percent
μl	Microlitres
OD	optical density
P	probability level
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
rpm	revolutions per minute
s	second(s)
WHO	World Health Organization
<i>s.l</i>	sensu lato
<i>s.s</i>	sensu stricto
NaOH	sodium hydroxide
HIV	Human Immune Virus

AIDS	Acquired Immune Deficiency Syndrome
RBM	Roll Back Malaria Programme
BHC	benzene hexachloride
GMC	Global Malaria Control Strategy
GST	glutathione S-transferase
AChE	Acetylcholinesterase
<i>Pf</i>	<i>Plasmodium falciparum</i>
PBS	phosphate buffered solution
KPL	Kirkegaard and Perry Laboratories
<i>Taq</i>	<i>Thermus aquaticas</i>
NA	naphthyl acetate
ELISA	Enzyme Linked Immunosorbent Assay
CDNB	1-chloro-2,4-dinitrobenzene
LC	larval collections
MBN	man-baited net
DHC	direct hand catches
NICD	National Institute for Communicable Diseases
NIHR	National Institute for Health Research
NGO	non-governmental organization
NMCP	National Malaria Control Programme
<i>kdr</i>	knockdown resistance
USA	United States of America
MTE	Masters in Tropical Entomology
PBO	piperonyl butoxide
DEM	diethyl maleate
CYP	Cytochrome P450
Tw	Tween
CS	Circumsporozoite
MO	Monooxygenases
ASCHI	Acetylthiocholine iodide

Chapter 1

Introduction

1.1 General overview

Malaria is the world's most important tropical parasitic disease killing more people than any other communicable disease except tuberculosis (Lambert, 2006). According to the World Health Organization (WHO) between 300 and 500 million people are infected each year, with 90% of these cases being recorded in sub-Saharan Africa. Deaths due to malaria are estimated to be 1.5-2.7 million each year with the majority of them occurring among children and pregnant women (WHO, 1978; 1985a). The effects of malaria extend far beyond direct measures of mortality and morbidity; it can reduce attendance at school and productivity at work and evidence suggests that the disease can impair intellectual development (Fernando *et al.*, 2003). Cerebral malaria can result in persisting developmental abnormalities (Carter *et al.*, 2005). The economic impact of malaria is immense and includes costs to health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism (WHO, 2004). It has been postulated that in the hardest hit countries, the disease may account for as much as 40% of public expenditure, 30-50% of in-patient admissions, and up to 50% of out-patients visits. Economists believe that malaria is responsible for a growth penalty of up to 1.3% per year in some African countries (Carter *et al.*, 2005).

On a global scale the geographical area affected by malaria is vast, spanning Central and South America, the Indian subcontinent, Southeast Asia, the Middle East, and Oceania. In developing countries, Africa in general and sub-Saharan countries in particular are the worst affected (WHO, 1986).

Malaria in humans is caused by four species of protozoan parasites belonging to the genus *Plasmodium*: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. *Plasmodium falciparum* is by far the most pernicious and causes most malaria-specific morbidity and

mortality (Collins and Paskewitz, 1995). The parasite, with the exception of a few cases of transplacental and blood transfusion associated transmissions, is exclusively transmitted to humans by infected female anopheline mosquitoes. There are almost 500 described anopheline mosquitoes but only 20% of these have been implicated as vectors of which most are of minor or incidental importance (Collins and Paskewitz, 1995). In sub-Saharan Africa where the majority of transmissions occur, three anopheline species, *Anopheles gambiae* Giles, *An. arabiensis* Patton and *An. funestus* Giles are the most important vectors (Gillies and Coetzee, 1987).

1.2 Malaria in Zimbabwe

In Zimbabwe malaria remains one of the most important infectious diseases affecting mainly the rural population with about 5.5 million people being at risk. In a medical surveillance conducted between 1992 and 2002, average clinical cases were recorded in 12% of the population (Figure 1.1), with a case fatality of 0.32% (Ministry of Health and Child Welfare, National Malaria survey, 2002). In 2002, 740 000 clinical malaria cases and 2 200 malaria deaths were reported. In the same period, 12% of outpatient attendances, 15% of inpatient admissions in hospitals, and 1% of all deaths were attributed to malaria (Midzi *et al.*, 2004)). The greatest burden of malaria occurs in children under five years, pregnant women and people living with HIV/AIDS.

Twenty-four of the country's 62 districts and representing three fifths of the country are malaria-endemic areas (Figure 1.2). However endemicity is altitude-dependent with very limited transmissions in high plateau regions. Areas of the country below 900 m are highly endemic for malaria, while those between 900-1200 m have low endemicity (Taylor and Mutambu, 1986; Govere, 2003). There are 43 documented anopheline species in Zimbabwe (Table 1.1) (Masendu *et al.*, 2005). Of these, *An. arabiensis* and *An. gambiae* often occur in sympatry and are the main malaria vectors with *An. arabiensis* being responsible for most of the transmissions (Mpofu, 1985; Masendu *et al.*, 2005). Other vectors such as *An. funestus* and *An. merus* Donitz occur sporadically and cause malaria in isolated incidences (Masendu *et al.*, 2005).

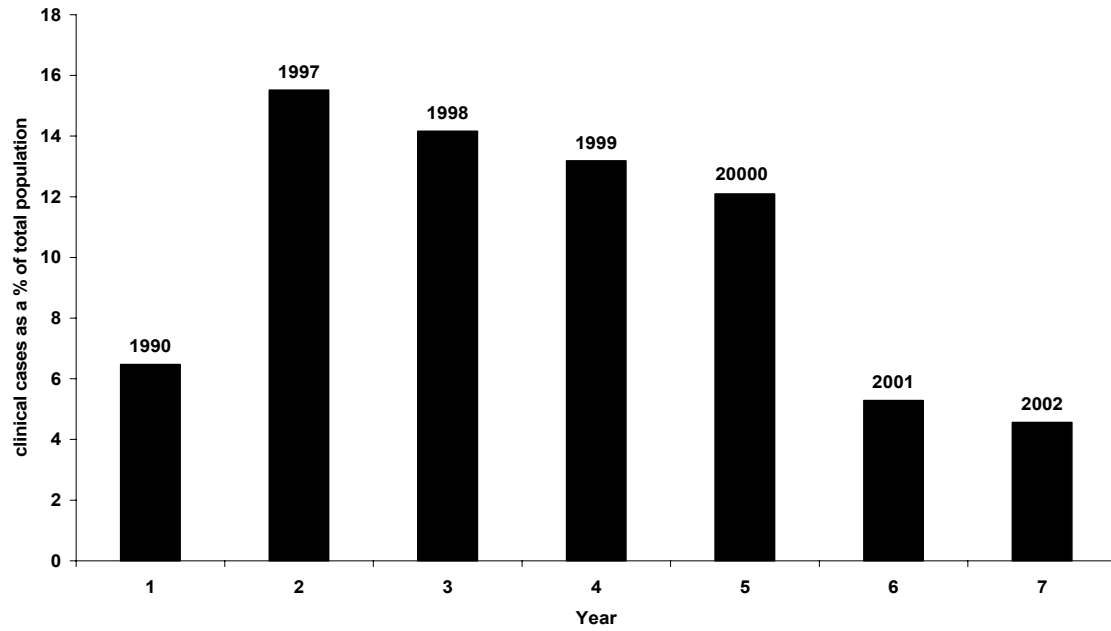


Figure 1.1: Malaria clinical cases in Zimbabwe (1990, 1997-2002)

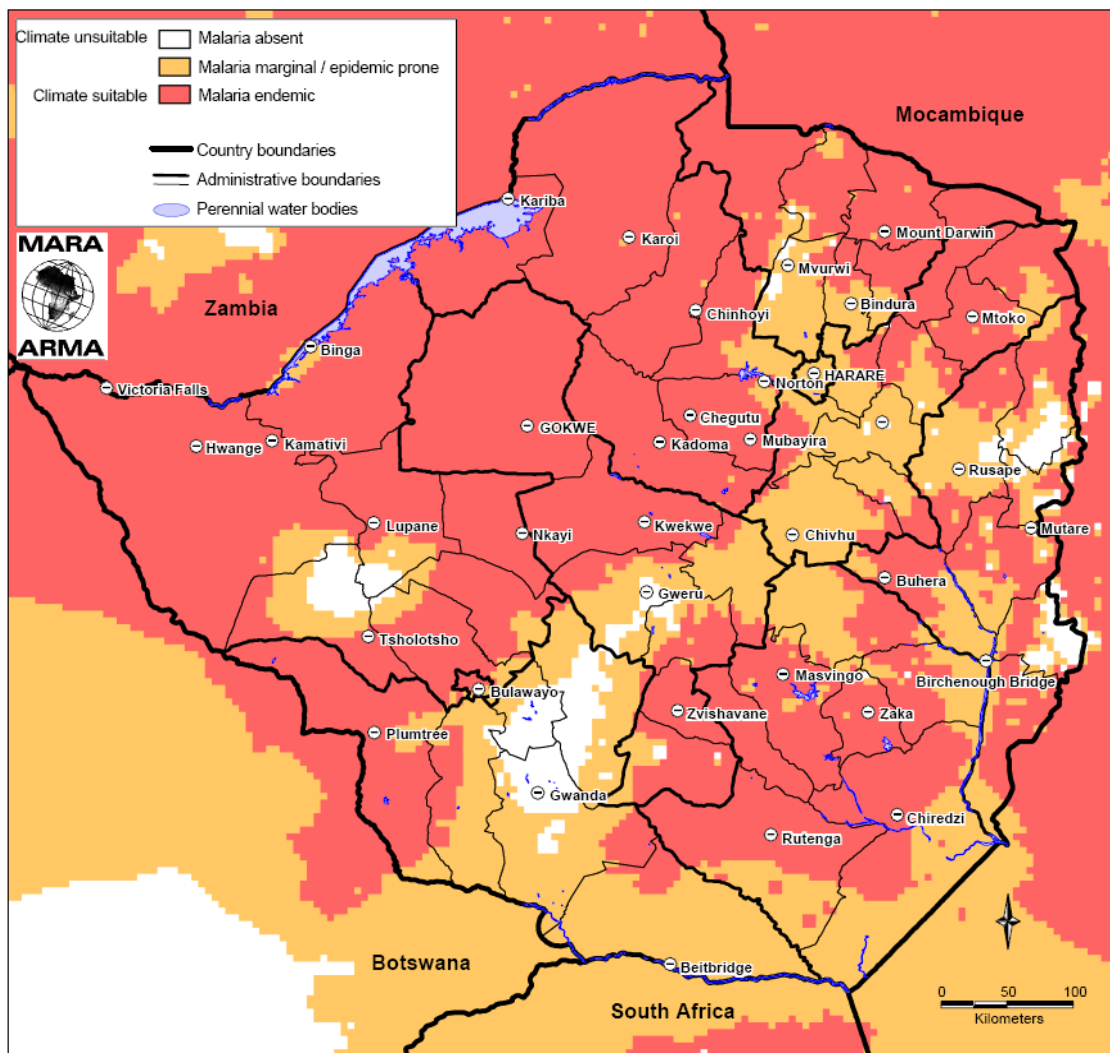


Figure 1.2: Distribution of malaria in Zimbabwe (adapted from MARA,2001)

The predominant parasite species is *P. falciparum* which accounts for over 97% of malaria cases while *P. malariae* accounts for about 2% and *P. ovale* for less than 1% (Crees and Mhlanga, 1985; Taylor and Mutambu, 1986). There are no recorded cases of malaria being caused by *P. vivax* in Zimbabwe.

The incidence of malaria in Zimbabwe is seasonal, occurring between December and April, with transmission ceasing in the cold dry months of May and June. However, in the Lowveld and the Zambezi valley, malaria transmission occurs throughout the year (Taylor and Mutambu, 1986). This seasonality of malaria transmission is mainly associated with the effect of temperature and rainfall on the survival and breeding of mosquitoes (Mpofu, 1985).

Table 1.1: Distribution of malaria vectors and other anophelines in Zimbabwe (adapted from Masendu *et al.*, 2005)

Province	District	Malaria vector	Other species
Matabeleland North	Binga Hwange Lupane Nkayi	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. rhodisiensis</i> <i>An. fuscivenosus</i>
Midlands	Gokwe North Gokwe South Kwekwe Mberengwa	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. squamosis</i> <i>An. coustani</i> , <i>An. rufipes</i>
Mashonaland West	Hurungwe Kadoma Kariba Makonde	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. marshallii</i> <i>An. pharoensis</i> , <i>An. listeri</i>
Mashonaland Central	Bindura Guruve Mazowe Muzarabani Rushinga, Shamva	<i>An. arabiensis</i> <i>An. merus</i> <i>An. gambiae</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. coustani</i>
Mashonaland East	Hwedza Mudzi UMP	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. coustani</i> , <i>An. vaneedeni</i> , <i>An. nili</i>
Manicaland	Chimanimani Chipinge Mutare Mutasa	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i> <i>An. marshallii</i>
Masvingo	Chiredzi Mwenezi	<i>An. arabiensis</i> <i>An. merus</i> <i>An. funestus</i>	<i>An. quadriannulatus</i> sp. <i>A</i> <i>An. pretoriensis</i>
Matabeleland South	Beitbridge Bulilamangwe Gwanda Insiza	<i>An. arabiensis</i> <i>An. merus</i>	<i>An. quadriannulatus</i> <i>An. pretoriensis</i> <i>An. listeri</i>

During October, rains begin as do isolated cases of malaria because suitable breeding sites are few and isolated. When rainfall reaches its peak in January the number of cases of malaria rises tremendously because of the prevalence of mosquito breeding sites and high humidity which favours mosquito survival (Taylor and Mutambu, 1986). However, high rainfall may cause flooding and wash away larvae. The malaria transmission peak is reached in March. From April, the incidence of malaria begins to decline as low temperatures that do not favour parasite development or mosquito habitat set in. The cold season reaches its peak in July and only isolated cases of malaria can be found as both temperature and dry conditions restrict mosquito biting activities and breeding success.

1.3 Malaria Control in Zimbabwe

The prevention of malaria transmission in Zimbabwe mainly relies on accurate diagnosis, prompt effective treatment of infection with a combination of chloroquine and sulphadoxine pyrimethamine for uncomplicated malaria, sulphadoxine-pyrimethamine alone for the prevention of malaria in pregnancy, quinine as the second line (treatment failure) drug recommended for severe malaria and by reduction of human-vector contact using residual chemical compounds (Ministry of Health and Child Welfare, 2006).

Intervention measures to restrict the transmission of malaria by controlling the vector population forms the main part of vector control in Zimbabwe. Of the vector control approaches available, indoor residual spraying forms the cornerstone due to its effectiveness in obtaining a rapid large scale impact at an affordable cost. It involves spraying of all the stable surfaces inside human habitations using an insecticide with residual action. Indoor residual spraying was initiated in 1949 with the use of benzene hexachloride (BHC). Use of this chemical was discontinued in 1974 after a malaria epidemic in the south eastern Lowveld pointed to resistance to BHC by *An. arabiensis* (Coetzee, 1987). This resulted in a shift in policy and indoor residual spraying with DDT was introduced with great success. Control operations were suspended during the war in 1976 but resumed in 1980. In order to manage bed bug resistance, DDT and deltamethrin were used interchangeably between 1987 and 1991. Following the world wide trend of shifting from persistent organochlorides to

synthetic pyrethroids, the use of DDT was discontinued. Currently, vector control is achieved by annual spraying of houses with synthetic pyrethroids including deltamethrin (15 mg/m²), lambda-cyhalothrin (25 mg/m²) and alphacypermethrin (30 mg/m²) in tobacco growing areas and a rotation of pyrethroids and DDT in non-tobacco growing areas (Ministry of Health and Child Welfare, unpublished data). The major problem that has evolved from residual house spraying in most countries is the development of insecticide resistance to DDT (WHO, 1986).

1.4 Justification of the Study

In the malarious areas of Zimbabwe, vector control is achieved mainly by indoor residual spraying with either DDT or pyrethroids (including deltamethrin, lambda-cyhalothrin and alphacypermethrin). In isolated incidences, vector control is based on the use of insecticide treated bed nets distributed by non-governmental organizations (NGOs) and private initiatives (Lukwa, personal communication). Owing to the health and financial implications of such control strategies, it is crucial that insecticides are used effectively against target species. However, the emergence of resistance to insecticides presents a threat to vector control programmes.

In order to design an appropriate and cost-effective control strategy it is imperative to have a comprehensive knowledge of the malaria vector species composition in the area of interest (WHO, 1995). In addition, the susceptibility of target mosquitoes to insecticides identified for use in control programmes needs to be established.

In Gokwe, despite decades of pesticide use in both agriculture and health, there is no conclusive data on the resistance status of vectors against insecticides being used for malaria control. Susceptibility tests have been conducted biannually by the National Institute of Health Research (NIHR) and more recently by Masendu and colleagues during a national anopheline survey between 1999 and 2002 (Masendu *et al.*, 2005). These studies gave conflicting results. Manokore *et al.* (2000) showed data indicating that malaria vectors were still susceptible to DDT and pyrethroids (deltamethrin, alphacypermethrin and lambda-cyhalothrin). On the other hand Masendu *et al.* (2005) discovered a population of *An.*

arabiensis showing evidence of resistance to DDT. Since then, no follow up studies have been completed. This study intends to cover this research gap.

The continued use of insecticides in public health and agriculture has to respond to problems associated with insecticide resistance. The phenomenon of cross resistance, where one insecticide selects for resistance not only to itself but other compounds to which insects may not have been exposed is well documented (Hemingway *et al.*, 1987). The identification of resistance mechanisms is necessary in order to predict the cross resistance spectrum, facilitate the choice of alternative insecticides and allow detailed mapping of areas with resistant populations (Brogdon and McAllister, 1998b). The aim of this study was to understand those mechanisms conferring resistance to insecticides being used by the NMCP. This information will be used in order to make rational decisions about insecticides to be used in control programmes in Zimbabwe.

1.5 Objectives

The aim of the project was to determine the malaria vector status and their susceptibility to insecticides.

The six main objectives of this study were:

- 1) To identify malaria vectors in Gwave, an endemic area in Zimbabwe.
- 2) To determine mosquito susceptibility levels to different classes of insecticides.
- 3) To determine mechanisms that confer insecticide resistance using biochemical assays.
- 4) To determine *P. falciparum* infectivity rate of the malaria vector species.
- 5) To assay for the presence of the *kdr* sodium channel gene mutation using molecular techniques.
- 6) To characterize carbamate and pyrethroid resistance in artificially resistant selected colonies.

Chapter 2

Literature Review

2.1 Malaria Vectors

Some anopheline mosquito species (Order: Diptera, Family: Culicidae, Sub-family: Anophelinae) are vectors of human malaria. The genus *Anopheles* has more than 400 known taxonomic species but only about 70 of these have been incriminated as vectors of malaria (Service, 1993a). In sub-Saharan Africa about 140 anophelines are known to be present with less than 20 of these capable of transmitting malaria (Hervy *et al.*, 1998). Five species, namely *An. gambiae* Giles, *An. arabiensis* Patton, *An. funestus* Giles, *An. moucheti* Evans and *An. nili* Theobald are considered to be major malaria vectors and are responsible for more than 95% of the total transmissions on the continent (Gillies and DeMillion, 1968; Mouchet *et al.*, 2004). The remaining 5% is transmitted by “secondary” vectors or vectors of local importance.

Vectors of human malaria often occur as sibling species (i.e., closely related species that are nearly indistinguishable morphologically) in a species complex. Members of the *An. quadrimaculatus* and *An. albimanus* complexes are vectors of malaria in North and South America, respectively. In Asia, members of the *An. dirus*, *An. culicifacies*, *An. maculatus* and *An. minimus* complexes are responsible for malaria transmission. In the African region south of the Sahara, the principal vectors of malaria are members of the *An. gambiae* complex, the *An. funestus* group, the *An. nili* complex and the *An. moucheti* group (Gillies and DeMellion, 1968; Gillies and Coetzee, 1987).

2.1.1 The *Anopheles gambiae* complex

The *An. gambiae* complex is comprised of seven morphologically identical sibling species, which can only be distinguished by means of chromosome banding patterns, allozyme analysis, chromatography of hydrocarbons, hybridization with DNA probes (Gillies and DeMillion, 1968; Gillies and Coetzee, 1987; Hunt *et al.*, 1998) and species specific

Polymerase Chain Reaction (PCR). Currently the PCR assay is the most convenient and rapid technique available. This assay was developed by Scott *et al.* (1993) and replaced the older methods mentioned above. Members of this complex include the major malaria vectors, *An. gambiae sensu stricto* (s.s) and *An. arabiensis*. They are two freshwater forms widely distributed throughout tropical Africa (Gillies and Coetzee, 1987). These two species sometimes occur in sympatry as they both have similar larval environmental requirements (Service, 1970; Service *et al.*, 1978; Gimnig *et al.*, 2001). Populations of *An. arabiensis* survive better in dry climatic conditions as compared to *An. gambiae* s.s whose preponderance in most areas peaks shortly after the onset of rains (White, 1972; Lemasson *et al.*, 1997).

The third freshwater form, the non-vector *An. quadriannulatus* Theobald, has a very disjunct distribution and tolerates relatively cooler conditions in comparison with the other two freshwater species (Gillies and Coetzee, 1987). It was only recently realized that this species should be regarded as comprising two distinct species: *An. quadriannulatus* species A, occurring in South Africa, Zimbabwe and Mozambique, and *An. quadriannulatus* species B, found in Ethiopia (Hunt *et al.*, 1998). *Anopheles melas* Theobald and *An. merus* Donitz, two saltwater breeding forms, are also members of this cryptic species complex and are minor malaria vectors in localized areas mainly along the West and East African coasts as well as in isolated inland areas where they are found in association with salt water pans (Gillies and Coetzee, 1987). *Anopheles bwambae* White, an extremely localized vector found breeding only in pools around the mineral springs of the Semiliki Forest in Uganda, completes the list of this important species complex (Gillies and Coetzee, 1987).

Members of the *An. gambiae* complex vary in their behavior, seasonal prevalence, and levels of vectorial capacity. *Anopheles gambiae* s.s and *An. arabiensis* have the widest distribution and occur in sympatry over extensive areas (Coluzzi, 1984). *Anopheles gambiae* s.s is highly anthropophilic whereas *An. arabiensis* is more opportunistic, feeding on either humans or animals, depending on the availability of hosts (Gillies and Coetzee, 1987). In Zimbabwe, members of the *An. gambiae* complex are almost always found in various combinations of sympatry (Masendu *et al.*, 2005).

2.1.2 The *Anopheles funestus* group

This group is comprised of ten species, nine of which are found in the Afrotropical region whilst the tenth species, *An. fluviatilis* James, was recorded in the Arabian Peninsula (Gillies and DeMellion, 1968; Gillies and Coetzee, 1987). This group cannot strictly be described as a cryptic complex because most members can be distinguished using morphological characters at certain stages of their development. Four members of the group, *An. funestus*, *An. aruni* Sobti, *An. parensis* Gillies and *An. vaneedeni* Gillies and Coetzee have almost identical morphology at all life stages (Gillies and DeMillion, 1968; Gillies and Coetzee, 1987; Constantini *et al.*, 1999). *Anopheles funestus* has a similar distribution over Africa as to that of *An. gambiae s.l.* Members of this group prefer to breed in large permanent water bodies. As a consequence adult densities of the species are less affected by rainfall compared to *An. gambiae s.l.* *Anopheles funestus* is highly anthropophilic and endophilic making it susceptible to control by indoor house spraying (Gillies and Coetzee, 1987).

2.1.3 *Anopheles nili* and *An. moucheti*

Anopheles nili has a wide distribution across most of West and Central Africa and also occurs rarely in Southern Africa (Gillies and Coetzee, 1987). It represents a group consisting of at least 3 species: *An. nili* s.s, *An. somalicus* and *An. carnevalei* (Awono-Ambene *et al.*, 2004). The *An. moucheti* group is very efficient forest vectors and consists of at least three taxa: *An. moucheti moucheti*, *An. moucheti nigeriensis* and *An. bervoetsi* (Fontenille and Carnevale, 2006).

2.2 Malaria Control

The goal of malaria control in endemic countries is to reduce as much as possible the health impact of malaria on a population using resources available, and taking into account other health priorities (WHO, 2001). The World Health Organization Global Malaria Programme recommends two primary interventions: diagnosis of malaria cases and treatment with effective medicines and vector control.

2.2.1 Malaria vector control

Vector control generally remains the most effective method to prevent malaria transmission and is therefore one of the four basic technical elements of the Global Control Strategy (GMC) (WHO, 2004). Vector control is any intervention designed to restrict the transmission of malaria by controlling vector populations.

The control of malaria vectors dates back to the 18th century when drainage of swamps for agricultural purposes resulted in a marked decrease in malaria cases in Northern Europe (Collins and Paskewitz, 1995). This environmental management approach mainly targeted larvae rather than adults. With the discovery of conventional insecticides such as pyrethrum (Persian insect powder) and Paris green, control of larvae shifted from environmental management to application of these chemicals onto water surfaces where mosquitoes bred. In the early 1930s discovery of the knockdown effect of pyrethrum extract on houseflies introduced a new concept of residual indoor spraying (Ross, 1936 as cited by Coetzee and Hunt, 1998). However, it was the discovery of the utility of DDT as an insecticide in 1942 by Paul Mueller that revolutionized control of insect vectors, especially mosquitoes, through residual indoor spraying (Collins and Paskewitz, 1995). Large scale spraying with DDT was implemented in the late 1940s in many countries. Success in countries such as Brazil in eradicating *An. gambiae* prompted the Eighth World Health Assembly to launch a malaria eradication campaign in 1955. The objectives of the eradication programme were to interrupt malaria transmission and to eliminate residual infection in the community (WHO, 1970). The major intervention method for the interruption of transmission during the eradication programme, apart from chemotherapy was indoor sprays with residual chemical insecticides such as DDT and benzene hexachloride. Setbacks to the ongoing eradication programme started occurring by 1966 mainly due to a short supply of DDT coupled with recurrent focal outbreaks. Continued efforts to eradicate malaria by different intervention methods were not successful for various administrative and technical reasons (WHO, 1992). Problems such as drug resistance, insecticide resistance, environmental changes, and breakdown of organized malaria control in developing countries, social and ecological problems were some of the factors which led to serious questions on the practicability of total malaria eradication. The eradication approach was finally abandoned and in 1969 the goal was revised to control.

Today it is recommended that an integrated approach should be used. This involves the utilization of all appropriate technological and management techniques to bring about an effective degree of vector suppression in a cost-effective manner. Malaria control through the use of insecticides still remains the favoured approach. There are four broad classes of insecticide of interest in malaria control. These are the organochlorines (including the cyclodienes and phenyl pyrazoles), organophosphates, carbamates and pyrethroids (WHO, 2003).

2.3 Mode of Action of Insecticides

All insecticides target the nervous system (Figure 2.1) and must first pass through the integument and arrive in sufficient concentration, either in an altered form or as an active derivative at the site of action (Narahashi, 1992).

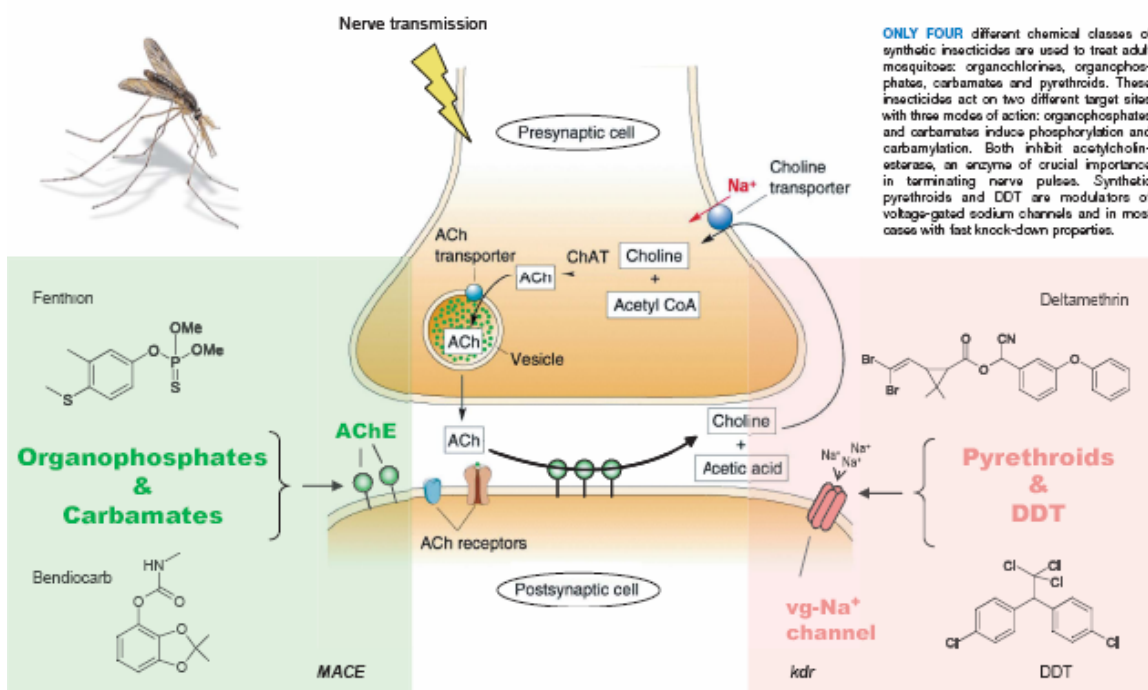


Figure 2.1: Biochemical target sites of synthetic insecticides (Nauen, 2006).

2.3.1 Action of organophosphates (OPs) and carbamates

OP's and carbamates target the cholinergic nerve junctions which are confined to the central nervous system. When a normally functioning motor nerve is stimulated, it releases the neurotransmitter acetylcholine, which transmits the impulse to a muscle or organ. Once the impulse is transmitted, the enzyme acetylcholinesterase immediately breaks down the acetylcholine in order to allow the muscle or organ to return to the relaxed state. OPs and carbamates disrupt the nervous system by forming a covalent bond through either carbamylation or phosphorylation with the site of the enzyme where acetylcholine normally undergoes hydrolysis (breakdown). The result is that acetylcholine builds up and continues to act so that nerve impulses are continually transmitted and muscle contraction continues (Corbett, 1974). This leads to death of the insect.

2.3.2 Action of pyrethroids

Pyrethroids are synthetic chemicals whose structure mimics the natural insecticide pyrethrin and constitute one of the most important classes of insecticides. It is estimated that over 25% of the insecticide market is dominated by pyrethroids (Georghiou, 1990). Pyrethroids are axonic poisons which act by binding to a protein in nerve cells called the voltage-gated sodium channel (Figure 2.1), thereby preventing it from closing normally resulting in continuous nervous stimulation. This explains the tremors exhibited by poisoned insects. They lose control of their nervous system and are unable to produce coordinated movement (Narahashi, 1992; Vijverberg *et al.*, 1982).

2.3.3 Action of organochlorines

The mode of action of organochlorines especially DDT is similar to that of pyrethroids. DDT and pyrethroids share many characteristics in their activity as insecticides and probably act on the same biomolecule but at different receptor sites (Miller and Saldago, 1985). The mode of action of DDT has never been clearly established, but in some complex manner, it destroys the delicate balance of sodium and potassium ions within the axons of the neurons in a way that prevents normal nerve impulses (Whiteacre and Ware, 2004). It acts on the

sodium channels to cause “leakage” of sodium ions. Eventually the affected neurons fire impulses spontaneously causing the muscles to twitch followed by convulsions and death (Busvine, 1951).

2.4 Insecticide Resistance

Insecticide resistance is defined as an inherited characteristic that imparts an increased tolerance to a pesticide such that the resistant individual survives a concentration of the compound(s) that would normally be lethal to the species (WHO, 1992). Insects are continuously confronted with lethal and non lethal stresses which include excesses in temperature and humidity, exposure to ultraviolet light, predation, parasitism, diseases, inter- and intra-species competition as well as toxic components such as pollutants, pesticides and plant allelochemicals. Resistance to pesticides is one of the several ways in which insects respond to a myriad of different environmental threat to their survival (Scott, 1995). Thus it is not surprising that they have evolved effective defense mechanisms for their continued survival.

Resistance is a selection by an insecticide which allows insects with resistance genes to survive and pass their resistant trait to their offspring. The percentage of resistant insects in a population thus continues to multiply while susceptible insects are eliminated. Eventually, resistant insects outnumber susceptible ones and the pesticide is no longer effective (Figure 2.2). How quickly resistance develops depends on several factors, including how quickly the insects reproduce, the migration and host range of the insect, insecticides exposed to it, rate of application and number of applications. Selection for resistance is thus greatly enhanced when the same insecticide is widely used in agriculture or in two or more types of applications, such as indoor spraying and larviciding, in the same area (WHO, 2003).

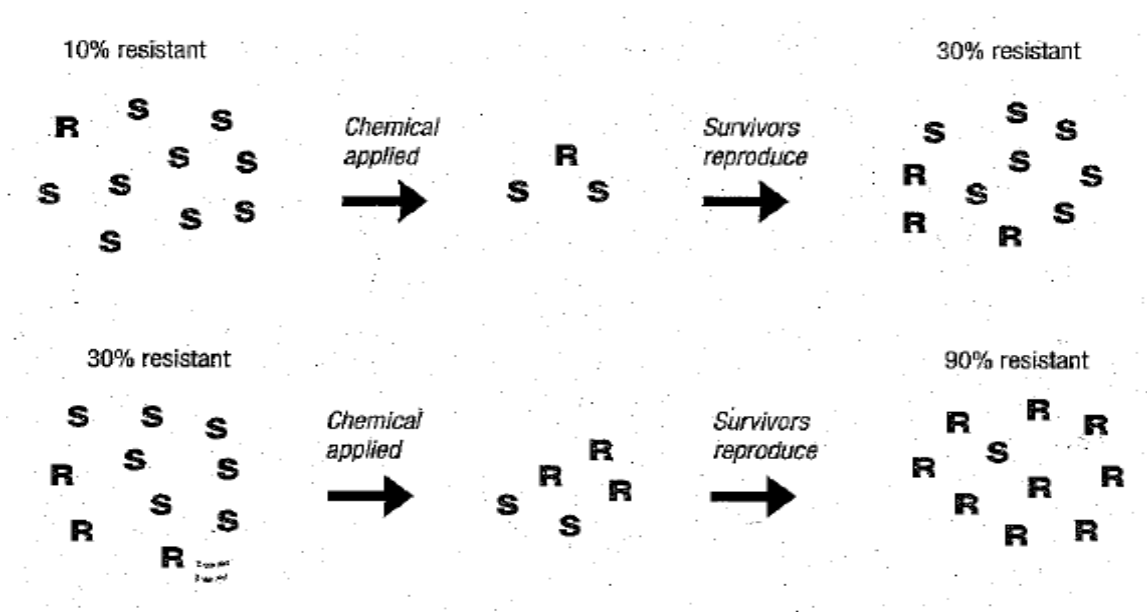


Figure 2.2: Diagrammatic representation of possible scenario for resistance development (R: resistant population, S: susceptible population) (adapted from Scott, 1995).

Several years of intensive use of organic insecticides to control arthropod pests and diseases vectors coupled with natural genetic mutations has led to a measurable shift in populations' susceptibility to insecticides due to the specific selection of these already preadapted individuals. The greatest increase in resistance has occurred during the last 40 years following the discovery and extensive use of synthetic insecticides (Brown, 1986).

The history of insecticide resistance dates back more than 80 years. The first recorded case of resistance was that of the "San Jose scale" to lime sulphur in 1908 (WHO, 1980). In mosquitoes more than 100 species are known to have developed resistance to one or more insecticides (Hemingway and Ranson, 2000), with the first case being reported in 1947 in the salt-marsh mosquitoes *Aedes taeniorhyncus* and *Ae. sollicitans* (Brown, 1986). In African malaria vectors, the first case of resistance was reported in northern Nigeria where a population of *An. gambiae* was reported to be resistant to cyclodiene insecticides (dieldrin and gamma-hexachlorocyclohexane), (Davidson, 1956; WHO, 1970). Almost 10 years later, the WHO Expert Committee on Insecticide Resistance reported in 1956 that there was evidence of organochlorine resistance in 20 insect species of public health importance. As

of 1992, the list of insecticide-resistance vector species included 56 anophelines and 39 culicines, body lice, bedbugs, triatomids, eight species of flea and nine species of ticks (WHO, 1992). The geographic distribution of resistance has continued to spread through Africa (Table 2.1), spanning from the well known documented case of *An. gambiae s.s kdr* based resistance in East and West Africa to pyrethroid and carbamate resistance in *An. funestus* from South Africa and Mozambique (Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Casimiro *et al.*, 2006). In Zimbabwe the first case of resistance to insecticides by mosquitoes was first recorded in 1974 in the southeastern lowveld of the country where a population of *An. arabiensis* showed evidence of resistance to benzene hexachloride (Green, 1981). Recently populations of *An. arabiensis* showed resistance to DDT in Gokwe (Masendu *et al.*, 2005).

Pesticide resistance in vectors continues to spread and affect disease control programmes in many countries. Resistance has now been recorded to all the commonly used classes of compounds: organochlorides (including DDT and dieldrin), organophosphates, carbamates and pyrethroids (Table 2.1). In addition, laboratory findings showed that resistance might develop to juvenile hormones, diflubenzuron, and even *Bacillus thuringiensis* serotype H-14 (WHO, 1986).

2.5 Resistance Mechanisms

Insecticide resistance is generally associated with behavioural, metabolic or physiological changes in insects, and results from three main types of mechanism: reduction in insecticide penetration, an increased metabolism of insecticide by esterase, monooxygenase or Glutathione-S-Transferases (GSTs) and modification of the insecticide target site (Hemingway, 1981; Mutero *et al.*, 1994).

2.5.1 Behavioural resistance

Insecticide avoidance, also called “behavioural resistance”, is the ability of some vectors to avoid contact with an insecticide. This is triggered from actions evolved in response to selective pressures exerted by the toxicant. Behavioural resistance can be stimulus-

dependent or stimulus-independent. Stimulus-dependent responses involve the detection of the toxicant and the avoidance of it. This is a common phenomenon among mosquitoes controlled by exposure to treated surfaces such as indoor residual spraying and insecticide treated bednets and curtains. Stimulus-independent resistance occurs when the target species starts inhabiting areas where the insecticide does not usually occur (Nauen, 2006).

Table 2.1: Status of insecticide resistance in the major malaria vectors in East and Southern Africa and their geographical distribution (Coetzee, 2000).

Species	Insecticide	Region
<i>An .gambiae s.s</i>	DDT	Zanzibar
	Dieldrin	Kenya and Madagascar
	Pyrethroids	Kenya and Zambia
<i>An. arabiensis</i>	DDT	Sudan, Ethiopia, Zanzibar, South Africa
	Dieldrin	Sudan, Ethiopia, Kenya, Madagascar, Zimbabwe
	Organophosphates	Swaziland, Sudan
<i>An. funestus</i>	Dieldrin	Kenya
	Pyrethroids	South Africa, Mozambique, Ghana
	Carbamates	South Africa, Mozambique, Ghana
	DDT	Ghana

2.5.2 Decreased insecticide penetration

A chemical toxicant can only exert its effect when it enters into the target organism; therefore the rate of insecticide penetration is an important determinant of insecticide toxicity (Plapp, 1976). Reduced penetration is when the composition of the insect's exoskeleton becomes modified in ways that inhibit insecticide penetration. Decreased penetration of insecticides would give ample time for detoxifying enzymes to metabolize the chemical and therefore make it less effective (Plapp, 1976). This kind of resistance was reported by Plapp and Hoyer (1968), who attributed decreased insecticide penetration as the

main causes of resistance to DDT and dieldrin by a strain of housefly (*Musca domestica*). Farnham (1971, 1973) showed that the mechanism is controlled by a gene that was given the name *pen* for penetration. Decreased cuticular penetration was also found in permethrin-selected strains of the housefly (DeVries and Georgiou, 1981). However this mechanism of resistance has not yet been attributed to malaria vector resistance despite evidence showing that decreased malathion absorption was responsible for malathion resistance in *Aedes aegypti* (Georgiou, 1980).

2.5.3 Metabolic resistance

Metabolic detoxification is one of the most common mechanisms of insecticide resistance (Hemingway and Karunaratne, 1998). Insects naturally possess metabolic enzymes that protect them from xenobiotics; therefore it is not surprising that metabolic detoxification may be a common mechanism of resistance to insecticides (Scott, 1991). 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 metabolic forms. The enzymes responsible for detoxification of xenobiotics are transcribed by three members of large multigene enzyme systems: cytochrome P450 Monooxygenases, non-specific esterase, and glutathione S-transferases (GSTs) (Brogdon *et al.*, 1998a). Their involvement in resistance is commonly identified by elevated enzyme levels and increase in the characteristic metabolites they produce. There are two major ways that the metabolic enzymes can produce resistance: firstly, the overproduction of the enzyme, leading to increased metabolism or sequestration, or secondly, an alteration in the catalytic centre of the enzyme unit that metabolizes the insecticides (WHO, 1998).

2.5.3.1 Glutathione-S- transferases (GSTs) and insecticide resistance

GSTs are a diverse family of enzymes found ubiquitously in aerobic organisms. They play a central role in the detoxification of both endogenous and xenobiotic compounds and are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (Enayati *et al.*, 2005). GSTs can metabolize insecticides by facilitating their reductive dehydrochlorination or conjugation reactions with reduced glutathione, to produce water

soluble metabolites that are more readily excreted. In addition, they contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Prapandathara *et al.*, 1995). Elevated GST activity has been associated with resistance to all the major classes of insecticides (Prapanthadara *et al.*, 1993). This elevation has been attributed to increase in one or more GST enzymes, either as a result of gene amplification or more commonly through increases in transcriptional rate, rather than qualitative changes in individual enzymes (Grant and Hammock, 1992; Ranson *et al.*, 2001).

Probably the well studied detoxification effect is demonstrated in the dehydrochlorination of DDT into DDE. This reaction is catalyzed by GSTs (Clark and Shamaan, 1984). Increased rates of DDT dehydrochlorination confer resistance to DDT in many insect species including houseflies and the mosquitoes *Ae. aegypti*, *An. gambiae*, and *An. dirus* (Grant *et al.*, 1991). In Southern Africa elevation of GSTs has been implicated in DDT resistance in *An. arabiensis* (Hargreaves *et al.*, 2003). Besides the well documented implication of GSTs in detoxifying other organochlorine insecticides, e.g. lindane, they have also been found to confer resistance to organophosphates (Haye and Wolf, 1988). However, GSTs have not yet been implicated in the direct metabolism of pyrethroid insecticides although theoretically they may play an important role in conferring resistance to this class of insecticides by detoxifying lipid per oxidation products induced by pyrethroids (Vontas *et al.*, 2001). This probably explains the lack of correlation between elevated GSTs and bioassays of pyrethroid resistant populations of *An. funestus* from Southern Africa (Brooke *et al.*, 2001).

2.5.3.2 Cytochrome P450 monooxygenases (CYPs) and insecticide resistance

P450 enzymes are a large complex superfamily of hydrophobic haem-containing enzymes found in most organisms including insects. These enzymes are involved in the metabolism of a wide variety of endogenous and exogenous compounds such as steroids, fatty acids and xenobiotics (Feyereisen, 1999; Hemingway and Ranson, 2000). Insect P450s have been implicated in insect growth, development, reproduction, insect resistance and tolerance to plant toxins (Hodgson and Klukarin, 1983; Feyereisen, 1999; Scott *et al.*, 1998), and are thus important in adaptation of insects to toxic chemicals.

Monooxygenases are involved in the metabolism of virtually all insecticides from the hydroxylation of DDT, the epoxidation of cyclodienes, the aromatic hydroxylation of the carbamates, carbaryl and oxidation of phosphorothioates (Hemingway, 1984; Feyereisen *et al.*, 1990). The cytochrome P450 enzymes confer insecticide resistance via an increased level of P450 activities resulting from elevated expression of P450 genes.

Currently this enzyme system is poorly studied in insect vectors such as mosquitoes. However, it is generally agreed that elevation of P450 enzymes is partly associated with pyrethroid resistance in *Anopheles* mosquitoes in Africa. Elevated monooxygenases activities are associated with pyrethroid resistance in *An. stephensi*, *An. subpictus*, *An. gambiae* and *An. funestus* (Elissa *et al.*, 1993; Vulule *et al.*, 1994; Hargreaves *et al.*, 2000; Brooke *et al.*, 2001). The involvement of P450s has primarily been documented on the basis of synergistic studies with the monooxygenase inhibitor, piperonyl butoxide (PBO), and detection of haem levels in resistant mosquitoes (Vulule *et al.*, 1999; Hargreaves *et al.*, 2000; Brooke *et al.*, 2001; Brooke *et al.*, 2006). In these studies, the resistance to pyrethroids was shown to be decreased by PBO.

2.5.3.3 Esterases

Esterases are a large group of enzymes which metabolize a wide variety of substrates (Scott, 1995). All esterases are able to hydrolyze ester bonds in the presence of water. Many insecticides, especially organophosphates and carbamates, contain ester bonds thus any elevation of esterase has been attributed to resistance (Fournier *et al.*, 1987; Chen and Sun, 1994). Esterase levels can be elevated by either gene amplification or altered gene expression (Figure 2.3) (Mouchès *et al.*, 1987; Field *et al.*, 1988; Poirie *et al.*, 1992).

The esterase-based resistance mechanisms have been studied most extensively in *Culex* mosquitoes and the aphid *Myzus persicae*. In these insects, the resistance esterase genes are highly amplified and up to 250 copies of the same gene may be found in a single individual (Mouchès *et al.*, 1986; Poirie *et al.*, 1992). All esterases are known to act by rapidly binding and slowly turning over the insecticide. They sequester rather than rapidly metabolize the pesticide (Hemingway and Ranson 2000).

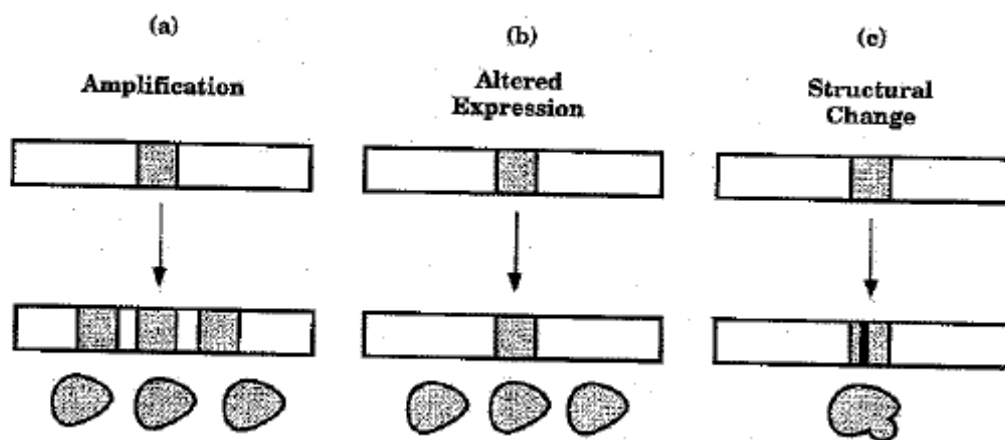


Figure 2.3: Graphical representation of ways in which esterase based resistance occurs: (a) gene amplification to increase amount of gene copies, (b) changes in the regulatory expression of gene, and (c) rewriting of genetic code to produce a structurally different product (Scott, 1995).

2.5.4 Target site and insecticide resistance

Target site insensitivity is usually correlated to a single amino acid substitution of the target protein which sufficiently alters the three dimensional structure of that protein so as to prevent or at least inhibit the binding of the insecticidal compound (Roush and McKenzie, 1987; Soderlund and Bloomquist, 1989). The target sites involved are the voltage-dependent sodium channels from nerve membranes for the pyrethroids and DDT, acetylcholinesterase for the organophosphates and carbamates and the GABA receptor for cyclodienes.

2.5.4.1 Sodium channels

Sodium channels are large complex membrane spanning proteins that generate the action potential in the neuronal membranes of insects that are responsible for conduction of electrical information throughout the nervous system (Vais *et al.*, 2001). Voltage-gated sodium channels are the target for both pyrethroid insecticides and DDT, where the insecticide operates by altering the functions of the channels in the nerve membranes of insects, preventing the repolarization phase of the action potentials (Satelle and Yamamoto, 1988; Narahashi, 1996). Mutations in the voltage-gated sodium channel gene have been

associated with knockdown resistance (*kdr*) to DDT and pyrethroid insecticides in several insect species (Williamson *et al.*, 1996; Brengues *et al.*, 2003; Enayati *et al.*, 2003). *Kdr* was first described in peach aphid, *Myzus persicae*, Diamond-back moth, *Plutella xylostella* and the German cockroach *Blatella germanica* (Miyazaki *et al.*, 1996; Martinez-Torres *et al.*, 1997). For the major malaria vector *An. gambiae s.s.*, two mutations at the domain II (Figure 2.4) of the voltage-gated sodium channel genes have been associated with high resistance to permethrin and DDT and induce cross resistance to pyrethroids (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Pinto *et al.*, 2006). The first mutation consists of a leucine-phenylalanine substitution and is widespread in West Africa at variable frequencies (Awolola *et al.*, 2003; Fanello *et al.*, 2003; Yawson *et al.*, 2004; Pinto *et al.*, 2006). The

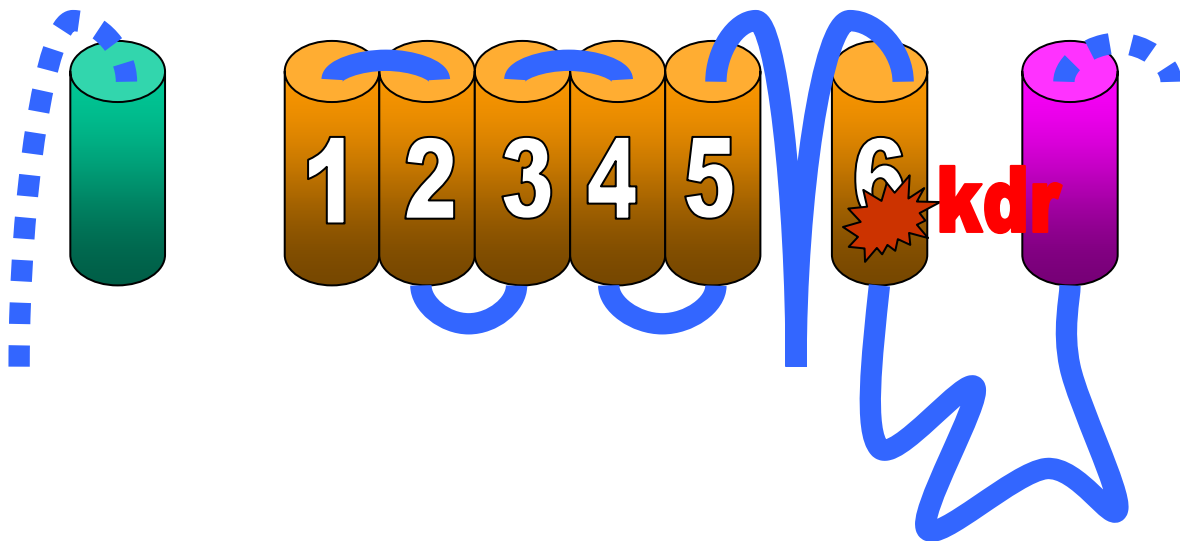


Figure 2.4: Diagrammatic representation of domainII of the sodium channel showing the position of mutation (*kdr*) that has been implicated in conferring pyrethroid resistance in *Cx. pipiens* and *An. gambiae s.s.* (Brogdon and McAllister, 1998a)

second mutation consists of a leucine-serine substitution at the same codon and is found mainly in East Africa (Ranson *et al.*, 2000). This substitution confers a lower resistance to permethrin and a high resistance to DDT. However, this geographic distribution is misleading as it is thought that this type of resistance is spreading to the other areas as can be exemplified by co-occurrence of both East and West Africa *kdr* resistance in a population of DDT-resistant *An. gambiae s.s.* from Uganda (Ranson *et al.*, 2000). While the *kdr* allele had

predominantly been found in *An. gambiae* s.s, several reports have also identified the presence of *kdr* in the sibling species *An. arabiensis* although these were not correlated to bioassay data (Ranson *et al.*, 2000; Diabate *et al.*, 2004; Matambo *et al.*, 2007). However, no published records have reported this mutation in the *An. arabiensis* sodium channel gene from Southern Africa despite decades of both DDT and pyrethroid usage. In this part of Africa, DDT and pyrethroid resistance in *An. arabiensis* resistance has been attributed to metabolic mechanisms rather than target site insensitivity (Hargreaves *et al.*, 2003).

2.5.4.2 Acetylcholinesterase (AChE)

AChE is a serine esterase in the alpha/beta hydrolase fold enzyme family (Ollis *et al.*, 1992). It plays a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. AChE also has a much less studied role in the development, maturation and maintenance of the nervous system (Grisaru *et al.*, 1999, Ranson *et al.*, 2002, Cousin *et al.*, 2005). AChE is the target for the largest group of insecticides, organophosphates and carbamates. Intensive use of these insecticides over the past 50 years has led to the development of resistance in many target species that are important in both agriculture and human health (Georghiou, 1990; Fournier and Mutero, 1994). The frequency involves changes in the AChE gene rendering it less sensitive to organophosphates and carbamates (Ayad and Georghiou, 1975). This is as a result of point mutations usually accompanied by a modification of the kinetic parameters of acetylcholine hydrolysis (Mutero *et al.*, 1994; Walsh *et al.*, 2001). These mutant forms of AChE have been characterized biochemically and can show widely differing spectra of insensitivity between species as well as marked range of insensitivity to different compounds within species (Devonshire and Moores, 1984). There are two types of AChE: AChE1 and AChE2 (encoded by *ace1* and *ace2* genes, respectively), (Bourgouet *et al.*, 1996b). Mutations in the *ace2* gene are responsible for resistance in higher dipterans such as *Drosophila melanogaster*; *Musca domestica* and *Bactrocea oleae* (Mutero *et al.*, 1994; Walsh *et al.*, 2001; Vontas *et al.*, 2002). In contrast, in mosquitoes, mutations in the *ace1* gene have been associated with resistance in *Culex pipiens* (Weill *et al.*, 2002), *An. albimanus* and *An. gambiae* Giles (Weill *et al.*, 2004).

2.5.4.3 Gamma-aminobutyric (GABA) receptors

The GABA receptor in insects is a heteromultimeric-gated chloride ion channel, a widespread inhibitory neurotransmitter channel in the insect's central nervous system and neuromuscular junctions (Bermudez *et al.*, 1991). The GABA receptor is implicated as a site of action for pyrethroids and avermectins as well as cyclodienes (Scott, 1995; Hemingway and Ranson, 2000). Most cases of cyclodiene resistance appear to be due to decreased sensitivity of the GABA subtype A receptor (Ffrench-constant *et al.*, 1991), an integral part of the chloride ion channel. Decreased sensitivity by GABA receptors is due to a structural change of the protein caused by a single point mutation which causes one specific amino acid to be substituted by another resulting in high levels of resistance to cyclodienes. This mutation has been associated with dieldrin resistance in various insect species and has been well studied in *D. melanogaster* where a single base pair mutation causing a single amino acid substitution (alanine to serine) within the second membrane-spanning region of the channel was found to be the difference between resistant and susceptible strains (Ffrench-constant *et al.*, 1993). This mutation directly affects the insecticide binding site and also allosterically destabilizes the insecticide preferred conformation of the receptor (Ffrench-constant *et al.*, 2000). For malaria vectors, no detailed work has been done on the mechanisms of resistance in the GABA receptor despite the prevalence of cyclodiene resistance which led to their withdrawal from use during the malaria eradication era. However Du *et al.* (2005) recently showed that a mutation conferring the substitution alanine²⁹⁶ to glycine is associated with dieldrin resistance in a laboratory strain of *An. gambiae*. They also showed that another mutation of the same codon conferring the substitution alanine to serine is associated with dieldrin resistance in a laboratory strain of *An. arabiensis*.

2.6 Detection of Resistance

During active malaria control programmes where insecticides are used against vector mosquito, it is important to detect and characterize an emerging resistance problem. This will ensure that future control strategies can be fine-tuned through optimizing current insecticide usage (Hemingway *et al.*, 1989). The initial step in identifying a potential

problem is to detect changes in the susceptibility of a population at very low level(s). There are various ways in which susceptibility can be identified, but whatever method is used, it should be fast, inexpensive, easy to use, and diagnostic for all types of resistance. Currently all available methods fall short of being ideal techniques. There are basically three methods for detecting changes in susceptibility of a population: bioassays, biochemical and molecular assays (WHO, 1998; Brogdon and McAllister, 1998b).

2.6.1 Bioassays

WHO has developed susceptibility bioassay tests available in kit form (Figure 3.4A), for mosquitoes, lice, bedbugs, reduviid bugs, cockroaches, black flies, houseflies, ticks and fleas (WHO, 1992). Bioassays are better able to detect the overall levels of resistance present in a population in a single test (Table 2.2). They can be divided into: time mortality bioassays which are based on insects being exposed by contact for different time periods to surface treated with a specific amount of insecticide (WHO, 1986, 1998), and dose mortality bioassays. Time-based bioassays have been further modified through the use of insecticide-coated glass bottles and solutions of standard grade insecticide or synergists. This approach simplifies the bioassay process and increases the amount of information that can be obtained from a limited pool of mosquitoes (Brogdon and McAllister, 1998a).

2.6.2 Biochemical assays

The assays are based on the principle that most resistance in insects is a result of over expression or overproduction of specific enzymes naturally involved in detoxification of allelochemicals. Biochemical assays can detect resistance mechanisms in individual mosquitoes (Brogdon and McAllister, 1998b).

2.6.3 Molecular assays

Molecular assays involve detection of resistance at molecular level, and are thus limited to known resistance genes (Table 2.2). However, molecular information on resistance mechanisms will increasingly be incorporated into diagnostic procedures. To date, target site

mechanisms such as *kdr* mutations have been detected by PCR amplification of specific alleles (WHO, 1998; Martinez-Torres *et al.*, 1998).

Table 2.2: Comparison of the advantages and disadvantages of detection methods that are based on either molecular or traditional technology (Scott, 1995).

Molecular Detection Assay	Bioassay
Can distinguish resistant genotypes even at low levels.	Less sensitive, only detects resistant phenotypes, not genotypes.
Distinguishes SS, SR, and RR genotypes reliably even when phenotypes are hard to distinguish.	Cannot distinguish SR and RR from each other unless the phenotypes are distinct from one another.
Generally greater accuracy and less variability because environmental components are eliminated.	Accurate and more variable because environmental components cannot be separated from genetic ones.
Fewer insects can yield more information because material from one insect can be used for several assays	Requires more insects to get the same data because the same individuals cannot be used in several bioassays.
Currently not adapted for field use.	Many are easily used in the field.
Material and equipment often costly	Generally inexpensive and simple to prepare and execute
More technical expertise often needed	
Limited to detection of known resistance genes	Can detect any type of resistance, even if the resistance gene is not known

Chapter 3

Materials and Methods

3.1 Study Area

The study was carried out in Gwave (17°55'S; 28°41'E), a village located in Gokwe South District, Midland's province of Zimbabwe. Gokwe is a semi-developed rural area with an economy primarily based on subsistence farming of mainly cotton and maize. The major rainfall season is November-February averaging 400 to 1200 mm annually with the highest monthly rainfall in either January or February. The rains are followed by a long period of dry and cold conditions. The highest mean monthly temperatures of 30°C occur in October and November. A high relative humidity of 65-85% is encountered between November and February.

The total population of Gokwe is about 500 000 people of which 15.5% are infected by malaria yearly (NIHR, National Malaria Survey, 1991). Mosquito breeding occurs throughout the year because of the easy availability of breeding sites which include artesian wells, shallow wells dug into river beds, small temporary rain puddles, pools at boreholes (Figure 3.1A) and large semi-permanent ponds (Figure 3.1B). These ponds are formed during the rainy seasons and can last up to 6 months.

Mosquitoes in this area are constantly under insecticidal selection pressure from insecticides employed in agriculture and public health. Large quantities of various insecticides covering all insecticide classes are applied for agricultural purposes. Chemical vector control through indoor residual spraying is the cornerstone of malaria control. DDT was the insecticide of choice from 1958 until 1987 when it was replaced by pyrethroids for commercial and environmental reasons. Currently, indoor residual spraying for malaria control with Icon® 10 WP (Lambda-cyhalothrin), a pyrethroid, is done once every year from November until January (Lukwa, personal communication).

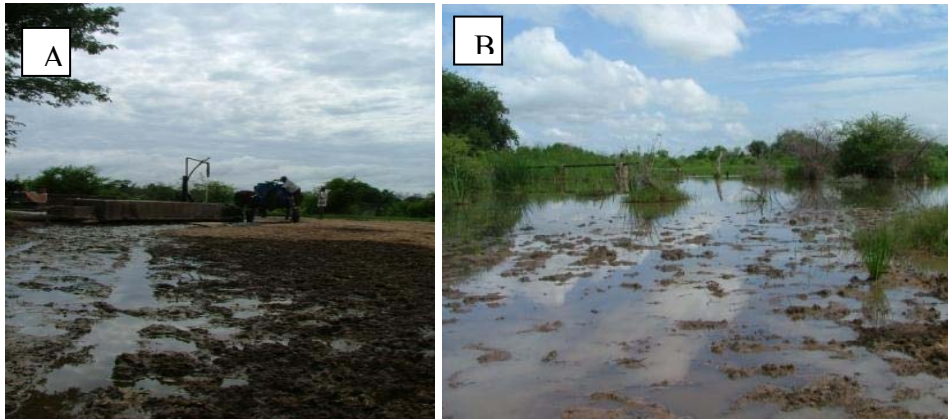


Figure 3.1: Breeding sites in study area **A:** pools at borehole sites;
B: large permanent ponds.

3.2 Study Outline

The study encompassed 5 days of field data collection and 8 months of laboratory analysis. Field work involved the capturing of adult *An. gambiae* complex mosquitoes using human-baited traps, hand searches in sleeping houses and larval collections. The collected *An. gambiae s.l* mosquitoes were assayed using WHO discriminating dosages of 0.75% permethrin, 4% dieldrin, 4% DDT, 0.1% bendiocarb and 5% malathion (WHO, 1998). The remaining live and all dead specimens were transported to the National Institute for Communicable Diseases (NICD) laboratory in South Africa. All dead mosquitoes from the insecticide exposures were identified to species level using the method described by Scott *et al.* (1993). Female survivors from the exposures were induced to lay eggs and their F₁ progeny were subdivided into batches depending on the number of adults emerging. One batch was stored at -70°C and subsequently used for biochemical analysis while the remaining batches were used for insecticide bioassays. During biochemical analysis, assays designed to quantify levels of monooxygenases, glutathione-S-transferases, esterases and altered acetylcholinesterases were performed. A susceptible laboratory colony of *An. arabiensis* (KGB) originating from Zimbabwe and maintained in the Botha DeMillion insectary since 1975 was used for comparative purposes. Molecular assays to establish the presence/absence of the knockdown resistance mutation (*kdr*) were performed on survivors of field exposures as well as the mothers of F₁ progeny survivors. Two laboratory colonies

designated MBN and Kwag, both originating from Mamfene, Kwazulu/Natal, South Africa were used to characterize carbamate and pyrethroid resistance by continuously putting these two colonies under 0.1% bendiocarb and 0.75% permethrin selective pressure, respectively.

3.3 Methods

3.3.1 Field collections and morphological identification

Adult anopheline mosquitoes were collected from the study area using human-baited trap catches (HBC) (as described by Mpofu and Masendu, 1986), larval collections (L.C), and direct hand catches (DHC) for indoor and cattle kraal resting mosquitoes. In HBC, a tailor-made tent with a gap between the bottom section of the tent and the ground was used (Figure 3.2A). People inside the tent acted as bait to attract host seeking mosquitoes. Using a flash light and an aspirator the mosquitoes were collected and placed into paper cups covered with netting material. Using the DHC method, mosquitoes were collected inside houses where people sleep early in the morning between 0700 hours and 1000 hours. Mosquitoes were also collected on and around a cattle kraal between 1800hrs and 2000hrs. Larval collections were conducted in stagnant ponds surrounding an aquifer using the dipping method (Figure 3.2B) as described by Service *et al.* (1978). Collected adult anophelines were identified morphologically as *An. gambiae s.l* using hand lenses and taxonomic keys (Gillies and DeMillion, 1968 and Gillies and Coetzee, 1987). Only mosquitoes conclusively identified as members of the *An. gambiae* complex were retained.

Wild caught larvae were reared through to adults by feeding them with larval food under ambient conditions (23-26°C). The wild collected adult mosquitoes and two day old adults reared from larval collections were maintained with a 10% sugar solution for 24 hours before being subjected to susceptibility tests.



Figure 3.2: Collection methods used: **A** - Human baited net trap, **B** - Larval collections from temporary rain puddles

3.3.2 Field bioassays

Insecticide susceptibility bioassays were conducted using WHO test kits (Figure 3.3A), for adult mosquitoes (WHO, 1981 revised in 1998). Samples of wild collected *Anopheles gambiae s.l* and those reared from larval collections were exposed to 0.75% permethrin, 4% dieldrin, 4% DDT, 0.1% bendiocarb and 5% malathion. For each insecticide test, 6 cylinders, 2 serving as controls and 4 as treatments, were used. Control cylinders contained plain filter papers, while treatments contained filter paper impregnated with the respective insecticide at a standard concentration. Twenty five to thirty sugar-fed female mosquitoes were allowed tarsal contact for 1 hour within the cylinder placed in a vertical position (Figure 3.3B). Mosquitoes were then transferred to clean holding tubes and provided with cotton pads soaked with a 10% sugar solution. Knockdown after one hour and mortality at 24 hour post exposure were recorded. Susceptibility status was determined according to WHO criteria: populations were considered resistant if more than 20% of individuals survived the diagnostic dose after 24 hours (WHO, 1998). After the exposures, dead mosquitoes were kept in 1.5 ml eppendorf tubes on desiccated silica for subsequent identification. The remaining surviving and dead specimens were transported to the National Institute for Communicable Diseases (NICD) laboratory in South Africa for species identification and further analysis.

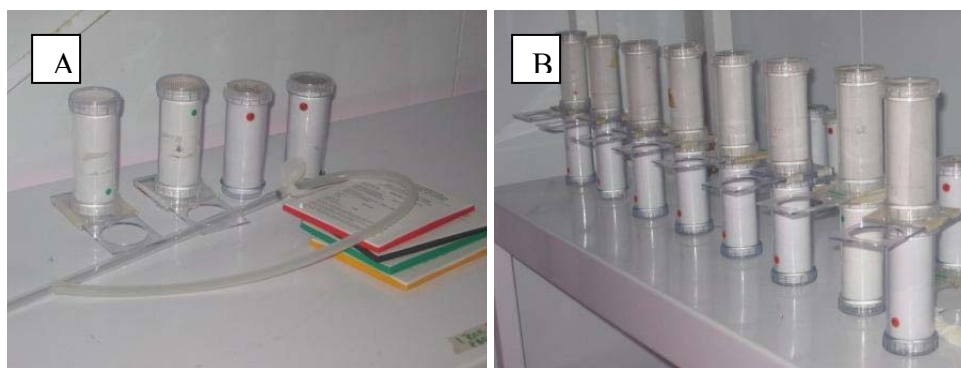


Figure 3.3: **A** - WHO test kit showing exposure tubes (cylinders with red stickers), holding tubes (cylinders with green stickers), manual aspirator (sucking tube) and insecticide impregnated papers (WHO diagnostic concentrations); **B** - Experimental set up for adult bioassays.

3.3.3 Mosquito rearing and maintenance

Live adult mosquitoes collected from the field and mosquitoes used in subsequent experiments were reared in the Botha DeMillion insectary at NICD in South Africa following the standard rearing procedure as described by Ford and Green (1972) and Hunt *et al.* (2005) with slight modifications. An insectary is a room where temperature, humidity and light are artificially maintained at constant values and is sufficiently screened to avoid mosquitoes from escaping.

Wild caught adult females were given a laboratory code and individually placed in glass vials lined with moist filter paper to induce laying of eggs. Egg batches from each female were washed separately three times with distilled water and transferred into 250 ml polythene plastic bowls (figure 3.4B). When larvae reached the third instar they were transferred to 2-litre plastic containers (Figure 3.4C), each filled with 1-litre of distilled water. Larvae were fed twice daily with a mixture of brewers yeast and finely ground dog biscuits prepared at a ratio of 1:3. Before pupation, the 4th instar larvae were transferred into 5-litre plastic containers (Figure 3.4D) containing 2.5-litres distilled water and covered with nets to prevent adult mosquitoes from escaping after emergence. After eclosion, adults were transferred using an aspirator into 10-litre plastic cages with sleeves for easy access (Figure

3.4E). A 10% sugar solution was made available. These adults were maintained on the sugar solution under standard insectary conditions of 25°C, 85% relative humidity and a photo period of 12: 12 hour light/darkness, with a 45-min dawn and dusk light regimen. To propagate the colony females were fed on guinea pig blood (Figure 3.5) four days after emergence. After 3 blood meals darkened Petri dishes acting as eggplates (Figure 3.4A) were placed into the plastic cages to facilitate egg oviposition. Eggs were washed as described above and the whole procedure was repeated in order to maintain the colony.

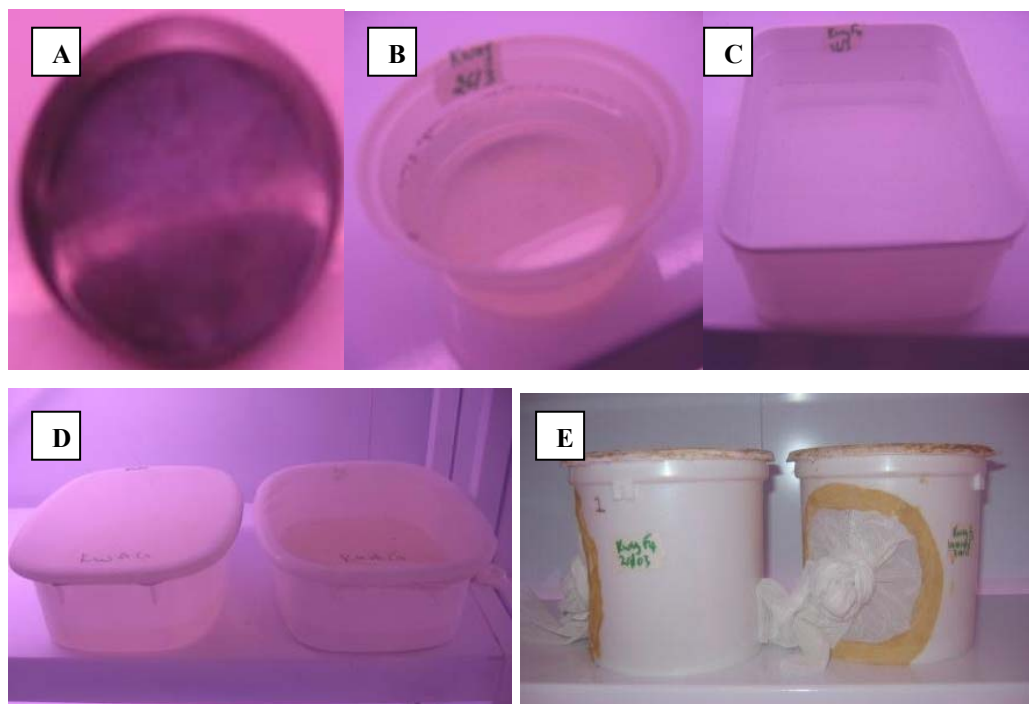


Figure 3.4: Summary of mosquito rearing: **A** - egg plate, **B** – 250 ml ice cream container for 1st instar larvae, **C** – 2 litre plastic container for rearing 2nd and 3rd instar larvae, **D** – 5 litre plastic container for rearing 4th instar larvae until adult emergence, **E** - Plastic cages for adults.



Figure 3.5: Mosquitoes being fed on guinea pigs

3.3.4 Species identification

The vectorial capabilities of malaria vector species are very different and vary within species and between localities. It is important to correctly identify those species involved in malaria transmission (Coetzee *et al.*, 1993; Hunt *et al.*, 1998; Koekmoer *et al.*, 2002). This is especially important in Southern Africa where the non-vector member of the *An. gambiae* complex, *An. quadriannulatus*, occurs in sympatry with the malaria vectors *An. gambiae s.s.*, *An. arabiensis* and *An. merus* (Coetzee and Hunt, 1998; Masendu *et al.*, 2005). The objective of the methods outlined in this section was to identify wild-caught mosquitoes of the *An. gambiae* complex into their respective sibling species.

3.3.4.1 Morphological identification

All the dead mosquitoes retained after field susceptibility tests and female survivors used to rear isolines were identified as belonging to *An. gambiae* complex using the standard morphological keys (Gillies and Coetzee, 1987) and were preserved individually on desiccated silica. Subsequently, each mosquito was identified to species level using the Polymerase Chain Reaction assay of Scott *et al.* (1993).

3.3.4.2 PCR species-specific identifications

3.3.4.2.1 *Anopheles gambiae* complex

Wild caught *An. gambiae* complex specimens retained after field susceptibility tests and females used to rear isolines were identified to species level using the polymerase chain reaction assay as described by Scott *et al.* (1993) and slightly modified by Van Rensburg *et al.* (1996). All primers and PCR reagents used were procured from Inqaba Biotech South Africa. Table 3.1 shows the primer sequences of all primers used for *An. gambiae* complex species-specific PCR identification.

One leg per specimen was dissected and placed in a micro centrifuge tube: 12.5 µl of a master mix containing 1.25 mM 10X PCR reaction buffer; 1.25 mM 10X dNTPs; 25 mM MgCl₂; 1.65 pmol *An. quadriannulatus* species A and 3.3 pmol each of *An. gambiae* s.s., *An. merus*, *An. arabiensis* and universal primers; 4.9 µl deionised water and 0.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase enzyme were added. The micro centrifuge tubes were placed into a centrifuge (Biofuge, Heraeus), and centrifuged at 13,000 rpm for 10 sec to release template DNA. The reaction mixture was then placed into a thermal cycler (Primus 96, MWG Biotech) with the following thermal profile: 30 cycles consisting of 94°C denaturation for 30 sec, 50°C annealing for 30 sec, and 72°C extension for 30 sec and a final autoextension step at 72°C for 5 minutes. Ten microlitres of the amplified products were loaded onto a 2.5% agarose gel, stained with ethidium bromide, submerged in 1X TAE buffer and subjected to electrophoresis at 100 V for 1 hour 30 minutes or until bromophenol blue was 3 cm from origin to allow for proper separation of amplicons. Each gel contained 4

positive control specimens drawn from known strains of *An. arabiensis*, *An. merus*, *An. quadriannulatus* spp A and *An. gambiae* s.s. The negative control consisted of the reaction mixture only. The gel was photographed in a gel documentation box (Syngene G-box, sydr4/1152). The amplified fragment sizes were 315 base pairs (bp) for *An. arabiensis*, 390 bp for *An. gambiae*, 153 bp for *An. quadriannulatus* spp A, and 466 bp for *An. merus*. These were compared to a 1 kilo base molecular marker (O'Gene ruler™, Fermentas Life Sciences. Cat no SM1153).

Table 3.1: Primer sequences used for *An. gambiae* s.l PCR reactions (Scott *et al.*, 2003)

Primer name*	Primer sequence (5' to 3')	Transcript length
ME	5' TGA CCA ACC CAC TCC CTT GA 3'	464 bp
GA	5' CTG GTT TGG TCG GCA CGT TT 3'	390 bp
AR	5' AAG TGT CCT TCT CCA TCC TA 3'	315 bp
QD	5' CAG ACC AAG ATG GTT AGT AT 3'	153 bp
UN	5' GTG TGC CCC TTC CTC GAT GT 3'	

* The UN primer anneals to the same position of rDNA for all five species, ME primer anneals to both *An. merus* and *An. melas*, GA primer anneals specifically to *An. gambiae* s.s, AR primer anneals to *An. arabiensis* and QD primer anneals to *An. quadriannulatus* sppA

3.3.4.2.2 *Anopheles funestus* group

Wild caught *An. funestus* group specimens retained after field susceptibility tests and females used to rear isolines were identified to species level using the polymerase chain reaction assay as described by Koekemoer *et al.* (2002) with slight modifications. All

primers and PCR reagents used were procured from Inqaba Biotech South Africa. Table 3.2 shows the primer sequences of all primers used for species-specific PCR identification.

DNA extraction

DNA was extracted from single mosquitoes using the standard procedure described by Collins *et al.* (1987) (see section 3.3.9.1). DNA was re-suspended in 200 µl 1X TE buffer and 0.5 µl was used for subsequent PCR amplification.

PCR amplification

Amplification was done in a 12.5 µl reaction volume containing 8.25 pmol each of the following primers: *An. vaneedeni*, *An. leeson*, *An. parensis*, *An. funestus*, *An. rivulorum* and a universal primer; 1.5 mM MgCl₂, 200 µl of each dNTP and 0.75 units of Taq DNA polymerase. The volume was made up to 12.5 µl with double distilled water. Amplification was carried out in a thermal cycler (Primus 96, MWG Biotech) with the following thermal profile: denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec. Each cycle was repeated 40 times followed by a final extension at 72°C for 5 minutes. Ten microlitres of the amplified products were loaded onto a 2.5% agarose gel, stained with ethidium bromide, submerged in 1X TAE buffer and subjected to electrophoresis at 100 V for 1 hour 30 minutes or until bromophenol blue was 3 cm from origin to allow for proper separation of amplicons. Each gel contained 5 positive control specimens drawn from known strains of *An. vaneedeni*, *An. leesoni*, *An. parensis*, *An. funestus* s.s and *An. rivulorum*. The negative control consisted of the reaction mixture only. The gel was photographed in a gel documentation box (Syngene G-box, sydr4/1152). The amplified fragment sizes were 500 base pairs (bp) for *An. funestus* s.s, 400 bp for *An. rivulorum*, 550 bp for *An. vaneedeni*, 250 bp for *An. parensis* and 153 bp for *An. leesoni*. These were compared to a 1 kilo base DNA ladder (O'Gene ruler™, Fermentas Life Sciences. Cat no SM1153).

3.3.5 Selection for resistant lines

Selection pressure on natural vector populations exposed to frequent contacts with residual chemicals during blood seeking activities and exposure to sprayed resting surfaces may increase the amount of detoxification enzymes responsible for insecticide resistance (Ferrari and Georgiou, 1990). For this reasons two *An. arabiensis* colonies originating from Mamfene area in KwaZulu/Natal colonized at the NICD and designated Kwag and MBN were selected as representative vectors for the study of development of pyrethroid and carbamate resistance respectively. MBN was colonized in 2002 from wild populations which showed low levels of carbamate resistance during the time of collections. Kwag was colonized in 2005 using F₁ progeny from wild caught females showing low levels of pyrethroid resistance (Mouatcho *et al.*, 2005, unpublished data).

To start the selections newly emerged male and female adults from the MBN and Kwag strains were placed into separate cages to ensure that mating did not take place prior to insecticide exposure. Selection for resistance to each insecticide began with exposure to 0.1% bendiocarb and 0.75% permethrin, respectively, for 20 minutes according to the standard WHO bioassay procedure (WHO, 1998). Averages of 25 individuals were used per exposure tube and at least 20 replicates were recorded for each generation. Male and female survivors 24 hour post- exposure were placed in a cage and left for 4 days to mate. Females were offered guinea blood meals three times per week. Eggs from each generation were reared through to adults and these were subjected to subsequent selection using 0.1% bendiocarb or 0.75% permethrin for each generation until mortalities below 50% were achieved. To check that the test papers had been correctly impregnated and that they remained insecticidal, all papers were tested, both prior to and after the exposures against an insecticide susceptible *An. quadriannulatus* spp A laboratory colony originating from Sangwe, Zimbabwe. After the fourth generation, half of each progeny cohort was kept and stored at -70°C for biochemical analysis. The pyrethroid selected line was named Kwag-Perm and the carbamate selected line was named MBN-Carb.

3.3.6 Indirect Enzyme-Linked Immunosorbent Assay (ELISA) for *Plasmodium falciparum*

The most important entomological factor in the epidemiology of human malaria is the entomologic inoculation rate (EIR) which provides a standard and relatively simple means of quantifying levels of exposure to infected mosquitoes (Burkot, 1995). The EIR uses an index of vector infectivity, the sporozoites rate and the human biting rate which expresses the degree of human-vector contact. The sporozoite rate, i.e. the proportion of mosquitoes with sporozoites in their salivary glands, also indicates whether a vector can transmit malaria. ELISA is a method which detects *P. falciparum*, *P. vivax*-210 and *P. vivax* 247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. The CS-ELISA method used was as described by Wirtz *et al.* (1987) with minimal modifications.

A total of 530 female mosquitoes from the field which were positively identified to species level had their heads and thoraces dissected and ground with Teflon pestles in 50 µl of grinding buffer (0.5% casein, 0.1 M NaOH, 0.002% phenol red and 0.1 M phosphate buffered saline [PBS], pH 7.4 containing 0.5% Nonidet P-40). After grinding, pestles were washed with 150 µl blocking buffer (0.5% casein, 0.1 M NaOH and 0.002% phenol red dissolved in 0.01 M PBS) and either tested immediately or stored at -70°C for later analysis. The wells of disposable polyvinyl chloride, flat bottomed microtiter plates (NUNC A/S, Denmark) were coated with a 50 µl volume of monoclonal antibody *P. falciparum* 2A10[Pf2A10] (2 µl/ml PBS) and covered with aluminium foil paper and held overnight at 4°C. The following day the plate contents were aspirated and the wells filled with blocking buffer and held for 1 hour in darkness at room temperature (23-26°C). After aspiration of the blocking buffer, 50 µl aliquots of mosquito triturate were added to the respective wells. These were covered and after 2 hours of incubation at room temperature well contents were aspirated and the plates washed twice with PBS-Tween 20, (PBS-Tw) before the addition of Peroxidase-labelled monoclonal antibodies (Pf2A10-CDC 15). After 1 hour of incubation in the dark at room temperature, the plates were washed three times with PBS-Tw, followed by addition of 100 µl/well of peroxidase substrate B solution [Kirkegaard and Perry Laboratories (KPL), Maryland, USA]. After 30 minutes of incubation in the dark, plates were read at 450 nm using an ELISA plate reader (Labsystems Multiskan RC, Genesis

software version 3.03). Negative controls consisted of seven 50 µl aliquots of triturate of laboratory-reared uninfected *An. arabiensis* and the positive control consisted of Nonidet P-40 treated salivary-gland sporozoites, recombinant *P. falciparum* CS protein (KPL, Maryland, USA).

Data Analysis

Results for *Plasmodium* sporozoite ELISA were first scored visually and then photometrically at 405 nm using an ELISA plate reader. Each positive sample was retested for confirmation; samples were considered sporozoite positive if absorbance exceeded twice the mean of seven negative controls (Beier *et al.*, 1988). Sporozoite infection rates, expressed as the proportion of *P. falciparum* positive of the total number of females tested were calculated per species per sampling method.

3.3.7 Biochemical assays

Biochemical assays were carried out on samples of F₁ progeny from wild caught females as well as the F₄ and F₃ generations of MBN-Carb and Kwag-Perm respectively. Glutathione S-transferase, general esterases activity, monooxygenases P450 and altered acetylcholine esterase (AChE) were assayed according to the method described by Penilla *et al.* (1998). For each microtitre plate, a batch of 47 mosquitoes (6 families with 6 mosquitoes each and 11 mosquitoes from KGB colony for comparison) was used. Mean enzyme activity absorbance was compared between the familial mosquitoes and the susceptible *An. arabiensis* (KGB) colony using a two sample *t*-test of means (Statistix 7.0, Analytical Software).

3.3.7.1 Homogenate preparation

Individual mosquitoes were transferred individually into microtitre wells using fine forceps and 50 µl of distilled water was added. The mosquitoes were thoroughly ground using a Teflon pestle. The crude homogenate was diluted to a volume of 200 µl per well using distilled water and used for enzyme assays as outlined below.

3.3.7.2 Glutathione-S-Transferase (GST) assay

The activity of GST was assayed using the method described by Lee, (1990) and Penilla *et al.* (1998). Using duplicates of each homogenate of a family line, 10 µl of the homogenate was placed in each well of a 96 well microtitre plate. This was followed by addition of 200 µl of: 10 mM reduced glutathione prepared in 0.1 M sodium phosphate buffer pH 6.5 and 65 mM of 1-chloro-2, 4-dinitrobenzene (CDNB) dissolved in methanol. Ten microlitres homogenate of a susceptible *An. arabiensis* laboratory colony originating from Kanyemba, Zimbabwe (KGB) was placed in separate wells of the same microtitre plate for comparative purposes. A well containing 10 µl of distilled water with 200 µl of CDNB was placed in a separate well to act as a negative control. The assay was then read kinetically on a plate reader (Labsystems Multiskan RC, Genesis software version 3.03) as a change in optical density for 5 minutes at 340 nm.

3.3.7.3 General esterase assay

The titration level of general esterases was determined. Two substrates: alpha and beta naphthyl acetate which are usually active in resistant insects, were used. Using the prepared homogenate, 2 x 20 µl replicates were pipetted into separate wells of a microtitre plate, 200 µl of alpha-naphthyl acetate solution [130 µl of 30 mM alpha-naphthyl acetate (NA) dissolved in 13 ml of 0.02 M sodium phosphate buffer (pH 7.2)] was added to one duplicate and 200 µl of beta naphthyl acetate solution [130 µl of 30 mM beta-naphthyl acetate also dissolved in 13 ml of 0.02 M sodium phosphate buffer (pH 7.2)] was added to the other. After incubating on ice for 30 minutes, 50 µl of: 0.023g fast blue salt dissolved in 2.25 ml distilled water plus 5.25 ml 5% sodium lauryl sulphate (SDS) dissolved in phosphate buffer (pH 7.0) were added to each well. A blank containing 20 µl distilled water, 200 µl of alpha-NA or beta-NA solution and 50 µl of stain were also placed in separate wells. Optical density was read at 570 nm as an end point.

3.3.7.4 Monooxygenases titration assay

The titration level of monooxygenases was determined per mosquito according to method described by Brogdon *et al.* (1988), Lee (1990) and Penilla *et al.* (1998). Using homogenate of individual mosquitoes 2 x 20 µl replicates of homogenate was transferred into separate wells of a microtitre plate, following which 80 µl of 0.0625 M potassium phosphate buffer (pH 7.2) was added to each replicate. This was followed by 200 µl of: 0.01g of 3, 3', 5, 5'-Tetramethyl Benzidine dissolved in 5 ml of methanol plus 15 ml of 0.25 M sodium acetate buffer (pH 5.0) added to each of the replicates. Finally 25 µl of 3% hydrogen peroxide was added to each well. Each plate was incubated at room temperature (23-26 °C) for 2 hours before optical density was read at 650 nm using a plate reader (Labsystems Multiskan RC, Genesis software version 3.03). Negative controls were run with 20 µl of buffer instead of the insect homogenate.

3.3.7.5 Insensitive acetylcholinesterase assay

The purpose of this assay is to detect the presence of an altered acetylcholinesterase following inhibition by the carbamate propoxur as described by Hemingway *et al.* (1986) and Penilla *et al.* (1998) with slight modifications.

Two x 25 µl replicates of crude homogenate per mosquito were placed in separate wells: 145 µl of Triton phosphate buffer (1% Triton buffer in 0.1 M phosphate buffer pH 7.8) was then added to each replicate and gently mixed to avoid bubble formation. A solution of 10µl of: [5, 5'-Dithio-bis (2-nitrobenzoic acid) DTNB in 2 ml of 0.1 phosphate buffer pH 7.0)] was added to each replicate. Finally, 25 µl of acetylthiocholine iodide (ASCHI) was added to one replicate while 25 µl of: ASCHI + 5 µl propoxur was added to the other replicate. A control was set up with blanks without any insect homogenate. For comparison a parallel run was done on the same plate using a susceptible laboratory colony (KGB) originating from Zimbabwe. The optical density was read using the Multiskan plate reader at 570 nm after 5 minutes of incubation.

3.3.7.6 Protein assay

The purpose of this assay is to measure the amount of total protein in each mosquito. A comparison between the means of each familial sample and the susceptible sample was then done in order to establish a correction factor that was used to adjust for the other assays.

Using a microtitre plate 2 x 10 µl replicates per mosquito were placed in separate wells and 300 µl of a BIO-Rad protein assay dye reagent concentrate (Bio-Rad Laboratories GmbH, Cat no. 500-0006) prepared as 1:4 dilution in distilled water was added. One blank was prepared with 10 µl of distilled water and 300 µl of BIO- Rad solution. The reaction was read on a plate reader (Labsystems Multiskan RC, Genesis software version 3.03) at 570 nm after incubation for 5 minutes.

Data analysis

Differences in enzyme level/activity between the susceptible (KGB) and family/selected strain samples were analyzed by Z-Student's *t*-test. All levels of significance were determined at $P < 0.05$.

3.3.8 Synergist assays

Synergists are compounds that are non-toxic, but serve to enhance toxicity of an insecticide with which they are combined. Insecticide synergists act by blocking the enzymes affecting insecticide detoxification.

3.3.8.1 Effect of PBO on carbamate and pyrethroid-resistant lines

The effect of piperonyl butoxide (PBO), an inhibitor of monooxygenase activity and a general insecticide synergist, was assayed in relation to the bendiocarb and permethrin resistance phenotypes. Three batches of 25-30 mosquitoes each were drawn from each colony. One sample was exposed to 4% PBO for 1 hour prior to exposure to insecticide for 30 minutes, whereas the second batch was only exposed to insecticide. The third batch,

which acted as a control, was exposed to PBO only. Mortality was scored after 24 hours post exposure. Following 5 replicates, mortalities between synergized and non-synergized samples were compared by replicate using Chi-square contingency tables.

3.3.8.2 Effect of DEM on carbamate and pyrethroid-resistant lines

The effect of diethyl maleate (DEM), an inhibitor of GST activity and a general insecticide synergist, was assayed in relation to the bendiocarb and permethrin resistance phenotypes. Three batches of 20-25 mosquitoes each were drawn from each colony. One sample was exposed to 8% DEM for 1 hour prior to exposure to insecticide for 30 minutes, whereas the second batch was only exposed to insecticide. The third batch, which acted as a control, was exposed to DEM only. Mortality was scored after 24 hours post exposure. Following 5 replicates, mortalities between synergized and non-synergized samples were compared by replicate using Chi-square contingency tables.

3.3.9 Molecular assay for knockdown resistance (*kdr*)

The mutation associated with knockdown resistance to pyrethroids/DDT was assayed for using the method previously described by Martinez-Torres *et al.* (1998) and Ranson *et al.* (2002).

3.3.9.1 Extraction of DNA

DNA was extracted from bioassay survivors of wild caught females and those mothers whose F₁ progeny was resistant to laboratory bioassays according to the method described by Collins *et al.* (1987). Mosquito abdomens retained after ELISA assay were individually placed into 1.5 ml eppendorf tubes and ground in 100 µl of grinding buffer (80 mM sodium chloride; 160 mM sucrose; 50.8 mM EDTA pH 8; and 130 mM Tris chloride pH 8), incubated at 70°C for 30 minutes then 14 µl of 8 M potassium acetate were added to each. The reaction mixture was then thoroughly mixed by inverting the eppendorf tubes before incubating on ice for 30 minutes. After incubation, samples were centrifuged at 13,000 rpm for 10 minutes at room temperature (23-26°C). Aliquots of the supernatant containing

suspended DNA were transferred into fresh tubes and incubated at -20°C for 1 hour followed by centrifugation at 13,000 rpm for 30 minutes. The supernatant was discarded and the remaining pellet was washed in 200 µl of ice cold absolute ethanol (99.8%) followed by centrifugation at 13,000 rpm for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol and air dried for 5 minutes at room temperature (23-26°C). The pellet was then resuspended in 100 µl of 1xTE.

3.3.9.2 Detection of *kdr* mutation

Three independent PCR assays were set up for each sample: the first contained the primers Agd 2 and Agd 4 which amplify a 137-bp product for the insecticide susceptible allele; the second contained primers Agd 1 and Agd 3 for amplifying a 195-bp product associated with the West African resistant allele and the third contained primers Ag1 and Agd5 for amplifying a 195-bp product associated with the East African resistant allele (Martinez-Torres *et al.*, 1998). The primers and PCR ingredients used were purchased from Inqaba Biotech South Africa and the primers sequences are tabulated in Table 3.2.

Using the suspended genomic DNA, 1 µl of DNA was pipetted into a reaction mixture containing 0.5 units of *Taq* DNA polymerase (Inqaba Biotechnical Industries, South Africa), enzyme buffer X1, 1.5mM MgCl₂, 0.1 mM dNTP, 40 pmol of Agd1, and 20 pmol of Agd3 for resistant allele or 40 pmol of Agd2 and 20 pmol of Agd4 for the susceptible allele making up a final volume of 12 µl with distilled water. The reaction mixture was placed in a thermal cycler (Primus 96plus MWG Biotech) with the following thermal profile: 94°C for 2 minutes, 40 cycles consisting of 94°C denaturation for 30sec, 50°C annealing for 30 sec, and 72°C extension for 30 sec and a final autoextension step at 72°C for 5 minutes to reduce primer dimers. Four microlitres of ficol loading dye was then added to 12 µl of the amplified PCR products and loaded on a 2.5% ethidium stained dye submerged in 1XTAE buffer and subjected to electrophoresis at 100V for 1 hour.

Table 3.2: Sequences of the primers used for *kdr* studies (Martinez-Torres *et al.*, 1998)

Primer	Sequence (5'- 3')
Agd1	5' ATAGATTCCCCGACCATG 3'
Agd2	5' AGACAAGGATGATGAACC 3'
Agd3	5' AATTTGCATTACTTACGACA 3'
Agd4	5' CTGTAGTGATAGGAAATTTA 3'
Agd5	5'-TTTGCATTACTTACGACTG-3'

3.3.9.3 Sequence analysis of the IIS6 domain

The 293bp fragment of the IIS6 domain containing the *kdr* mutation was amplified using Agd 1 and Agd 2. This fragment was sent to Inqaba Biotechnical Industries, South Africa for sequencing inorder to verify PCR results.

Data analysis

Kdr mutations results were analyzed using Statistix 7.0 Analytical Software and the sequences were analyzed using Lasergene package (DNASTAR version 7, SeqMan program, Inc, Madison, WI). Multiple sequence alignment was generated using the ClustalW (<http://www.ebi.ac.uk/cgi-bin/clustalw>).

Chapter 4

Results

4.1 Mosquito collection and identification

Total numbers of adult anophelines collected in the field using the various collection methods are presented in Table 4.1. In total, 648 anophelines belonging to four different taxa were collected. The *An. gambiae* complex comprised 98% of the overall collection. Other taxa collected included *An. squamosis* group (0.9%), *An. coustani* (0.5%) and *An. funestus* group (0.3%). Two specimens of *An. funestus* group were identified as *An. funestus* using the PCR assay as described by Koekemoer *et al.* (2002).

Table 4.1: Anopheline species collected in Gwave Village.

Collection method	<i>An. gambiae</i> complex	<i>An. squamosis</i> complex	<i>An. coustani</i> complex	<i>An. funestus</i> group	Total (%)
Cattle kraal	554	6	3	2	565 (87.2)
Larval catches	46	0	0	0	46 (7.1)
Man baited	37	0	0	0	37 (5.7)
Indoor resting	0	0	0	0	0
Total (%)	637 (98.3)	6 (0.9)	3 (0.5)	2 (0.3)	648

The overwhelming majority (87%) of these mosquitoes were collected using the cattle kraal collection method. Larval collections contributed 7.1% of the total catch while 5.7% were captured using man-baited net trap. The highest taxa diversity was recorded in the cattle kraal collections while larval and man-baited net trap collections produced the lowest species richness. Indoor resting catches were unsuccessful and no adults were collected after searching in 25 houses.

Table 4.2 shows details of the *An. gambiae* complex species caught and their abundance in relation to the various collection methods. A total of 637 members of the *An. gambiae* complex were caught. PCR assays (Figure 4.1) confirmed the identity of 573 of them. Of these 472 (72.8%) were *An. arabiensis*, 79 (12.8%) were *An. quadriannulatus* spp A and 22 (3.4%) were *An. merus*. The remaining 64 (10%) could not be identified because either the DNA had degraded, or because technical problems arose during assays or specimens were morphologically misidentified. *Anopheles arabiensis* was the dominant species across all the collection methods. Proportional distribution of the three species was similar between the cattle kraal and larval collections with *An. quadriannulatus* species A always dominating *An. merus*. On the other hand in man-baited net trap collections *An. merus* dominated *An. quadriannulatus* spp A.

Table 4.2: Proportions of *An. gambiae* complex species caught at Gwave.

	N	Habitat type					
		Cattle kraal		Larval		MBN	
		n	%	n	%	n	%
<i>An. arabiensis</i>	472	422	84.4	36	83.7	14	46.7
<i>An. quadriannulatus</i> species A	79	66	13.2	6	14.0	7	23.3
<i>An. merus</i>	22	12	2.4	1	2.3	9	30.0
Total	573	500	87.2	43	7.5	30	5.2

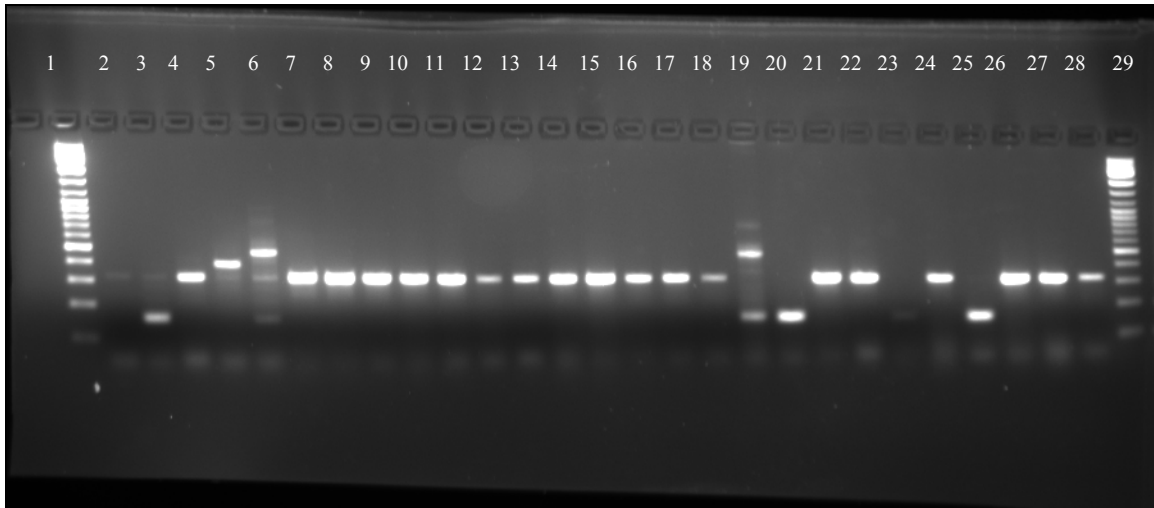


Figure 4.1: *Anopheles gambiae* species specific PCR. Lane1 and 29: 1 kilobase gene ruler molecular marker; lane 2: negative control; lane 3: *An. quadriannulatus* spp A positive control; lane 4: *An. arabiensis* positive control; lane 5: *An. gambiae* positive control; lane 6: *An. merus* positive control; lanes 7-18, 21, 22, 24, 26-28: *An. arabiensis*; lane 19: *An. merus*; lane 20, 25: *An. quadriannulatus* species A and lane 23: no amplification.

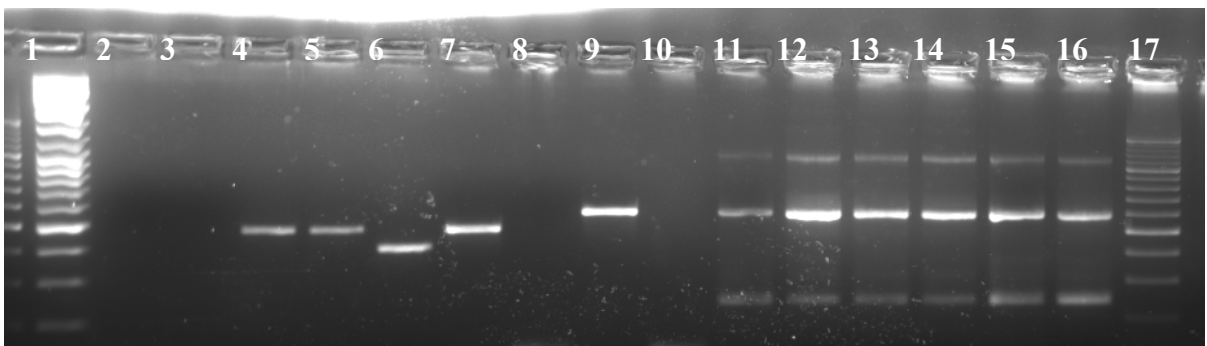


Figure 4.2: *Anopheles funestus* species specific PCR. Lane 1 and 17: 1 kilobase gene ruler DNA ladder; lane 2: negative control; lane 3, 8 and 9: no amplification; lane 4, 5, and 7 *An. rivulorum* positive control; lane 9: *An. funestus* positive control; lane 11-16: *An. funestus*.

4.2 Mosquito Infectivity

Table 4.3 summarizes infectivity rates of *An. gambiae* complex mosquitoes tested for *P. falciparum* infection. A total of 530 anophelines were examined by ELISA for *P. falciparum* CSA. All specimens giving positive ELISA results were re-tested to confirm results. Only two specimens (0.4%) were positive. These two females were identified as *An. arabiensis* giving an overall infection rate of 0.5% for *An. arabiensis*. Analysis per collection method per sibling species gave an infection rate of 14.3% for *An. arabiensis* collected by MBN catches. The other sibling species were not infected.

Table 4.3: Comparison of *Plasmodium falciparum* circumsporozoite antigen rates for the *An. gambiae s.l.* collected from Gwave.

Collection method	<i>An. arabiensis</i>		<i>An. merus</i>		<i>An. merus</i>		Total	
	total	+ve	total	+ve	total	+ve	total	+ve
	tested	n (%)	tested	n (%)	tested	n (%)	tested	n (%)
Cattle kraal	422	0 (0.0)	12	0 (0)	66	0 (0)	500	0 (0)
Man-baited net	14	2 (14.3)	9	0 (0)	7	0 (0)	30	2 (6.7)
Total	436	2 (0.5)	21	0 (0)	73	0 (0)	530	2 (0.4)

4.3 Bioassays

4.3.1 Field susceptibility tests

Table 4.4 shows WHO susceptibility tests carried out on wild-caught *Anopheles gambiae* complex as well as adults reared from larval collections. Susceptibility tests included the following insecticides: 4% DDT, 0.75% permethrin, 4% dieldrin, 5% malathion and 0.1%

bendiocarb. Results revealed that the wild populations were completely susceptible to malathion, bendiocarb and dieldrin but showed resistance to permethrin (a pyrethroid) and DDT according to WHO criteria (WHO, 1998). After PCR identifications, results showed that 78 out of 110 (70.9%) and 41 out of 87 (47.1%) *An. arabiensis* were susceptible to 4% DDT and 0.75% permethrin respectively. *Anopheles quadriannulatus* spp A showed susceptibility level of 70% against 4% DDT and 66.7% against 0.75% permethrin (Table 4.5). *Anopheles merus* showed evidence of resistance to both DDT and permethrin with susceptibility levels of 40% and 75% respectively. All the three sibling species were completely susceptible to 4% dieldrin, 5% malathion and 0.1% bendiocarb (Table 4.5).

Table 4.4: Summary of susceptibility status of *An. gambiae* complex from Gwave to insecticides.

Insecticide	Mortality 24 hr post exposure			
	(n) tests	sample size (n)	mean % mortality	WHO interpretation
0.75% permethrin	4	107	53	Resistant
4% DDT	8	193	72	Resistant
4% dieldrin	2	59	100	Susceptible
0.1% bendiocarb	2	53	100	Susceptible
5% malathion	2	58	100	Susceptible

4.3.2 F₁ progeny susceptibility tests

Table 4.6 details WHO susceptibility test results of exposures done using 0.75% permethrin and 4% DDT on F₁ progeny of families reared from wild caught *An. arabiensis*.

Table 4.5: Insecticide susceptibility status of wild caught *An. gambiae* sibling species.

Species	Insecticides									
	Permethrin		DDT		dieltrin		bendiocarb		malathion	
	Total (n)	(%) mort	Total (n)	(%) mort	Total (n)	(%) mort	Total (n)	(%) mort	Total (n)	(%) mort
<i>An. arabiensis</i>	87	47*	110	68.4*	37	100	40	100	52	100
<i>An. merus</i>	12	66*	5	40*	-	-	-	-	3	100
<i>An. quadrinnulatus</i>	4	75*	20	70*	4	100	6	100	11	100

* Resistant according to WHO criteria

After exposure to 0.75% permethrin for 1 hour mortalities ranged between 0% and 100%. There was evidence of permethrin resistance in 21 out of 37 families (56.8%) and average mortality across family was 69.8% (Table 4.7). After 1 hour exposure to 4% DDT mortalities ranged between 28.6% and 100% and there was evidence of resistance in 16 out of 59 families (25.4%) exposed. The average mortality across family was 85.9%. Families 27, 64, 94, 118, 144, and 156 showed cross resistance to both DDT and permethrin exposures.

Table 4.6: WHO insecticide susceptibility test results on 1-3 day old F₁ *An. arabiensis* from Gwave. Results expressed as % mortality 24 hr post exposure. (* indicates families showing cross resistance to DDT and permethrin).

4% DDT						
Fam.	(n)	% mort		Fam.	(n)	% mort
27*	7	28.6		103	9	100
33	11	45.5		104	8	87.5
37	11	72.7		108	7	100
38	9	100		109	8	100
41	18	66.7		111	11	100
46	5	100		113	10	60
48	10	100		116	10	100
50	9	88.9		117	9	100
53	11	100		118*	9	66.7
57	7	100		120	10	100
60	11	81.8		127	9	100
62	13	100		134	7	100
63	10	100		135	11	100
64*	12	33.3		136	9	100
66	7	100		139	11	100
68	7	100		142	7	100
74	12	16.7		144*	14	78.6
75	6	83.3		149	11	54.6
77	14	100		150	5	40
79	9	88.9		153	12	91.7
80	10	100		156*	13	69.2
82	9	100		169	10	100
84	10	90		166	8	100
85	13	100		178	7	100
90	8	100		180	9	100
94*	8	62.5		187	10	100
95	13	69.2		189	9	88.9
96	9	88.9	191	6	100	
97	11	81.8	194	9	66.7	
101	9	100				

0.75% Permethrin						
Fam.	(n)	% mort		Fam.	(n)	% mort
27*	13	53.9		144*	15	66.7
37	11	81.8		149	10	100
38	12	66.7		154	8	87.5
46	8	0		156*	11	45.5
48	10	70		166	12	83.3
53	12	100		169	10	60
57	15	46.7		178	9	55.6
64*	8	50		180	9	100
66	11	45.5				
74	12	100				
77	11	63.6				
80	10	90				
82	8	63.6				
84	11	54.5				
85	10	50				
90	10	100				
94*	9	44.4				
95	6	100				
97	13	92.3				
101	11	18.2				
103	10	90				
108	8	100				
111	11	72.7				
104	12	50				
113	4	100				
118*	6	16.7				
120	10	100				
135	10	80				
139	6	66.7				

Table 4.7: Summary of WHO insecticide susceptibility tests using F₁ *An. arabiensis* reared from wild caught females.

Insecticide	Mortality 24 hr post exposure		
	families tested (n)	sample size (n)	% mortality across family
0.75% permethrin	37	372	69.8
4% DDT	59	567	85.9

4.3.3 Bioassays for resistance selections

In order to fully understand mechanisms conferring carbamate and pyrethroid resistance in *An. arabiensis*, artificial selections were done to two laboratory colonies originating from KwaZulu/Natal, South Africa.

4.3.3.1 Selection for carbamate resistance (MBN-Carb)

Anopheles arabiensis originating from Kwazulu-Natal designated MBN-Carb was exposed to the diagnostic dosage of 0.1% bendiocarb using the WHO procedure at four different exposure periods in order to obtain survivors. These were then used to produce subsequent generations. Figure 4.3 shows the results of WHO susceptibility tests against the base line colony. After exposing the MBN baseline colony (F₀) to 0.1% bendiocarb for 20, 30, 40, and 60 minutes, mean mortalities 24 hour post exposure were recorded as 72%, 87%, 94% and 100% respectively. Based on these results an exposure period of 20 minutes was chosen in order to select a carbamate resistant line.

Bioassay data by generation (F₀-F₆), based on selection for resistance to 0.1% bendiocarb, are shown on Figure 4.4. Successive selections for resistance showed a steady decline in mortality across the generations. Mortality decreased from 71% in the F₀ generation to 39% in the F₆ cohorts. A comparison of bendiocarb tolerance was assayed between the selected F₆

cohorts and the MBN baseline colony (F_0). The results of this comparison are summarised graphically in Figure 4.5. The predicted exposure time causing 50% mortality (LT_{50}) was 6.9 minutes for the baseline colony and 68.8 minutes for the bendiocarb selected samples showing a 10 fold increase in the selected cohorts. However, following a 40 minute exposure, average mortality in the selected cohorts was 50% while the baseline colony showed 100% mortality.

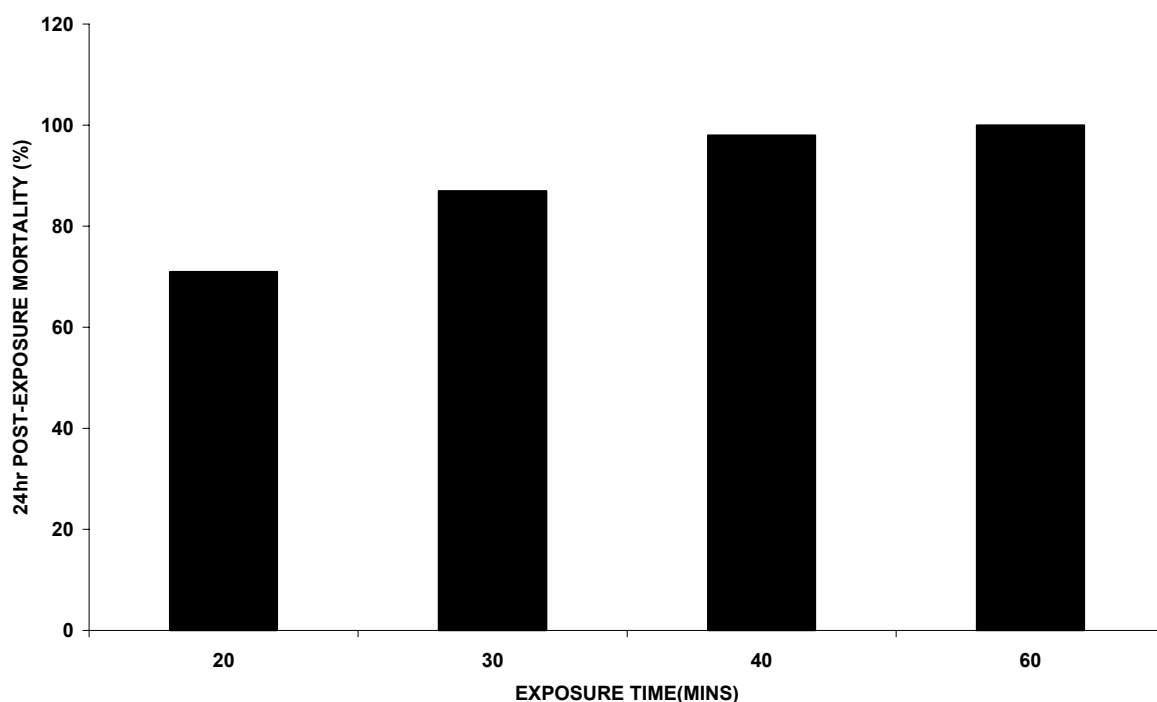


Figure 4.3: WHO bioassay results of 0.1% bendiocarb exposures against the MBN baseline colony at different exposure times.

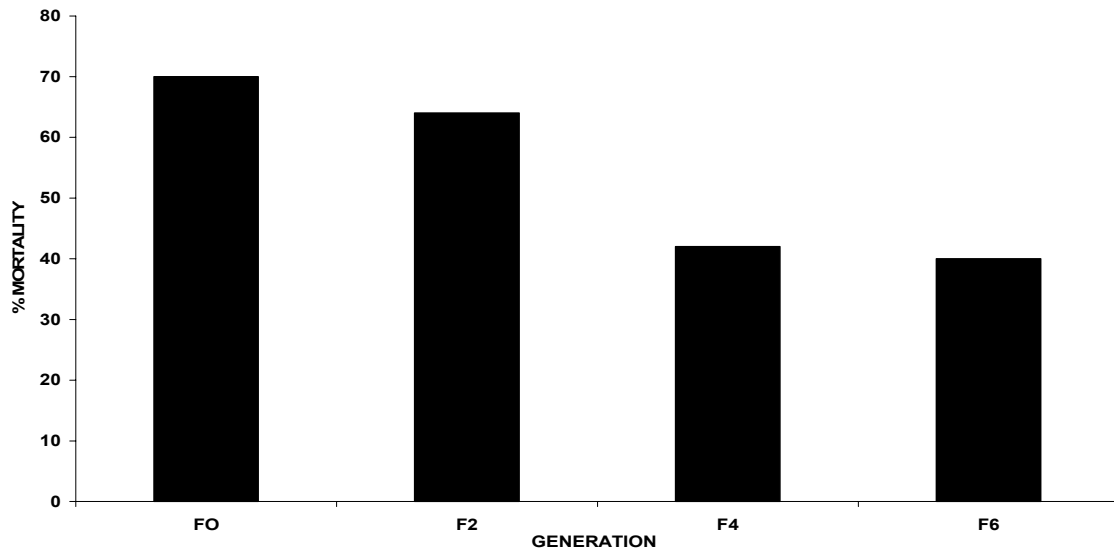


Figure 4.4: Results of susceptibility bioassays against the baseline control population, F_0 and carbamate resistance selected generations F_2 , F_4 , and F_6 . *Anopheles arabiensis* adults exposed to 0.1% bendiocarb for 20 minutes.

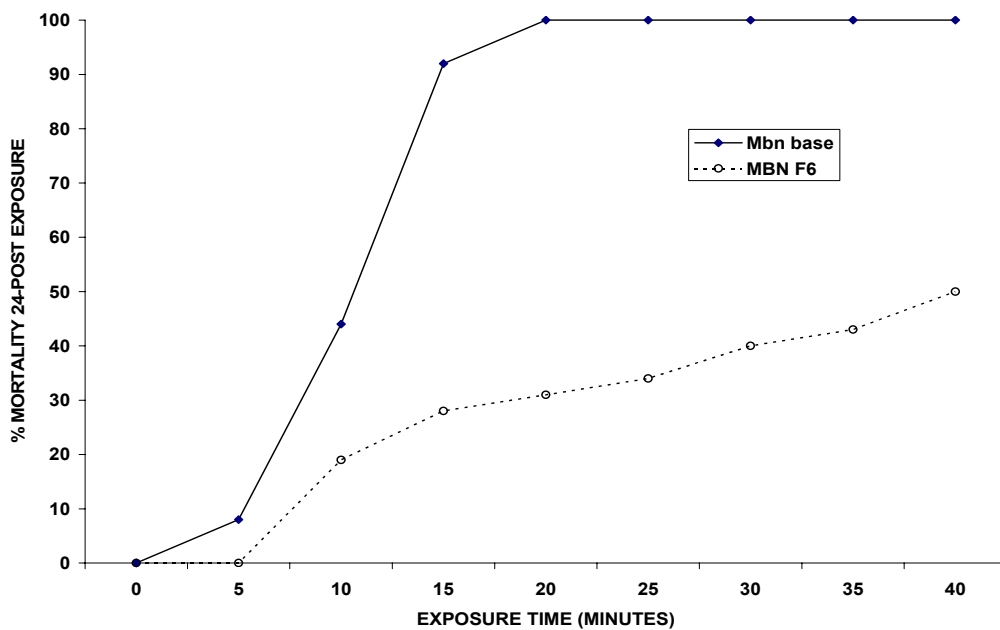


Figure 4.5: Time-mortality curve based on a range of 0.1% bendiocarb susceptibility tests using bendiocarb resistance selected cohorts and base line colony.

4.3.3.2 Selection for pyrethroid resistance (Kwag-Perm)

Selections started with exposures of *An. arabiensis* baseline colony originating from Kwazulu/Natal designated Kwag-Perm to 0.75% permethrin at four different exposure times (20, 30, 40 and 60 minutes). Mean percentage mortalities 24 hour post exposure for the respective exposure times are summarised in Figure 4.6. Average mortalities were 68%, 77%, 100% and 100% respectively. An exposure period of 30minutes was subsequently chosen in order to select a pyrethroid resistant line.

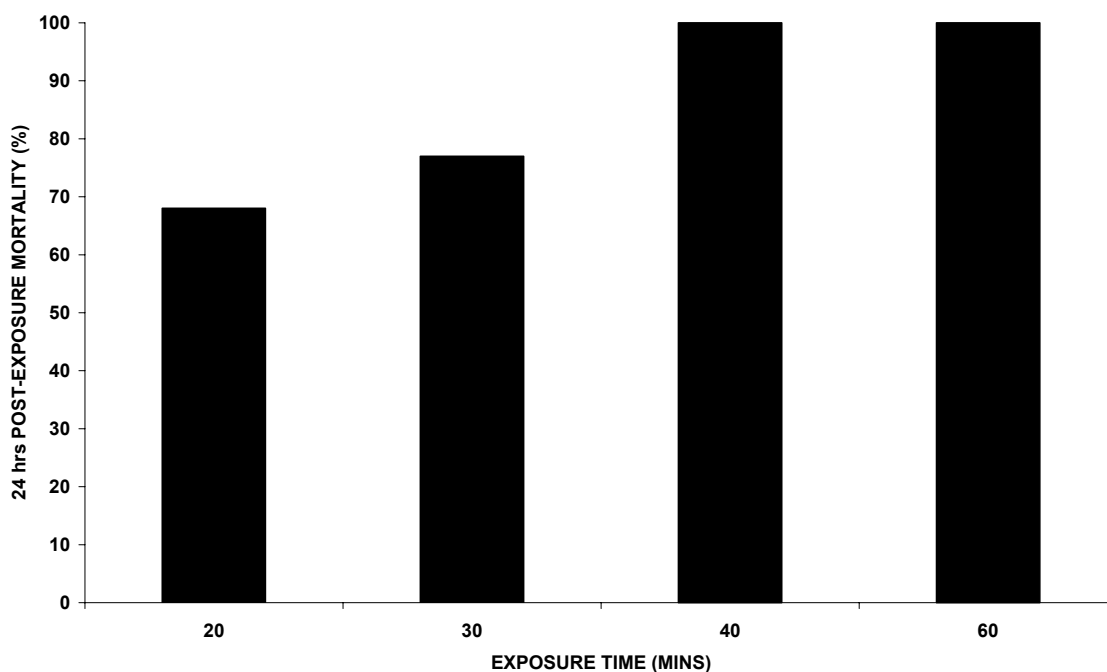


Figure 4.6: WHO bioassay results using 0.75% permethrin exposures at 4 different exposure times against the F_0 generation of the Kwag *An. arabiensis* colony.

Susceptibility test results by generation (F_0 - F_4), based on selection for pyrethroid resistance using 0.75% permethrin are summarised in Figure 4.7. Successive selections showed a decline in mortality across generations starting from a mortality of 70% in the F_0 to 35% in the F_4 cohorts. A comparison of mortalities at different exposure times between the baseline colony and the F_4 selected generation showed highly significant difference in observed mortalities between the two cohorts ($P < 0.000$). The predicted exposure time causing 50%

mortality (LT_{50}) was 7.3 minutes for the baseline colony and 34.4 minutes for the F_4 selected cohorts. Following 30 minutes exposure, mortality in the selected cohorts was 54% as opposed to 100% in the baseline colony (Figure 4.8).

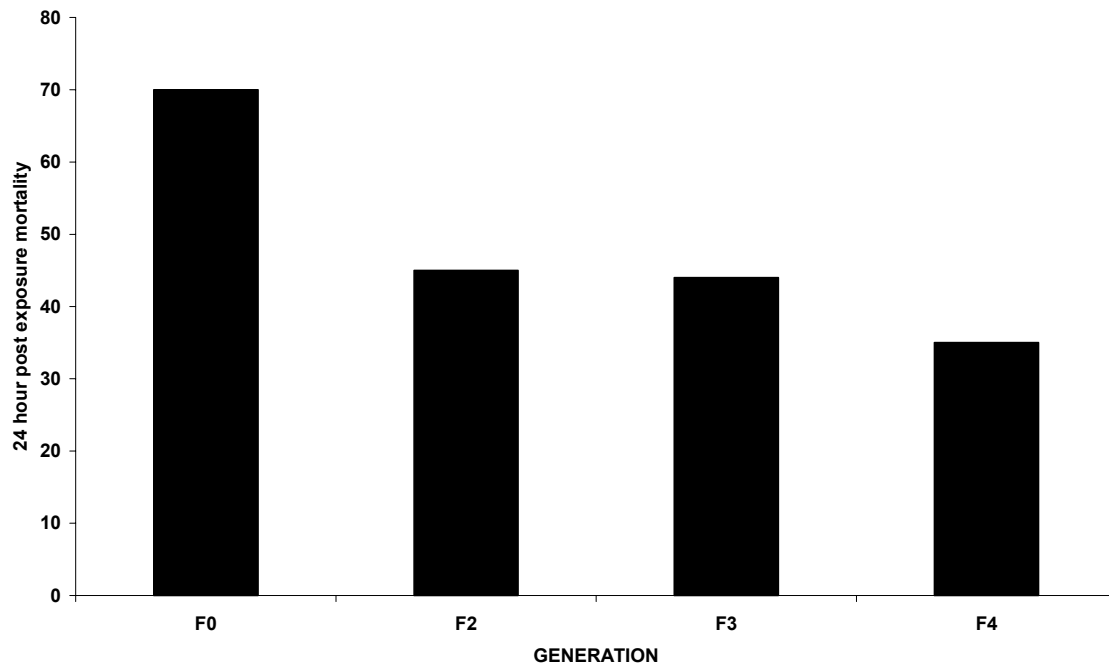


Figure 4.7: Results of 0.75% permethrin susceptibility bioassays against the *An. arabiensis* baseline control Kwag (F_0) and pyrethroid selected F_2 , F_3 , and F_4 generations.

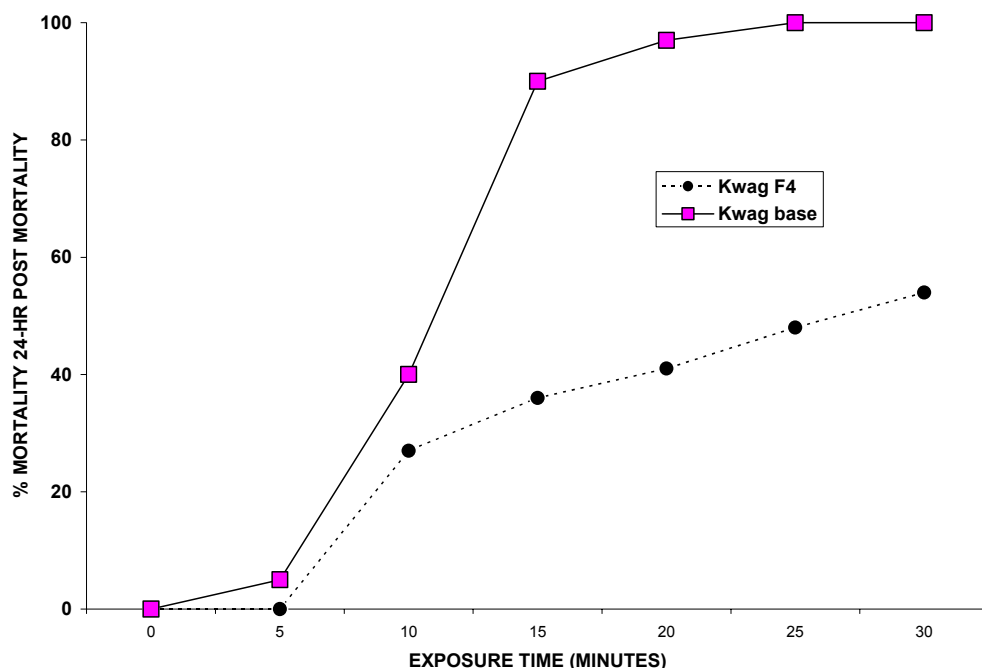


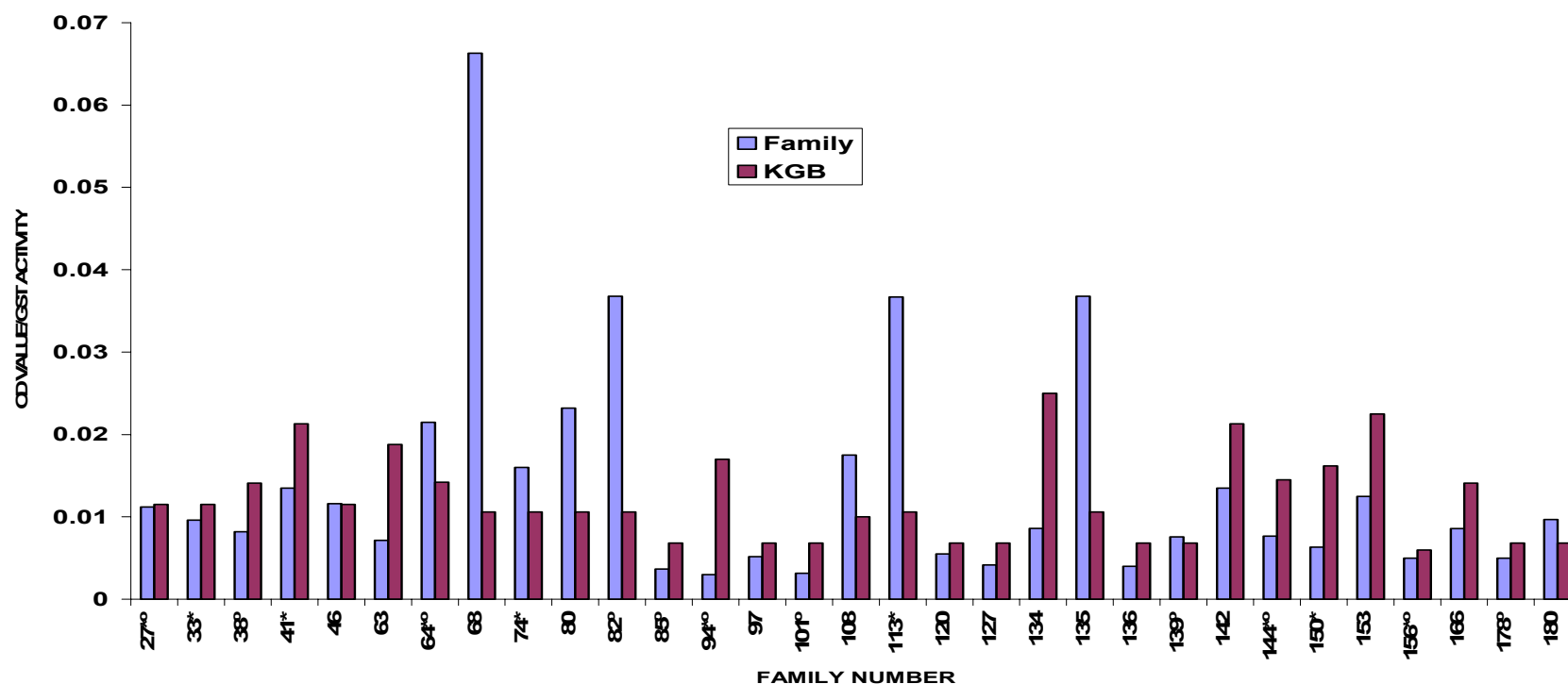
Figure 4.8: Exposure time using 0.75% permethrin vs. mortality for *An. arabiensis* Kwag base and selected KwagF₄ strain.

4.4 Biochemical assays

4.4.1 Family lines

4.4.1.1 Glutathione-S-transferase (GST)

Figure 4.9 shows the average level of GST activity/family of F₁ progeny of adult *An. arabiensis* females collected from Gwave compared with the standard susceptible KGB strain assayed on the same plate. Families 64, 68, 74, 80, 82, 108, 113 and 135, i.e. 8/31 (25.8%), showed significantly higher levels of GST activity ($P < 0.05$) than their corresponding control (KGB), based on two sample *t*-tests assuming unequal variances. Of the 8 families which showed elevated GST activity, 3 families (64, 74 and 113) showed correlation with DDT bioassay mortality data and 2 families were correlated with permethrin bioassay mortality data. Families 38, 85, 94, 142, 144, 150, 153 and 166, i.e. 8/30, showed significantly lower levels of GST activity ($P < 0.05$) compared to their



* Family resistant to DDT exposures

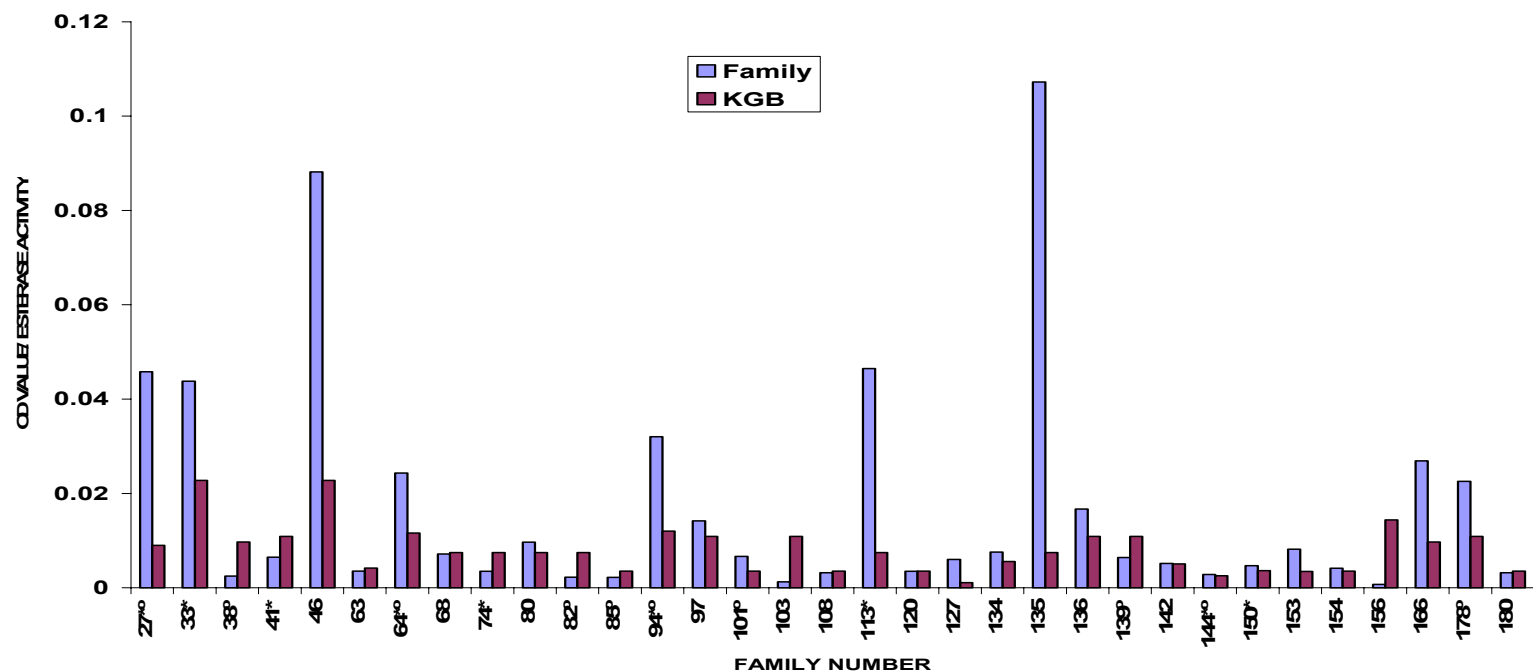
° Family resistant to permethrin exposure

Figure 4.9: Average levels of glutathione-S-transferase (GST) activity in *An. arabiensis* F₁ progeny, by family, and corresponding GST activity means for susceptible *An. arabiensis* (KGB) samples assayed simultaneously.

corresponding control (KGB). Of the families with suppressed GST activities families 94, 144 and 150 were resistant to DDT exposures while families 38, 85, 94 and 144 were resistant to permethrin exposures. Of the families showing cross resistance to both DDT and permethrin only family 64 had elevated GST. The other families, 94 and 144 had suppressed GST activity. Families 27 and 156 showed no significant difference in GST activity compared to the corresponding KGB control.

4.4.1.2 General esterase assay

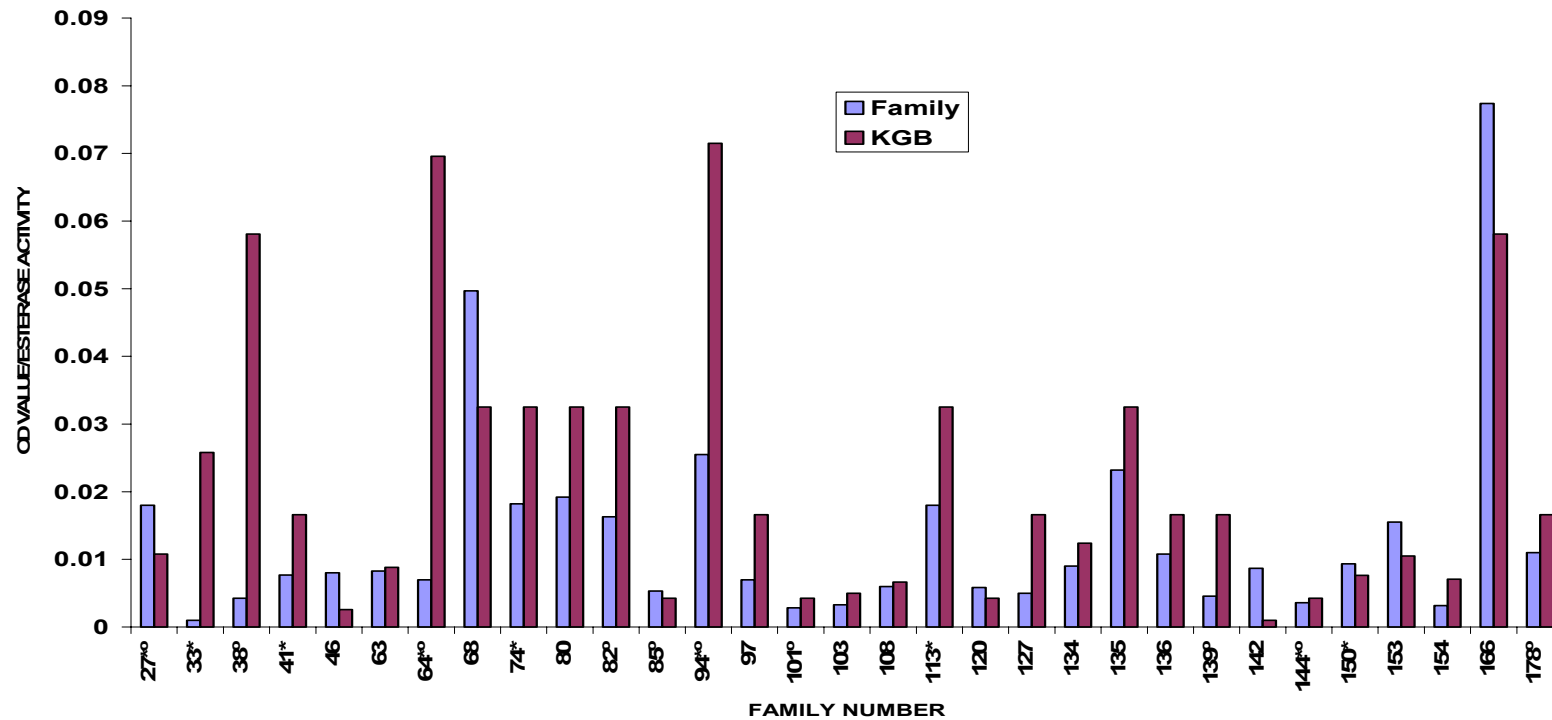
Figures 4.10 and 4.11 show the average levels of non specific esterase activity using alpha and beta-naphthyl acetate as substrates in *An. arabiensis* F₁ progeny as well as corresponding esterase activity means for the susceptible *An. arabiensis* (KGB) strain. A significant increase in esterase activity using the substrate alpha-naphthyl acetate was observed in families 27, 33, 46, 64, 94, 113, 135, 166 and 178, i.e. 9/33 families assayed (27.3%) ($P < 0.05$). Families 38, 41, 103, 139, and 156 gave activities significantly lower than the reference colony. All the other families showed no significant differences in activity compared to their corresponding KGB ($P > 0.05$) strain. Non-specific esterase activity based on beta-naphthyl acetate as a substrate showed that only two families (6.5%) gave an increased esterase activity while 14/31 (45.2%) showed activities significantly lower than the reference strain (KGB). There was significant correlation between the general esterase activities using the substrate alpha-naphthyl acetate. Families with elevated esterase activity using alpha-naphthyl as a substrate were either resistant to pyrethroids or DDT except for families 46, 135 and 166 which were susceptible to both DDT and permethrin. Three of the families (27, 94 and 64) showing cross resistance to both DDT and permethrin had elevated esterase activities using alpha-naphthyl acetate as a substrate. Family 156 which also showed cross resistance to both DDT and permethrin had suppressed general esterase activity.



* Family resistant to DDT exposures

° Family resistant to permethrin exposure

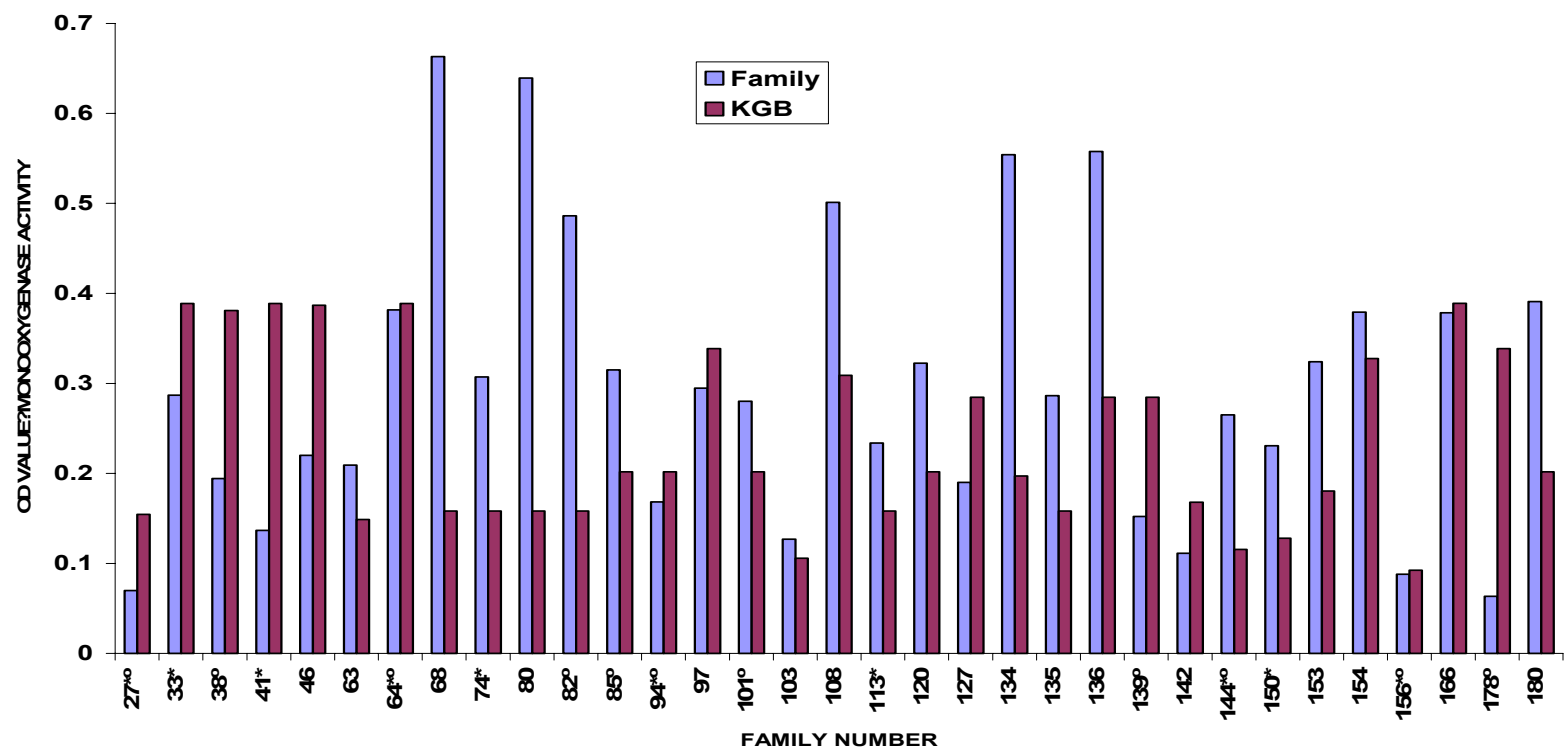
Figure 4.10: Average levels of non-specific esterases using alpha-naphthyl acetate as a substrate in F₁ progeny of wild caught *An. arabiensis*, by family, and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) samples assayed simultaneously.



* Family resistant to DDT exposures

° Family resistant to permethrin exposure

Figure 4.11: Average levels of non specific esterase using beta-naphthyl acetate as a substrate in F₁ progeny of wild caught *An. arabiensis*, by family, and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) assayed simultaneously.



* Family resistant to DDT exposures

° Family resistant to permethrin exposure

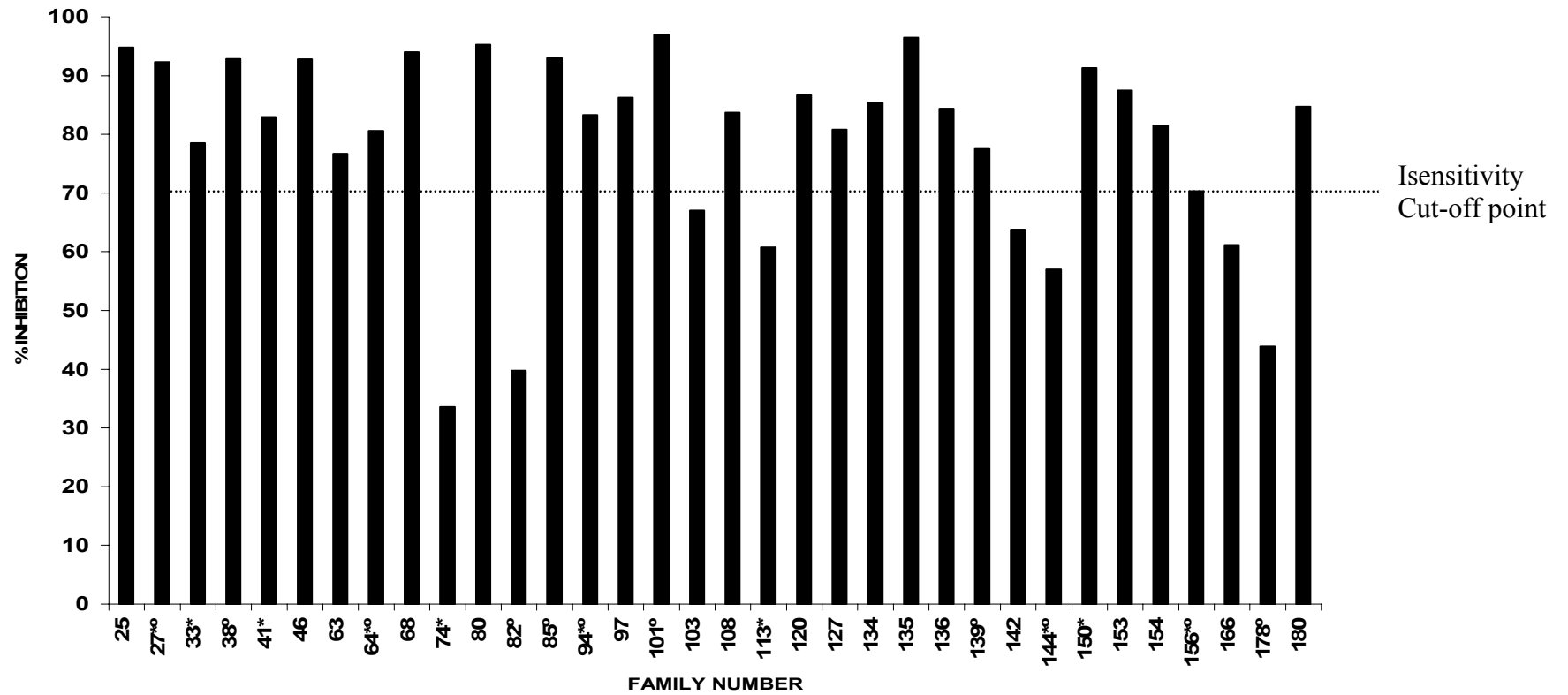
Figure 4.12: Average levels of monooxygenase activity in F₁ progeny of wild caught *An. arabiensis*, by family line, and corresponding means for the *An. arabiensis* strain (KGB) assayed simultaneously.

4.4.1.3 Monooxygenase assay

Figure 4.12 shows the average level of monooxygenase activity/family of F_1 progeny of adult females collected from Gwave compared with the standard susceptible KGB strain assayed on the same plate. Families 68, 74, 80, 82, 85, 101, 108, 113, 120, 134, 135, 136, 144, 150, 153 and 180 i.e. 16/33 families assayed (48.5%), showed significantly higher levels of monooxygenase activity ($P < 0.05$) than their corresponding controls (KGB), based on two sample t -tests assuming unequal variances. Seven families (27, 33, 38, 41, 46, 127, 139 and 142) showed monooxygenase levels significantly lower than the reference susceptible colony KGB. The remaining families (10/33) showed no significant difference in monooxygenase activity compared to the susceptible KGB colony. There was no significant correlation between monooxygenase activity and bioassay mortality data across families ($P > 0.05$). Of the 16 families showing elevated monooxygenases only four families (82, 85, 101, and 144) were resistant to permethrin exposures. Families 74, 113, 144 and 150 were resistant to DDT exposures and had elevated monooxygenases. Families 68, 80, 108, 120, 134, 135, 136, 153, and 180 had elevated monooxygenases despite being susceptible to both DDT and permethrin.

4.4.1.4 Insensitive acetylcholinesterase assay

The mean percentage propoxur inhibitions of AChE for the familial F_1 progeny of wild caught *An. arabiensis* are given in Figure 4.13. AChE inhibition rates by propoxur ranged from 33.6% to 97% for familial F_1 progeny compared to an average of 93% for the susceptible colony (KGB). The parameters of this assay are set so that percentage inhibition by propoxur less than 70% indicates the presence of an altered AChE. By this criterion, 8 families (Families 74, 82, 103, 113, 142, 144, 166 and 178), i.e 8/34 assayed, showed evidence of insensitive AChE despite absence of carbamate and organophosphates resistance in the bioassays.



* Family resistant to DDT exposures

° Family resistant to permethrin exposure

Figure 4.13: Mean acetylcholinesterase percentage inhibition by propoxur in F₁ progeny of *An. arabiensis* reared from wild-caught females.

4.4.2 Carbamate-resistant selected line (MBN-Carb)

Table 4.8 summarizes enzyme activities between the base line colony (F_0) and the F_4 generation of the MBN carbamate selected strain. Results showed that there were significant increases in non-specific esterase and GST activities, and no significant increase in monooxygenase activity. Levels of non-specific esterase using beta-naphthyl acetate as a substrate showed a highly significant difference between the selected and unselected cohorts (student t -test; $P < 0.05$, assuming unequal variance), increasing 5 fold. There was no significant difference in mean non-specific esterase levels using alpha-naphthyl acetate as a substrate ($P > 0.05$, assuming unequal variances). The mean level of GST activity was significantly elevated in the selected cohorts ($P < 0.05$) increasing two fold from the baseline colony.

Acetylcholinesterase assays gave no suggestion of reduced propoxur inhibition with an average inhibition of 88%. This was lower than the percentage inhibition in the baseline colony which was 92%. There was no significant difference in inhibition between the two generations ($P > 0.05$).

Table 4.8: Comparison of specific activities of alpha and beta non-specific esterases, monooxygenases, GSTs and insensitive acetylcholinesterase from the *An. arabiensis* MBN baseline and the 0.1% bendiocarb selected test strain.

Generation	α -esterases	β -esterases	MFO	GST	AChE
	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean % Inhibtn. $\pm$ SD
F_0	0.027 $\pm$ 0.016	0.003 $\pm$ 0.001	0.810 $\pm$ 0.347	0.547 $\pm$ 0.3	92 $\pm$ 6
F_4	0.037 $\pm$ 0.008	0.014 $\pm$ 0.003	0.615 $\pm$ 0.249	1.186 $\pm$ 0.552	88 $\pm$ 5.2
p -value	0.0643	0.0000	0.1408	0.0091	0.321

4.4.3 Pyrethroid-resistant selected line (Kwag-Perm)

Table 4.9 shows detoxifying enzyme levels of Kwag-Perm base line cohorts compared to the F₃ generation of a 0.75% permethrin selected strain. A significantly higher level of monooxygenase was detected in the selected (F₃) strain compared to the unselected parent colony (F₀) ($P = 0.0008$) showing a 5-fold increase in the selected strain. The mean level of glutathione S-transferase activity of the selected strain was not significantly higher than that of the unselected sample ($P > 0.05$). Levels of non-specific esterases using beta-naphthyl acetate as a substrate showed significant differences between the selected and unselected cohorts ($P < 0.05$). The baseline samples showed higher activity than the selected cohorts. However, there was no significant difference in mean non-specific esterase levels using alpha-naphthyl acetate as a substrate ($P > 0.05$). The percentage inhibition ranged from 75 % to 94% with an average of 83.5% for the selected cohorts while the baseline population showed inhibition ranging from 84% to 98% with an average of 91%. This shows no indication of the presence of an altered AChE in the permethrin selected strain.

Table 4.9: Comparison of specific activities of alpha and beta non-specific esterases, monooxygenases and GST between the *An. arabiensis* susceptible baseline strain and the 0.75% permethrin selected strain. *p*-values are based on 2-sample *t*-tests.

Generation	α -esterases	β -esterases	MFO	GST	AChE
	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean OD value $\pm$ SD	Mean % Inhibtn. $\pm$ SD
F ₀	0.032 $\pm$ 0.018	0.012 $\pm$ 0.002	0.101 $\pm$ 0.039	0.697 $\pm$ 0.419	91 $\pm$ 7
F ₃	0.019 $\pm$ 0.016	0.007 $\pm$ 0.003	0.512 $\pm$ 0.027	0.861 $\pm$ 0.429	83.5 $\pm$ 8.5
<i>p</i> -value	0.0804	0.011	0.0008	0.4054	0.172

4.5 Synergists bioassays

In order to confirm involvement of a specific upregulated enzyme system synergists studies were done. Synergist act by blocking the active site of an enzyme responsible for insecticide detoxification. This has an effect of fully exposing the respective insect to the toxic effect of an insecticide.

4.5.1 Effect of PBO on carbamate and pyrethroid-resistant lines

The effect of the monooxygenase synergist PBO on a 0.75% permethrin and a 0.1% bendiocarb resistance selected line is detailed in Table 4.10 and 4.11, respectively. Exposure to 4% PBO for one hour followed by 40 minutes exposure to 0.1% bendiocarb gave 100% mortality compared to an average of 55% for the unsynergized sub sample. The difference in mortality (24 hour post-exposure) between synergized and unsynergized samples was statistically significant ($\chi^2 = 18$; $P = 0.0000$).

Exposure to 4% PBO for one hour followed by 30 minutes exposure to 0.75% permethrin gave 100% mortality compared to an average of 59% for the unsynergized sub samples. The difference in mortality (24 hour post-exposure) between synergized and unsynergized samples was statistically significant ($\chi^2 = 15.73$, $P = 0.0013$).

4.5.2 Effect of DEM on carbamate and pyrethroid-selected lines

The effect of DEM, a GST synergist on a 0.75% permethrin and a 0.1% bendiocarb selected line is detailed in Table 4.12 and 4.13. Samples that were exposed to permethrin after DEM exposure showed higher mortalities averaging 78.9% compared to the mean mortality of 55.7% in samples exposed to permethrin alone ($\chi^2 = 14.42$, $P = 0.0007$). Exposures of samples to 8% DEM followed by 30 minutes exposure to 0.1% bendiocarb (synergized) gave 100% mortality while the unsynergized sample gave a mean mortality of 81%. The

difference in mortality between the two sub-samples was significant ($\chi^2 = 12.03$, $P = 0.0073$).

Table 4.10: Comparison of synergized and unsynergized mean percentage mortality 24 hour post exposure for F_4 generation of *An. arabiensis* (MBN) selected using 0.1% bendiocarb ($\chi^2 = 18$; $P = 0.0000$).

Treatment	n	% mortality $\pm$ SD (24 hour post-exposure)
4% PBO+0.1% bendiocarb	138	100 $\pm$ 0
0.1% bendio only	138	55.0 $\pm$ 3.5
4% PBO only	150	0.0

Table 4.11: Comparison of synergized and unsynergized mean percentage Mortality 24 hour post-exposure for F_3 generation of *An. arabiensis* (Kwag) selected using 0.75% permethrin. ($\chi^2=15.73$; $P = 0.0013$).

Treatment	n	% mortality $\pm$ SD (24 hour post-exposure)
4% PBO+0.75% permethrin	101	100 $\pm$ 0.00
0.75% permethrin only	111	59.0 $\pm$ 22.00
4% PBO only	150	0.00

Table 4.12: Comparison of synergized and unsynergized mean percentage mortality 24 hour post exposure for F₄ generation of *An. arabiensis* (Kwag) selected using 0.75% permethrin ($\chi^2 = 14.42$, P = 0.0007).

Treatment	n	% mortality $\pm$ SD (24 hour post-exposure)
8% DEM + 0.75% permethrin	57	78.7 $\pm$ 2.7
0.75% permethrin only	61	55.7 $\pm$ 3.7
8% DEM only	150	0.0

Table 4.13: Comparison of synergized and unsynergized mean percentage mortality 24 hour post exposure for F₇ generation of *An. arabiensis* (MBN) selected using 0.1% bendiocarb ($\chi^2 = 12.03$, P = 0.0073).

Treatment	n	% mortality $\pm$ SD (24 hour post-exposure)
4% DEM + 0.1% bendiocarb	83	100 $\pm$ 0
0.1% bendiocarb only	79	81.0 $\pm$ 5.2
8% DEM only	88	0.0

4.6 Molecular assay for *kdr*

4.6.1 PCR assay

Table 4.14 summarizes *kdr* genotype frequencies for wild collected *An. arabiensis* using PCR assay (Figure 4.14). A total of 22 individual mosquitoes resistant to DDT and 27 resistant to permethrin were assayed; 18 mosquitoes, (six from the DDT resistant samples and 12 from the permethrin resistant samples) consistently failed to amplify. The *kdr* mutation was absent (SS) in 68.2% of the DDT survivors assayed, while the frequency for heterozygotes (RS) was 22.7% and 9.1% for resistance homozygotes (RR). PCR assays for permethrin resistant samples showed that the *kdr* mutation was absent in 77.8% of those assayed while the frequency of resistance homozygotes and heterozygotes was 11.1% in both cases. Analysis of families showing cross resistance showed that four of the families were heterozygotes (RS) while two families family 118 and 144 were homozygous resistant (RR).

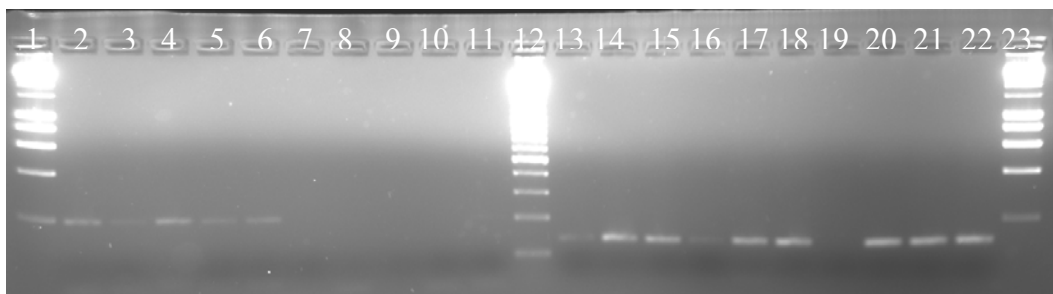


Figure 4.14: Results of PCR-based diagnostic test for *kdr* detection. PCR products separated on a 2.5% agarose gel stained with ethidium bromide. Lane 1, 12 and 23: 1 kilobase gene ruler molecular marker; Lane 2, 3, 4, 5 and 6: mosquito samples with *kdr* allele; lane 13-18, 20-22: susceptible samples; Lanes 7-11 and 19: no amplification.

4.6.2 Sequence analysis of the II S6 domain

Figure 4.15 shows the consensus sequence of 35 samples sequenced (all samples were sequenced once). Using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information GenBank (<http://www.ncbi.nlm.nih.gov/Genbank>),

the consensus sequence showed a 99% sequence identity (227/228 base pairs) to the *An. arabiensis* para-type sodium channel gene (accession number DQ2637491). Figure 4.16 is a cut-down chromatogram of the sodium channel gene region showing the location of the codon associated with the *kdr* mutation. After alignment of this region in individual sequences the *kdr* “Leu-Phe” mutation was absent in all the DDT and permethrin resistant mosquitoes screened.

Table 4.14: Frequencies of *kdr* genotypes in bioassay survivors to DDT or permethrin of *An. arabiensis* samples from Gwave, Zimbabwe.

Insecticide	Bioassay Phenotype	<i>kdr</i> genotype	(n)	Frequency
DDT	Resistant	SS	15	68.2
	Resistant	RS	5	22.7
	Resistant	RR	2	9.1
	Resistant	*	6	-
Permethrin	Resistant	SS	21	77.8
	Resistant	RS	3	11.1
	Resistant	RR	3	11.1
	Resistant	*	12	-
DDT+Perm	Resistant	RS	4	66.7
	Resistant	RR	2	33.3

* samples failed to amplify

```

Consensus      -----
AATGTCACGTGA 12
An. arabiensis TGAATCTGTAAAATTCCATCTTGGCAGATCATGGTCGGGGAATCTATAATGTCA-TGTA 59
                *****  ****

Consensus      AAAACGATCTTGGTCCATGTTAATTTGCATTACTTACGACTAAATTCCTATCACTACAG 72
An. arabiensis AAAACGATCTTGGTCCATGTTAATTTGCATTACTTACGACTAAATTCCTATCACTACAG 119
                *****

Consensus      TGGCCAAGAAAAATGGTATGCAGGATACATCACCGACAAGCATAACAATCCCACATTGATT 132
An. arabiensis TGGCCAAGAAAAATGGTATGCAGGATACATCACCGACAAGCATAACAATCCCACATTGATT 179
                *****

Consensus      CAATCCATTCTCCGCATAGCACACGGAACACAATCATGAAGGAATGCATGAAATCTGTAA 192
An. arabiensis CAATCCATTCTCCGCATAGCACACGGAACACAATCATGAAGGAATGCA----- 227
                *****

Consensus      AATTCCATCTTGGCAGATCATGGTCGGGGAATCTAT 228
An. arabiensis -----

```

Figure 4.15: Comparison of the consensus nucleotide sequence of the sodium channel gene of *An. arabiensis* with that deposited in the NCBI GenBank (accession number DQ263749). Identical positions are indicated by an asterisk (*) and (-) indicates missing nucleotides. Multiple sequence alignment was generated using the ClustalW (<http://www.ebi.ac.uk/cgi-bin/clustalw>).

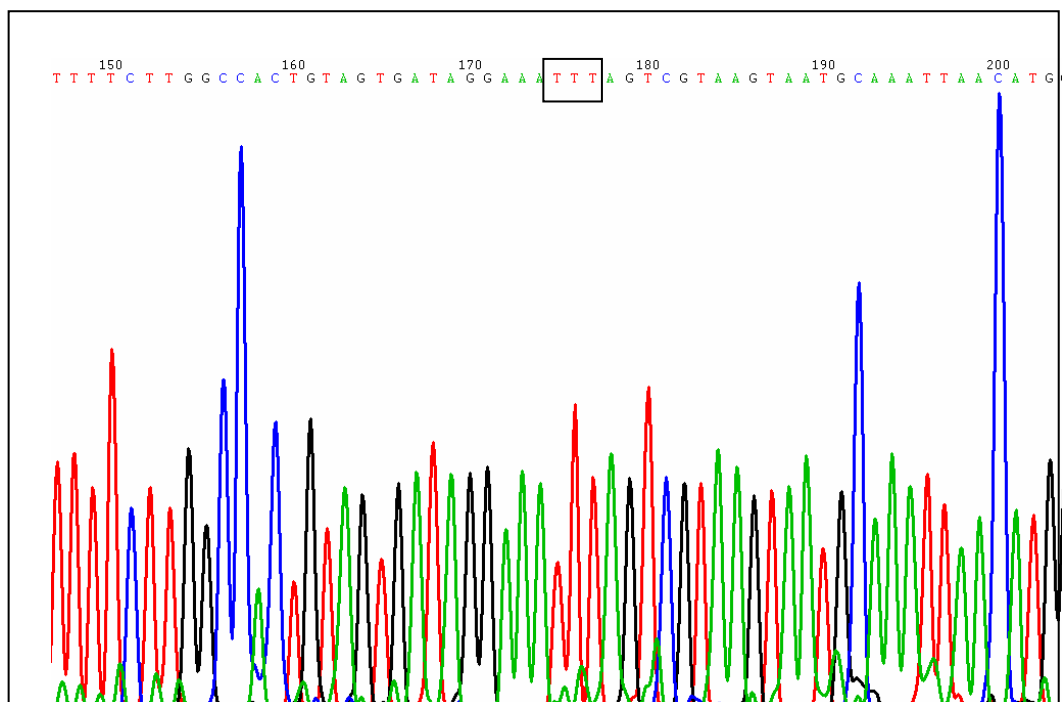


Figure 4.16: Cut-down chromatogram of sodium channel gene showing the key location where base substitution occurs resulting in *kdr* mutation.

4.7 Cross-resistance

Table 4.15 summarizes bioassays, biochemical assays and molecular assays for *kdr* mutation done to families showing cross resistance to DDT and permethrin. Bioassay results showed that all the six families were resistant to both DDT and permethrin exposures. Biochemical results showed that only family 144 had elevated monooxygenase activity. Family 64 had elevated GST activity. Families 27, 64 and 94 had elevated esterase activity using alpha-naphthyl acetate as a substrate. PCR assays to detect *kdr* mutation showed that families 64, 94, 118 and 144 were heterozygous resistant and families 27 and 156 were homozygous resistant. Sequence analysis of the II 6 domain sodium channel gene showed complete absence of the *kdr* mutation.

Table 4.15: Summary of bioassays, biochemical analyses and molecular assays done to families showing cross-resistance to DDT and permethrin exposures.

Fam.	Bioassay		Biochemical assay				<i>kdr</i> mutation	
	DDT	Perm.	MFO	GST	α -est.	β -est.	PCR	seqn.
27	R	R	-	=	+	+	RR	SS
64	R	R	=	+	+	-	RS	SS
94	R	R	-	-	+	-	RS	SS
118	R	R	N/A	N/A	N/A	N/A	RS	SS
144	R	R	+	-	=	=	RS	SS
156	R	R	=	-	=	=	RR	SS

R-resistant; -: suppressed enzyme activity; +: upregulated enzyme activity =: no changes in enzyme activity

Chapter 5

Discussion

5.1 Mosquito collections

In Zimbabwe, 43 anopheline taxa have been recorded, of these, seven are known to occur within Gokwe district (Crees and Mhlanga, 1985; Mouchet *et al.*, 2004; Masendu *et al.*, 2005). Despite the limited scope and time of our collections 4 anopheline taxa; *An gambiae* complex, *An. funestus* group, *An. coustani* complex and *An. squamosis* complex were collected tallying with species ranges previously recorded in this area. Four collection methods, man-baited net trap, larval collections, cattle kraal collections and indoor house searches were used. Mosquito sampling was carried out for 5 days. The total number of mosquitoes collected was far below similar studies done in the same area. This might be due to a number of factors. Firstly our collection methods were limited as alluded to earlier. Collections of adult mosquitoes could have been accomplished by using a variety of other available methods. These include traps such as light traps baited with carbondioxide, window exit traps, and pit trips. Another contributing factor may be spraying which had been done a month earlier. During the period of our collections 72% of the village had been covered by the NMCP spraying team. All homesteads visited had been sprayed with Icon[®] 10 WP. This spraying significantly affected indoor resting catches to the extent that 25 homesteads were visited and no individual anopheline was collected and therefore this collection method was abandoned.

Highest species richness was recorded in the cattle kraal collections. This should be expected as most of the anophelines caught are highly zoophilic and exhibit exophilic behavior. The exophilic behavior of *An. coustani* and members of the *An. gambiae* complex caught are well documented (Gillies and Coetzee, 1987). Cattle kraal collections also collected relatively more mosquitoes than the other collection methods indicating that it is a much better method of sampling in this particular area. However, its main disadvantage is that it mainly collects zoophilic mosquitoes and is thus highly biased.

Transmission of malaria is complicated by the number of vector species involved. Studies carried on the African continent indicate that at least two of the main vector species often occur in sympatry (Gillies and Coetzee, 1987; Coluzzi, 1984). Most of these vectors also occur as members of cryptic or sibling species complex that include both vector and non-vector species. Members of the *An. gambiae* complex constitute the primary vectors of malaria in Zimbabwe. Four species *An. gambiae* s.s, *An. arabiensis*, *An. merus* and *An. quadriannulatus* occur in various sympatric combinations. *Anopheles quadriannulatus* is always found in sympatry with one or both of the malaria vectors (Taylor *et al.*, 1986; Crees, 1996; Masendu, 1996; Masendu *et al.*, 2005).

During this study 98.3% of the total catches constituted members of the *An. gambiae* complex as collections were targeted towards members of the *An. gambiae* complex. Identification by rDNA species-specific PCR showed that three members of the *An. gambiae* complex; *An. arabiensis*, *An. merus* and *An. quadriannulatus* were captured. In all the collections methods used these three species were found in sympatry. This is consistent with previous studies done in the same area (Manokore *et al.*, 2000, Masendu *et al.*, 2005). In Gokwe district four members of the *An. gambiae* complex *An. gambiae* s.s, *An. arabiensis*, *An. merus* and *An. quadriannulatus* species A have been reported to occur in various combinations of sympatry (Mpofu *et al.*, 1986; Masendu *et al.*, 2005). Absence of *An. gambiae* s.s in our collections probably points to the effectiveness of the indoor residual spraying done yearly in this area. *Anopheles gambiae* s.s is highly anthropophagic and exhibit endophilic behavior and is thus highly susceptible to indoor residual spraying (Gillies and Coetzee, 1987).

Analysis of distribution of the three species in each collection method showed that *An. arabiensis* predominated the other species contributing on average 82% of larval and cattle kraal collections and 46% of MBN collections. Overall, *An. arabiensis* contributed 72.8% of the total sample size. This concurs with similar studies carried out in this area which showed that *An. arabiensis* is the predominant sibling species in Gokwe district (Mpofu *et al.*, 1986; Manokore *et al.*, 2000; Masendu *et al.*, 2005). Cattle kraal collection constituted 65% of *An. arabiensis* collected and is expected as it has been shown that a high proportion *An. arabiensis* will readily feed on animal hosts (Coetzee, 1987). The ratio of *An. arabiensis*

to *An. quadriannulatus* species A from this study (1:7) differs from previous studies White, (1974) found the ratio of *An. arabiensis*: *An. quadriannulatus* species A approximated 1:1 in animal shelters and outdoor resting sites.

The presence of *An. quadriannulatus* species A, a zoophilic species in MBN collections is not a new phenomenon and has been recorded by Hunt and Mahon, (1986). In this study *An. quadriannulatus* species A was collected in houses occupied by humans at three localities; Mathlanga, Muhupe and Deka in Zimbabwe and Marigoma in South Africa. Indoor resting collections of *An. quadriannulatus* species A were also made by Sharp *et al.* (1984) in South Africa.

The presence of a small percentage of *An. merus* was also not surprising. Though this salt water breeder is found mainly in coastal areas of Kenya and Tanzania it has been recorded far inland in Zimbabwe and parts of South Africa (Coetzee *et al.*, 2000). This is mainly because the species is also capable of surviving in salt pans (Gillies and Coetzee., 1987), and the area where mosquitoes were collected is characterized by high flouride content thus saline (MTE class, unpublished data). The sympatry of *An. merus* with the other 2 sibling species in the larval collections suggests the tolerance of this species to freshwater as reported by Coetzee *et al.* (1993). The other species recorded during this study were *An. squamosis* complex, *An. coustani* complex and *An. funestus* group. Both *An. squamosis* and *An. coustani* are species complexes, but are not known to play any role in malaria transmission in southern Africa (Gillies and Coetzee, 1987). The presence of *An. funestus* in cattle kraal captures, a vector which had long been decimated by indoor residual spraying, warrants further investigation to find out whether this small population is a geographically isolated one or is a population which has migrated from Mozambique where it occurs abundantly (Casimiro *et al.*, 2006). Failure to capture it indoor might suggest that it has now adopted a highly exophilic behaviour to avoid sprays.

5.2 Sporozoites infection

Gokwe district is one of the areas in Zimbabwe where malaria transmission is stable and endemicity is high. Exposure to infective bites occurs during the transmission season.

Sporozoite infection rate is one of the factors used in studying the dynamics of malaria. The sporozoite rates for the major malaria vectors in the study area were recorded previously by Mpofu (1985). Infection rate for *An. arabiensis* ranged from 0.5% to 2% depending on time of collections. The infectivity rate was high during March, the peak of malaria transmission in Zimbabwe.

During this study, an overall infectivity rate of 0.5% for *An. arabiensis* was recorded. This is comparable to the study done by Mpofu in 1985. Analyzing sporozoite rate per species and per collection method showed an abnormally high figure of 14% for the MBN collected *An. arabiensis*. This can be attributed to a number of factors. Firstly the collections were done during the peak of malaria transmission. Another contributing factor is probably due the small sample size (n=14). A final contributing factor might be the methodology used to determine the sporozoites rates. ELISA based assays detect sporozoites that are obtained from the head and thoraces of female mosquitoes. This assay does not detect sporozoites directly from the saliva glands and therefore the sporozoites rates obtained could be slightly elevated. It would be interesting to repeat the study in the dry season with a relatively larger sample size and doing salivary dissections to determine infectivity rate. The absence of infections in the other species suggests that they might not be playing any role in malaria transmission in Gwave. The absence of *An. arabiensis* infected with sporozoites in outdoor collections (cattle kraal) may indicate effectiveness of residual spraying for controlling mosquitoes. Pyrethroids are known to have excito-repellent effects which divert mosquitoes to feed on domestic animals thereby avoiding being infected with the human malaria parasite.

5.3 Susceptibility of *An. gambiae* complex to insecticides

Several susceptibility tests have been carried out in Gokwe (Murahwa, 1995; Manokore *et al.*, 2000; Govere, 2003). All screening tests done to date indicate susceptibility of *An. gambiae s.s* and *An. arabiensis* to DDT and synthetic pyrethroids including deltamethrin, lambdacyhalothrin and permethrin. The only deviation from this was the detection of a population of DDT resistant *An. arabiensis* in the Gwave gardens in Gokwe South (Masendu *et al.*, 2005).

During this study all the tested wild populations and F₁ progeny were fully susceptible to 4% dieldrin, 0.1% bendiocarb and 5% malathion. This may be due to sample area having no history of use of these classes of insecticides and therefore resistant individuals have not yet been selected for. A susceptibility level of 72% for DDT and 53% for permethrin was observed in the wild population. While 56.7% families reared from wild collected *An. arabiensis* and 25.4 % families showed evidence of resistance to permethrin and DDT respectively. After PCR identifications were carried out, DDT and pyrethroid resistance was confirmed in all the three sibling species.

The evidence of resistance of the species to DDT is not surprising in view of the long history of its use in both agriculture and health. Selection for resistance might have started as far back as 1970 when DDT was used to control tsetse flies. During these early years DDT was applied once yearly at a rate of 237 g/ha (Matthiessen, 1985). In agriculture chlorinated compounds such as Dichlorvos, Bexadust (benzene hexachloride), used to control aphids and Dicofol used to control red spider mites in tomatoes and cotton might have contributed to the selection pressure resulting in DDT resistance. This effect of agricultural insecticides on selection for resistance in malaria vector is probably evident in resistance observed in *An. quadriannulatus* a species which rarely rests indoor.

In this study, resistance to permethrin a pyrethroid shown in three sibling species *An. arabiensis* (47.7%), *An. merus* (75%) and *An. quadriannulatus* (66.7%) of wild population *An. gambiae* complex collected and *An. arabiensis* of F₁ progeny of wild collected adults. Such resistance levels were not expected. This observation is the first of its kind for this district and contrasts with those of Manokore *et al.* (2000), Govere (2003) and Masendu, *et al.* (2005). These researchers performed insecticide bioassays on *An. arabiensis* using the pyrethroids; 0.025% deltamethrin and 0.1% lambdacyhalothrin and concluded that the resident mosquitoes were still susceptible to these insecticides. The discovery of populations which are resistant to pyrethroids has serious implications on malaria control strategies. Pyrethroids (deltamethrin and lambdacyhalothrin), are the main insecticides being used by the NMCP and is one of the classes recommended by WHO for vector control especially for bed net impregnation. In this area the development of resistance may be due to high selective pressure imposed on mosquitoes through the indiscriminate usage of pyrethroids by villagers

to control agricultural crop pests and at times indoor spraying of bedbugs. A brief investigation of chemical usage in this area revealed high usage of pyrethroids during cotton growing season. In a pesticide usage survey done by Masendu and colleagues in 1999 pyrethroids were ranked highly in the classes of insecticides used in Gokwe (Masendu *et al.*, 2005). Residues of pyrethroids sprayed on cotton and rice crops have long been suggested as the source of selection favoring the emergence of pyrethroid resistance (Lacey and Lacey, 1990; Ellisa *et al.*, 1993; Martinez-Torres *et al.*, 1998).

5.4 Biochemical assays

Insect populations have evolved a variety of mechanisms to counter effect of toxic chemical compounds. Metabolic detoxification of exogenic compounds using specialized enzyme systems is one of the common ways in which insects respond to foreign substances. Increased production and over expression of monooxygenases, non-specific esterases and GSTs have been associated with insecticide resistance (Vulule, 1996; Penilla *et al.*, 1998; Brooke *et al.*, 2001; Enayati *et al.*, 2003).

Biochemical assays of various enzyme systems in the F₁ progeny of wild caught females showed evidence of elevation in a number of enzymes. Increased levels of monooxygenase and esterase activity were found in the F₁ progeny of the wild-caught *An. arabiensis* assayed (42% of the families tested positive for monooxygenase; 27.3% and 6.5% for general esterases assayed using alpha and beta-naphthyl acetate as substrates). This suggests that these enzymes may account for the pyrethroid resistance observed during bioassays. Several reports have demonstrated that elevated levels of monooxygenases are responsible for pyrethroid resistance in *An. gambiae* and *An. funestus* (Brogdon *et al.*, 1988, Vulule, 1999; Enayati *et al.*, 2003; Casimiro, 2006). However, our results are not conclusive as there was no correlation between mortality data and enzyme activity. Of the nine families which were assayed for monooxygenase activity only three families (17.4%) were resistant to permethrin exposures. General esterase activity showed a better correlation with bioassay data, of the nine families which were resistant to permethrin exposures 6 families (66.7%) had elevated esterases using alpha-naphthyl acetate as a substrate. It is highly likely that esterases play significant role in pyrethroid detoxification in this population of *An. arabiensis*. General

esterases are involved in pyrethroid metabolism in several insects. Vulule and colleagues showed that esterases were elevated in *An. gambiae* selected for permethrin resistance compared to their unselected strains (Vulule *et al.*, 1999).

An increased level of GST activity was found in 8 of the 31 families assayed. Presence of elevated GSTs in 3 families may be explained by evidence of DDT resistance observed in bioassays done to these families. Resistance to DDT is frequently conferred by increased production of GST enzymes which catalyze DDT detoxification reactions (Clark and Shamaan, 1984; Brooke *et al.*, 2001). Nevertheless, as was the case with monooxygenase assay no correlation between bioassay mortality data and enzyme activity was observed. The present results can not be regarded as conclusive as GSTs have also been known to catalyze other non resistant related reactions such as biosynthesis of hormones and protection against oxidative stress (Enayati *et al.*, 2005). This probably explains elevation of GST in three families which were neither DDT nor permethrin resistant.

Data from insensitive acetylcholinesterase assays done suggest the presence of altered AChE resistance gene in the population. This was not expected as organophosphates and carbamates have never been used for vector control in this area. AChE is the molecular target for organophosphates and carbamates. However, the intensive use of carbamates in agriculture may have resulted in the appearance of mosquitoes possessing altered AChEs that are less sensitive to propoxur. Use of organophosphates and carbamates is relatively prevalent in Gokwe. Spraying of cotton with Rogor[®] (Dimethoate), carbaryl and benfuracarb is widespread. The low frequency occurrence of insensitive AChE suggests a very recent event. This study might serve as evidence that mechanisms conferring carbamate and organophosphates resistance are now present in *An. arabiensis* from Gokwe though at very low frequencies which can not be detected by bioassays. Any changes to carbamates by the NMCP should be done after careful consideration of the above fact.

5.5 Selection for resistance

Selection to both permethrin and bendiocarb showed a marked increase in survival from the F₂ to the F₄ generation. The LT₅₀ of the carbamate selected cohorts increased 10 fold while

that of pyrethroid selected cohorts increased 4.5 times suggesting that the insecticide selections done increased the proportion of resistant individuals. In this laboratory study selection for carbamate resistance was more rapid compared to pyrethroid selection. This observation clearly mimics the effect of continual exposure to the same insecticide in the field can result in selection of resistant individuals. Therefore, there is a need to continuously carry out susceptibility tests especially in areas where one particular insecticide is being used continuously. This is more so applicable to Gwave village where evidence of pyrethroid resistance has been discovered in this study yet indoor residual spraying is still being done using pyrethroid. This will consequently result in the rapid increase of pyrethroid resistance.

Female cohorts in all generations showed markedly higher levels of resistance than male counterparts. This observation is not a new phenomenon as Hunt *et al.* (2005) in their study of pyrethroid-selected laboratory *An. funestus* observed a similar trend. This was attributed to a sex-linked factor associated with resistance in mosquitoes.

A significant increase in monooxygenase titers was recorded in the pyrethroid selected cohorts compared with their corresponding unselected cohorts suggesting that this enzyme group is associated with pyrethroid resistance. Demonstration of complete synergism of resistance phenotype by piperonyl butoxide and incomplete synergism by DEM suggests that pyrethroid resistance is essentially mediated by monooxygenase detoxification with the possible involvement of elevated GST activity as a cofactor (Brooke, personal communication). GST involvement in pyrethroid resistance has been demonstrated in the plant hopper *Nilaparvata lugens* by Vontas *et al.* (2001). Vontas and colleagues postulated that elevated GSTs may protect tissues from oxidative damage associated with exposure to pyrethroids. Esterase assays showed no significant difference between the OD values of selected resistant and baseline susceptible individuals when alpha and beta naphthyl acetate was used. This suggests that pyrethroid resistance in this population of *An. arabiensis* is not conferred by metabolic detoxification using esterases.

The carbamate resistant selected line showed significant increases in GSTs and esterases using the substrate beta-naphthyl acetate. Involvement of GSTs was confirmed by synergist studies using DEM. Significantly higher mortalities rates (100%) occurred when the selected

strains were synergized with DEM before exposure to 0.1% bendiocarb. Presumably suppression of GST activity by DEM indicates role GSTs in carbamate resistance in the tested strain. Involvement of GSTs in carbamate resistance has never been recorded. Further investigations need to be carried out to ascertain why GSTs are being elevated when this population is exposed to bendiocarb. The increase in esterase activity is not a new phenomenon; it has been shown before in other arthropod strains where esterase levels are elevated in organophosphate and carbamate resistant individuals. Increased esterase activity with beta naphthyl acetate has been recorded in both *Myzus persicae* Sulzer (Needham and Sawicki, 1971) and *Culex quinquefasciatus* (Georghiou, 1990). A surprising observation was the complete reversal of bendiocarb resistance after synergizing with PBO; this was not expected as biochemical assays did not show any elevated monooxygenases. Probably monooxygenases are playing a secondary role in carbamate detoxification. Monooxygenases are known to give protection to a variety of insecticides including chlorinated hydrocarbons, organophosphates, pyrethroids and carbamates (Scott, 1995).

5.6 Distribution of *kdr* allele and cross-resistance

Knockdown resistance (*kdr*) has been detected in *An. gambiae* s.s and *An. arabiensis* (Martinez-Torres *et al.*, 1998; Ranson *et al.*, 2000; Awolola *et al.*, 2003; Diabete *et al.*, 2004; Matambo *et al.*, 2007). This target site resistance has been closely associated with cross resistance to DDT and pyrethroids in several *An. gambiae* s.s populations (Martinez-Torres *et al.*, 1988; Diabate *et al.*, 2002; Awolola *et al.*, 2003; Fanello *et al.*, 2003). However, of all the *kdr* mutations reported in *An. arabiensis* none has showed any correlation with bioassay mortality data. Matambo *et al.* (2007) attributed the *kdr* mutation observed in *An. arabiensis* from Sudan to an independent development while Stump *et al.* (2004) proposed inheritance through introgression with *An. gambiae* for the *kdr* mutation observed in a population of *An. arabiensis* from Kenya.

In this study, the Leu-Phe mutation in the sodium channel gene was detected by polymerase chain reaction. Confirmation tests using sequence analysis showed complete absence of the *kdr* allele. This confirms the widely held view that mutation-specific PCR assays, developed to detect single nucleotide polymorphism, are often difficult to optimize and may not be as

reliable as other methods (Pinto *et al.*, 2006, Koekemoer, personal communication). Despite repeated efforts to optimize this assay our results have shown that this has not been completely achieved. Alternative assays for the detection of *kdr* mutation have been shown to be more effective. For example methods such as Hot Oligonucleotide Assay (HOLA), FRET/Melt Curve analysis have been developed (Lynd *et al.*, 2005; Verhaeghen *et al.*, 2006). Currently pyrosequencing as an alternative method is being investigated within VCRU.

Absence of *kdr* mutation from sequencing results does not in any way signify absence of target site resistance mechanisms. Evidence of a target site resistance mechanism is overwhelming and can be supported on two fronts. Firstly, both pyrethroid and DDT resistance was observed during field and laboratory bioassays. Bioassays done on F₁ progeny of wild caught *An. arabiensis* showed 6 families carrying cross-resistance to permethrin and DDT. This is a strong indication of resistance being conferred by a common mechanism in these 6 families. Secondly, this suspected common resistance mechanism can not be attributed to metabolic detoxifications. Biochemical assays done on the six families did not give any common enzymes involved in conferring resistance to DDT and permethrin. Only general esterases using alpha-naphthyl acetate as a substrate were elevated in three of the families. Non-specific esterases have never been known to confer cross-resistance to DDT and pyrethroids. A possible way forward therefore will be to sequence the segment 6 of domain II of the sodium channel gene of these 6 families and compare it with the sequence of a known susceptible *An. arabiensis* strain. It is possible that alternative substitutions in the *An. arabiensis* sodium channel other than those found in *An. gambiae* s.s are responsible for resistance to DDT and permethrin.

Chapter 6

Summary and recommendations

Three members of the *An. gambiae* complex, *An. arabiensis*, *An. merus* and *An. quadriannulatus* occur in sympatry. *Anopheles arabiensis* is the major malaria vector as exemplified by high *Plasmodium* infection rates. The sympatric and ubiquitous occurrence of *An. quadriannulatus* underscores the need to carry out specific species identification in any malaria vector studies in this area. The occurrence of *An. merus* in fresh water breeding sites necessitates studies on distribution and ecological behaviour of this sibling species and its role in malaria transmission in Gokwe. The presence of *An. funestus* albeit in low proportions might indicate reemergence of a vector which had been successfully eliminated by residual indoor house spraying. Further investigations need to be carried out to verify its presence.

The combined biochemical and bioassay data show a potential of broad spectrum resistance. The findings of this study cannot be extrapolated generally to the susceptibility status of Gokwe since the sampling was limited to one locality Gwave village and the sample size was too small to make conclusions. However evidence of DDT and pyrethroid resistance is overwhelming and need to be taken into consideration with regards to malaria control in Zimbabwe, since malaria control strategies rely heavily on the use of these classes of insecticides. The way forward is careful consideration of the use of mosaic insecticides or rotational use of insecticides to slow down the widespread development of pyrethroid resistance. Careful and routine detection and monitoring of insecticide susceptibility levels of vector populations in malaria endemic areas covering a wider geographical area is also recommended.

The presence of families showing cross-resistance to DDT and permethrin needs to be investigated further. Our results did not give any clue at why this cross resistance occurred. Biochemical results and PCR results were inconclusive. Sequencing of the whole sodium channel gene should be done on the samples which showed cross resistance as mutation might have occurred at any of the target site for these two insecticides. The presence of

insensitive acetylcholinesterase at low frequency in the population of *An. arabiensis* from Gwave has implications for the spread of the gene across the whole district. Any changeover to carbamates and organophosphates should be after careful consideration of the impact it might cause in selecting for resistance.

The selections done in the laboratory gave an insight of the resistance mechanisms conferring insecticide resistance in *An. arabiensis* in Southern Africa. Resistance is mainly conferred by metabolic based resistance mechanisms. Elevation of monooxygenases, general esterases and GST enzymes being the main enzymes involved. Elevated GSTs found in the carbamate selected strains warrants further investigations as GSTs have never been implicated in carbamate resistance. *Anopheles arabiensis* colony from Zimbabwe needs to be established and detailed studies on DDT and pyrethroid resistance done.

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