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**Munhenga, Givemore**

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# Characterisation of resistance mechanisms in the major malaria vector *Anopheles arabiensis* from southern Africa

**By**
**Givemore Munhenga**

**Thesis submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Doctor of Philosophy.**

**Johannesburg, 2010**

# DECLARATION

I declare that this thesis hereby submitted by me for the Degree of Doctor of Philosophy at the University of Witwatersrand, Johannesburg is my own independent work and has not been previously submitted for any degree or examination in any other University. I further more cede copyright of the thesis in favour of the University of Witwatersrand.

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# ABSTRACT

The continual increase in insecticide resistance in malaria vector mosquitoes is threatening the efficacy and sustainability of most malaria control programmes. The major problem facing malaria vector control programmes is lack of scientific information to assist in resistance management strategies. The objective of this thesis is to elucidate insecticide resistance mechanisms in *Anopheles arabiensis* from southern Africa. This information is necessary to predict cross resistance spectrum, facilitate choosing alternative insecticides and assist in mapping areas with resistant populations. Field studies to update data available on insecticide susceptibility and characterise the resistance mechanisms were carried out in Gokwe, a malaria endemic area in Zimbabwe. In addition this study reports changes in insecticide resistance levels, detoxification enzymes and P450 gene profile following artificial permethrin and bendiocarb selection of laboratory colonies originating from Mamfene, South Africa.

A total of 943 anophelines belonging to four different taxa were collected over a two year period with the majority (98.8%) being members of the *An. gambiae* complex. Species in the *An. gambiae* complex were identified by polymerase chain reaction (PCR) and *An. arabiensis* (72.0%) predominated over all the other sibling species. Among the *An. arabiensis* females 0.5% and 4.8% was positive for *Plasmodium falciparum* in 2006 and 2008 respectively. WHO diagnostic tests on wild *Anopheles arabiensis* populations showed resistance to permethrin at a mean mortality of 47% during 2006 and a mean mortality of 69.4% in 2008. DDT resistance (68.4% mean mortality) was present in 2006; however in 2008 a mean mortality of 94.8% was recorded. Insecticide susceptibility tests on $F_1$ *An. arabiensis* families showed that 25.4% (n = 59) and 14.2% (n = 14) of the families were resistant to DDT in 2006 and 2008 respectively. For permethrin exposures 56.8% (n = 37) and 78.8% (n = 14) families were resistant in 2006 and 2008 respectively. Eight families were resistance to both DDT and permethrin during the two collection periods. Biochemical assays of $F_1$ *An. arabiensis* families reared from 2006 collections revealed comparatively high levels of monooxygenase (48%.5% of families tested, n = 33, $p<0.05$), glutathione S-transferase (26.7% of families tested, n = 30, $p<0.05$) and

general esterases activity compared to the reference colony. No knockdown resistance (*kdr*) and ace-$1^R$ mutations were found.

After 12 generations of bendiocarb and permethrin selections of *An. arabiensis* mosquitoes from Kwazulu/Natal there was approximately a 2.8-fold and 3.8-fold respective increase in the $LT_{50}$ compared to the parental colony. Selections resulted in increased levels of non-specific esterase and monooxygenase activity for the permethrin selected cohorts, and elevated glutathione S-transferases and general esterases for the bendiocarb colonies. Involvement of monooxygenase and glutathione S-transferase in pyrethroid and bendiocarb resistance was confirmed by synergist studies using piperonyl butoxide and diethyl maleate respectively.

P450 gene profiling of the permethrin selected line showed that 4 genes CYP6Z1 (4.67-fold), CYP6Z2 (1.72-fold), CYP6M2 (2.24-fold) and CYP4G16 (1.39-fold) were over expressed in the resistant cohorts.

Continued use of pyrethroid insecticides by the Zimbabwean National Malaria Control programme (NMCP) is likely to further select resistant vectors. Use of insecticides such as organophosphates and carbamates, mosaic insecticides or rotation of insecticides is recommended. These results contribute to our limited knowledge of metabolic resistance mechanisms and provide useful information for future studies to come up with new insecticides and diagnostic tools for detection and management of resistance.

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# PRESENTATIONS

## Oral

1) **Givemore Munhenga,** Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, Maureen Coetzee and Lizette L Koekemoer. Characterisation of resistance mechanisms in the major malaria vector *Anopheles arabiensis from* southern Africa. **September 2009**. *38th Annual Conference of the Parasitological Society of southern Africa, Johannesburg, South Africa.*

2) **Givemore Munhenga,** Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, and Lizette L Koekemoer Studies on a major vector *Anopheles arabiensis* from Gwave, a malaria endemic area in Zimbabwe," **July, 2008**. *XXIII International Congress of entomology, Durban, South Africa.*

3) **Givemore Munhenga,** Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, and Lizette L Koekemoer Malaria vector status and insecticide susceptibility studies in Zimbabwe," **May, 2007** *National Institute for Communicable Diseases Scientific Day Presentations, Sandringham, Johannesburg, South Africa.*

## Poster

1) **Givemore Munhenga,** Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, Maureen Coetzee and Lizette L Koekemoer. Studies on a major vector *Anopheles arabiensis* from Gokwe, a malaria endemic area in Zimbabwe," **November, 2009**. *5th MIM Pan-African Malaria Conference, Nairobi, Kenya.*

2) **Givemore Munhenga,** Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, Keith Hargreaves, Maureen Coetzee and Lizette L Koekemoer. Changes in Insecticide Resistance Levels and Detoxification Enzymes Following Insecticide Selection in *Anopheles arabiensis* (Diptera: Culicidae),'' **August 2008**. *Faculty of Health Sciences Research Day, University of Witwatersrand, Johannesburg, South Africa.*

## Peer reviewed publications

1) **Givemore Munhenga**, Hieronymo T Masendu, Basil D Brooke, Richard H Hunt, Lizette L Koekemoer (2008) Pyrethroid resistance in the major malaria vector *Anopheles arabiensis* (Diptera: Culicidae) from Gwave, a malaria endemic area in Zimbabwe. *Malaria journal* **7**: 247

2) Joel C. Mouatcho, **Givemore Munhenga**, Keith Hargreaves, Basil D. Brooke, Maureen Coetzee and Lizette L. Koekemoer (2009) Pyrethroid resistance in a major African malaria vector, *Anopheles arabiensis*, from Mamfene, northern Kwazulu/Natal, South Africa. *South African Journal of Science* **105**, 127-131

3) **Givemore Munhenga**, Richard. H. Hunt, Maureen Coetzee, Keith Hargreaves and Lizette .L. Koekemoer. Differential expression of P450 genes in *Anopheles arabiensis* following permethrin selection, In Prep.

## Technical reports

1) N. Lukwa, V. Gunguluzi, A. Makuwaza, L. Chibarinya, T. Dube, C. Kasambare and **G. Munhenga.** Supporting the National Malaria Control Programme through bioassays and insecticide resistance testing. *Zimbabwe National Institute for Health Research Technical Report 2008.*

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# ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| **%** | percent |
| **µl** | microlitres |
| **AChE** | acetylcholinesterase |
| **ACT** | artemisinin based combination therapy |
| **AIDS** | acquired immune deficiency syndrome |
| **ASCHI** | acetylthiocholine iodide |
| **BHC** | benzene hexachloride |
| **BLAST** | basic logic alignment search tool |
| **bp** | base pair |
| **CDNB** | 1-chloro-2,4-dinitrobenzene |
| **CS** | circumsporozoite |
| **CYP** | cytochrome P450 |
| **DDT** | diethyl diphenyl trichloroethane |
| **DEM** | diethyl maleate |
| **DEPC** | diethyl pyrocarbonate |
| **DNA** | deoxyribonucleic acid |
| **dNTPs** | deoxynucleotide triphosphate |
| **EC** | emulsion concentrate |
| **ELISA** | enzyme linked Immunosorbent assay |
| ***et al*** | and others |
| **GABA** | gamma –aminobutyric acid |
| **GMC** | global malaria control strategy |
| **GST** | glutathione S-transferase |
| **HIV** | human Immune virus |
| **hr** | hour |
| **IGS** | Intergenic spacer region |
| **IRS** | Indoor residual spraying |
| **ITN** | insecticide treated net |
| **ITS** | Internal transcribed spacers |

| | |
|---|---|
| **IUPAC** | International Union of Pure and Applied Chemistry |
| **kb** | kilo base |
| ***kdr*** | knockdown resistance |
| **KPL** | Kirkegaard and Perry laboratories |
| **LC** | larval collection |
| **LSDI** | Lubombo spatial development initiative |
| **M** | molar |
| **MBN** | man-baited net |
| **mg** | milligram |
| **$MgCl_2$** | magnesium chloride |
| **min** | minutes |
| **ml** | millilitre |
| **mm** | millimetre |
| **mM** | millimole |
| **MO** | monooxygenase |
| **NCBI** | National Centre for Biotechnology Information |
| **NA** | naphthyl acetate |
| **NaOH** | sodium hydroxide |
| **ng** | nanogram |
| **NGO** | non-governmental organization |
| **NICD** | national Institute for communicable diseases |
| **NIHR** | national Institute for health research |
| **nm** | nanomole |
| **NMCP** | national malaria control programme |
| **ºC** | degrees celsius |
| **OD** | optical density |
| **OP** | organophosphate |
| **P** | probability level |
| **PBO** | piperonyl butoxide |
| **PBS** | phosphate buffered solution |
| **PCR** | polymerase chain reaction |

| | |
|---|---|
| **Pf** | *Plasmodium falciparum* |
| **pH** | potential of hydrogen |
| **qPCR** | quantitative polymerase chain reaction |
| **RBM** | roll back malaria programme |
| **RNA** | ribonucleic acid |
| **rpm** | revolutions per minute |
| **s** | second(s) |
| ***s.l*** | sensu lato |
| ***s.s*** | sensu stricto |
| **SARN** | southern Africa regional network |
| **SNP** | single nucleotide polymorphism |
| ***Taq*** | *Thermus aquiticas* |
| **TPP** | triphenyl phosphate |
| **Tw** | tween |
| **UNICEF** | United Nations Children's Fund |
| **USA** | United States of America |
| **VCR** | vector control reference unit |
| **WHO** | World Health organisation |
| **WP** | wettable powder |

# CHAPTER 1

## GENERAL INTRODUCTION

Malaria remains the most important insect transmitted human parasitic disease despite decades of concerted efforts to control it. Use of insecticides to control malaria vectors remains at the forefront in the fight against malaria. However, continual use of insecticides on a focal population will most likely result in the selection or possibly development of insecticide resistance, forcing a change over to new insecticides. Unfortunately, the continual use of insecticides in both agriculture and public health has resulted in the increase in insecticide resistant malaria vector species. Insecticide resistance in malaria vectors can lead to malaria control failure.

Development of new insecticide classes to keep pace with insecticide resistance is proving difficult and it is unlikely that new products will be available in the near future. The feasible strategy is to prevent or retard development of resistance to the currently available insecticides. The development of insecticide resistance can be delayed through resistance management strategies such as rotational use of different classes of insecticides or use of a mosaic spraying regime (Hemingway *et al.,* 1997). In order for such resistance management strategies to be effectively implemented, information on the susceptibility status of malaria vectors should be known. Regular monitoring of vector populations for insecticide resistant phenotypes and possible underlying resistance mechanisms should also form part of a resistance management programme.

## 1.1 Malaria burden

Infectious diseases continue to impose an enormous burden on the world population. Insect-borne diseases significantly contribute to this burden particularly in Africa. Malaria is among the top five diseases causing major public health problems (WHO, 2004).

It is estimated that three billion people worldwide are at risk of malaria infection. Of these, 300-500 million people are infected annually, leading to 1 to 2 million deaths. These deaths are particularly recorded among children under 5 years and pregnant women mostly in Africa (The Prescriber, UNICEF publication, 2000, http://www.rbm.who.int).

The effects of malaria extend beyond measures of mortality and morbidity. The economic impact of malaria is immense and includes costs of 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). Indeed, it is postulated that malaria control can be used as the precursor for development (Gallup and Sachs, 2001). It is against this background that some successful collaborative malaria control programmes

have been initiated such as the Lubombo Spatial Development Initiative (LSDI) (Sharp *at al.,* 2007) and Bioko Island malaria control programme (Kleinschmidt *et al*., 2007).

Human malaria is caused by infection with the protozoan parasite of the genus *Plasmodium*. There are four recognized human malaria species in the genus *Plasmodium*: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. It has now been realised that a fifth species, *P. knowlesi,* commonly associated with primate malaria in Southeast Asia, is capable of human infections (Singh *et al.,* 2004). *Plasmodium falciparum* is by far the most virulent. In 2006 alone, 91% of an estimated 247 million malaria cases were attributed to *P. falciparum* infections (WHO, 2008). *Plasmodium malariae* and *P. ovale* infections cause low but significant rates of morbidity (Collins and Paskewitz, 1995). *Plasmodium vivax* infections are more chronic and account for about 10% of malaria in sub-Sahara Africa (Mendis *et al.,* 2001).

## 1.2 Malaria in southern Africa

The burden of malaria does not spare southern Africa, where it is estimated that 36% of the 145 million people in this region live in malarious areas. Malaria is thus rated as the second leading cause of mortality and morbidity resulting in 300, 000 to 400, 000 deaths out of the 20 million malaria episodes recorded annually (SARN, 2008).

Malaria transmission in southern Africa is seasonal (Coetzee and Hunt, 1998; Maharaj *et al.,* 2005). The malaria season begins in October and carries through to May with transmission peaks being recorded between February and May (Coetzee *et al.,* 2000). Because of the seasonal nature of most of the transmission in the region, individuals

living in malaria areas do not build up high levels of semi immunity to the disease, as happens in high transmission areas such as West and East Africa. The seasonal nature means that the region is prone to epidemics, often spreading rapidly causing intense outbreaks and high case fatality. Thus the importance of malaria control in southern Africa can never be overemphasized.

The main strategy for malaria control in southern Africa is indoor residual spraying supplemented by insecticide treated bed nets. Individual countries differ in the type of insecticide used for IRS. The Zimbabwe National Malaria Control Programme (ZNMCP) alternates between DDT and pyrethroids. The South African malaria control programme uses DDT to spray traditional houses and pyrethroids are used for westernised houses (Maharaj *et al.,* 2005). Southern African countries have realised that control activities are best achieved if there is cooperation between countries. There is a lot of cross border movement between southern African countries which in some instances results in imported malaria cases between countries. The first inter-country malaria control initiative was the LSDI which involves South Africa, Mozambique and Swaziland. Over the past seven years, LSDI initiatives resulted in the reduction of malaria by 90% in the implementing regions of South Africa and Swaziland and 70% in the implementing regions of Mozambique (Sharp *et al.,* 2007). This success has prompted the SADC Malaria Technical Committee to implement another cross-border initiative in the Trans-Zambezi region which will involve six countries namely Angola, Botswana, Democratic Republic of Congo, Namibia, Zambia and Zimbabwe (SARN, 2008).

## 1.3 Malaria vectors

Three species of *Anopheles*, namely *An. gambiae* Giles, *An. arabiensis* Patton and *An. funestus* Giles are considered to be major malaria vectors and are responsible for more than 95% of the total transmissions on the African continent (Gillies and DeMeillon, 1968; Gillies and Coetzee, 1987; Mouchet *et al.,* 2004). The remaining 5% is transmitted by "secondary" vectors such as *An. moucheti* Evans, *An. merus* Donitz *An. melas* Theobald, *An. bwambae* White and *An. nili* Theobald. These vectors are normally important in localized areas (Gillies and Coetzee, 1987).

This study focuses on *An. arabiensis,* the major vector in southern Africa in the absence of *An. funestus.* Therefore the review of the literature on malaria vectors is confined to the *An. gambiae* complex.

### 1.3.1 *Anopheles gambiae* complex

The *An. gambiae* complex mosquitoes are the most common vectors of malaria in sub-Saharan Africa (White, 1974; Gillies and Coetzee, 1987), and consist of *An. gambiae* s.s, *An. arabiensis, An. merus*, *An. melas*, *An. quadriannulatus* species A Theobald and B Hunt, Fettene and Coetzee and *An. bwambae* (Gillies and DeMeillon, 1968; Gillies and Coetzee, 1987; Hunt *et al.,* 1998). As a result of their different biologies, these species differ in their ability to transmit malaria.

#### 1.3.1.1 *Anopheles gambiae* sensu stricto

*Anopheles gambiae* s.s is the most efficient malaria vector amongst the members of the complex (Gillies and Coetzee, 1987; Della Torre *et al.,* 2002). It is widely distributed across Africa with appreciable numbers occurring in West and East Africa (Gillies and Coetzee, 1987). Such distribution is thought to be climate dependent. *Anopheles gambiae* survive best in humid areas. In a laboratory experiment it was shown that *An. gambiae* can out-compete *An. arabiensis* at high relative humidity of 80-90% (Gillies and Coetzee, 1987). Such rainfall dependent behaviour is exhibited in savannah zones where population numbers of *An. gambiae* start to rise explosively soon after the first rains, reaching a peak in the middle of rains and declining steadily thereafter (Gillies and De Meillon, 1968). Larval habitat of *An. gambiae* varies from permanent wells and irrigation channels to sunlit temporary pools.

*Anopheles gambiae* s.s is further divided into chromosomal forms based on differences in frequencies of paracentric inversion polymorphisms on chromosome arm 2 (2R), geographical distribution and ecological data such as aridity and breeding sites patterns (Coluzzi *et al.,* 1979). There are five chromosomal forms named by the non-linnean nomenclature as Mopti, Bamako, Bissau, Forest and Savanna (Coluzzi and Petrarca 1985).

Based on the genotyping of the X-linked rDNA, *An. gambiae* s.s can be further classified into two distinct molecular forms M and S. This classification is based on the sequence analysis of the intergenic spacer region (IGS) (Favia *et al.,* 2001) and the internal

transcribed spacers (ITS) (Gentile *et al.,* 2001). The relationship between the molecular forms and the chromosomal forms varies according to ecological and geographical distribution (Della Torre *et al.,* 2002). The S-form is predominant in many areas where it proliferates during the rainy season. It can be found breeding in rain-depended pools and temporary sunlit puddles (Pinto *et al.,* 2007). The M-form is mainly found in West Africa and Central Africa where it is associated with man-made long-lived water bodies such as rice fields and water canals used for irrigation (Della Torre *et al.,* 2005). Unlike the S-form, the M-form is known to survive in dry climatic conditions which might explain its unusual occurrence in northern Zimbabwe an area which is very arid (Masendu, 2004). The distribution of M-form in West Africa extensively overlaps that of the S-form (Pinto *et al.,* 2007). Difference in vectorial competency between the two has not yet been established. However, theoretically it is postulated that the M-form should be a more efficient vector. In experimental studies done by Diabate *et al.* (2008) they showed that the M-form has better reproductive output and longevity compared with the S-form. Other factors which determine vectorial capacity such as prevalence of *Plasmodium* infection and anthropophilic and endophilic behaviour were found to be similar. Contrary to their conclusions, earlier studies of malaria transmission in rice fields dominated by M-form were found to be low (Diuk-Wasser *et al.,* 2005; Manoukis *et al.,* 2006). This suggests that other factors might be involved in determining vectorial capacity of these two species.

*Anopheles gambiae* is highly anthropophilic feeding preferentially on humans (White, 1974; Gillies and Coetzee, 1987). In exceptional cases, *An. gambiae* has been found

feeding on other animals (Diatta *et al.,* 1998; Bogh *et al.,* 2001); however, even in such situations the human blood index was still high. This anthropophagic behaviour creates an intimate association between human parasite reservoir and vector explaining why *An. gambiae* always has higher sporozoite rates compared to other members of the complex.

After blood feeding the majority of *An. gambiae* mosquitoes rest indoors making them vulnerable to control measures such as IRS. In fact in areas where there are organized malaria control programmes, *An. gambiae* populations have been significantly reduced. For instance in Zimbabwe before the introduction of indoor residual spraying (IRS) *An. gambiae* was found in several localities (Mahon *et al.,* 1976). However after decades of IRS a nationwide anopheline survey showed that its distribution is now patchy (Masendu *et al.,* 2005).

#### 1.3.1.2 *Anopheles arabiensis*

*Anopheles arabiensis* is a fresh water species with the same larval habitat requirements as *An. gambiae s.s.* Its range and relative abundance is mainly influenced by climatological factors (Lindsay *et al.,* 1998). This species tend to survive better in dry conditions and is tolerant to higher temperatures (Gillies and Coetzee, 1987). The exact way in which *An. arabiensis* survive these conditions remains enigmatic. It has been suggested that adult females tend to lay their eggs on damp surfaces, rather than water, with hatching being delayed in a proportion of eggs (Coluzzi, 1965), and females aestivate during periods of prolonged dryness (Omer and Cloudsley-Thomson, 1970). This behaviour probably explains its preponderance over *An. gambiae* in drier areas such as Botswana (Abdulla-

Khan, 1998), South Africa (Hargreaves *et al.,* 2003), Zimbabwe (Masendu *et al.,* 2005), Namibia (Ntomwa *et al.,* 2006), and Sudan (Abdalla *et al.,* 2008).

The feeding pattern of *An. arabiensis* is opportunistic, feeding on both humans as well as animals depending on the availability of both hosts. *Anopheles arabiensis* exhibits both exophilic and endophilic behaviours (Gillies and Coetzee, 1987). This variable behaviour makes them incompletely vulnerable to house spraying with insecticides. This species remains the main malaria vector in southern African countries where well managed control programmes have been implemented such as Namibia, South Africa and Zimbabwe (Hargreaves *et al.,* 2000; Masendu *et al.,* 2005; Ntomwa *et al.,* 2006).

#### 1.3.1.3 *Anopheles quadriannulatus*

*Anopheles quadriannulatus* is the third fresh water species in the *An. gambiae* complex. It is distributed in three disconnected geographical areas, Ethiopia, Zanzibar and the whole of southern Africa (Gillis and Coetzee, 1987). It is further classified into two distinct species; sibling species A and B. Species A represents the nominal *An. quadriannulatus* and is widespread in South Africa, Swaziland, Mozambique and Zimbabwe (Hunt *et al.,* 1998), while species B has mainly been found in Ethiopia (Hunt *et al.,* 1998). *Anopheles quadriannulatus* species B tolerates cooler conditions compared to the other three fresh water species. In most parts of southern African species A is found in sympatry with the malaria vectors *An. gambiae* s.s, *An. arabiensis* and *An. funestus* (Coetzee *et al.,* 2004; Masendu *et al.,* 2005). This sympatric occurrence with vectors and its morphological similarity with other members of the *An. gambiae* complex make accurate species identification a requirement in any control programme especially

so in southern Africa. In Zimbabwe *An. quadriannulatus* has been found in sympatry with *An. merus* (Masendu, 2004), this, despite the fact that the former require fresh water while the later proliferates in salty water.

*Anopheles quadriannulatus* is mainly zoophilic and exophilic. In rare cases it has been found resting indoors (Sharp and Quicke, 1984; Hunt and Mahon 1986). Laboratory studies have shown that *An. quadriannulatus* is susceptible to *Plasmodium falciparum* infection (Takken *et al.,* 1999). However, this species is not known to play any significant epidemiological role in malaria (Gillies and Coetzee, 1987) because of its strong zoophagic, exophagic and exophilic behaviour.

#### 1.3.1.4 *Anopheles merus*

*Anopheles merus* is a saltwater breeding member of the *An. gambiae* complex. It is found in localized areas mainly along the East African coast as well as in isolated inland areas where they are found in association with salt water pans (Gillies and Coetzee, 1987). *Anopheles merus* has been recorded in inland Mozambique and South Africa (Coetzee *et al.,* 1993), Swaziland (La Grange, 1995), Zambia (Kloke, 1997) and Zimbabwe (Coetzee *et al.,* 1993; Masendu *et al.,* 2005).

*Anopheles merus* is rated as a minor vector of malaria in localized areas (Gillies and Coetzee, 1987). It is known to prefer feeding on animals if both human and animal hosts are present (Gillies and Coetzee, 1987). In areas where *An. merus* occur in large numbers sporozoites rates as high as 11.6% has been recorded (Temu *et al.,* 1998) signifying their role as vectors in localised areas. *Anopheles merus* has also been incriminated as one of

the vectors of Bancroftian filariasis in coastal East Africa (Bushrod, 1981; Mosha and Petrarca, 1983).

Although *An. merus* has been found in southern Africa its role in malaria transmission is not well understood. In Zimbabwe's Masakadza area *An. merus* was found resting indoors and outnumbering other sibling species of the *An. gambiae* complex. Unfortunately, the infectivity rate was not determined (Masendu, 2004). In Mozambique an infection rate of 0.067% was found in field collected *An. merus*, which was comparatively higher than those of *An. arabiensis* (0.002%), and *An. funestus* (0.016%), probably indicating a role in malaria transmission in this country (Sharp *et al.,* 2007).

#### 1.3.1.5 *Anopheles melas*

*Anopheles melas* is the second saltwater member of the *An. gambiae* complex. Just like *An. merus* its distribution is localized. It is widely distributed along the West Africa coastline where it is associated with saline waters around tidal areas (Gillies and DeMeillon, 1968) and has been found associated with mangrove swamps on the Atlantic coast of Cameroon (Wondji *et al.,* 2005). This species has also been recorded in Senegal (Gillies and DeMeillon, 1968; Diop *et al.,* 2002), coastal region of Equatorial Guinea (Moreno *et al.,* 2004) and The Gambia (Jawara *et al.,* 2008). There are no records of this species in southern Africa.

The feeding pattern of *An. melas* is variable depending on the availability of hosts. A human blood index of 36% was recorded in The Gambia from specimens collected in houses without domestic animals, indicating its readiness to feed on humans (Bogh *et al.,*

2001). It was shown that in the presence of both humans and domestic animals it can equally feed on humans (Muirhead-Thomson, 1948). However, it is usually associated with areas having large numbers of domestic animals (Gillies and DeMeillon, 1968).

*Anopheles melas* is an important malaria vector along the West African coast (Gillies and Coetzee, 1987). It has been implicated as a vector in Senegal (Diop *et al.,* 2002), Equatorial Guinea (Moreno *et al.,* 2004) and The Gambia (Jawara *et al.,* 2008). Infectivity with *P. falciparum* varies, with the majority of tests done showing low sporozoites rates. However, this low infectivity rate is usually compensated with their occurrence in large numbers when it comes to efficiency in malaria transmission (Gillies and DeMeillon, 1968).

#### 1.3.1.6 *Anopheles bwambae*

*Anopheles bwambae* is an extremely localized vector found breeding only in pools around the mineral springs in the Semliki forest in Bwamba County, Uganda (Gillies and Coetzee, 1987). Its biting and resting behaviour is very similar to *Aedes africanus* resting on fallen logs, sticks and dry leaves (White, 1985). However, this species is readily attracted to humans compared to the former which exclusively feed on monkeys (Haddow and Dick 1948). In this regard *An. bwambae* is taken as an anthropophilic species with endophilic tendencies. *Anopheles bwambae* is susceptible to infection by *P. falciparum* therefore it is regarded as a malaria vector. *Plasmodium falciparum* infection rates of 0.7% have been recorded in indoor collections from Bwamba, Uganda (White,

1985). This species has also been implicated as a possible transmitter of *Wuchereria bancrofti* (White, 1985).

## 1.4 Malaria control

The goal of malaria control is to reduce as much as possible the health impact of malaria on a population using available resources, and taking into account other health priorities (WHO, 2001a). Technically this can be achieved by a combination of multiple interventions that disrupts the parasite-vector-human cycle at several points. The World Health Organization Global Malaria Programme recommends two primary control strategies: diagnosis and treatment of malaria cases with effective medication preferably artemisinin based combination therapy (ACT) and malaria prevention through vector control (WHO, 2008).

### 1.4.1 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). Malaria vector control is any intervention designed to restrict the transmission of malaria by suppressing vector populations.

Large scale control of malaria vectors dates back to the early 19$^{th}$ 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 (DeMeillon, 1936). However, it was the discovery 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). This led to the adoption of large scale spraying with DDT during the malaria eradication era (WHO, 1970). Malaria vector control through the use of dieldrin and gamma BHC soon followed, but development of resistance and toxicological concern led to the withdrawal of these two insecticides (Davidson, 1958). Currently chemicals recommended for vector control are pyrethroids DDT, carbamates and organophosphates (WHO, 2008).

Despite concerns of insecticide resistance, malaria vector control through the use of insecticides still remains the favoured and most effective approach.

## 1.5 Insecticides used for vector control

Vector control strategies mostly rely on the usage of natural and synthetic chemical molecules which have the potential to kill the target insects. Presently different formulations of synthetic chemical insecticides are in use for vector control. Wettable powder (WP) formulations are used for indoor residual spraying while emulsion concentrate (EC) are used for larval control. For IRS, insecticides in use include DDT 50% WP, Malathion 25% WP and synthetic pyrethroids. Synthetic pyrethroids include

deltamethrin 2.5% WP, cyfluthrin 10% WP, lambdacyhalothrin 10% WP, alphacypermethrin 5% WP, Etofenprox 10% WP and Bifenthrin 10% WP. Synthetic pyrethroid insecticides are also used for bed nets (WHO, 2001b).

### 1.5.1 Insecticide classes and mode of action

Almost all insecticides used for malaria vector control belong to four main classes of insecticides.

#### 1.5.1.1 Pyrethroids

Pyrethroids are synthetic analogues of the natural insecticide pyrethrin. They are made more photostable than their natural counter parts. Pyrethroids constitute one of the most important classes of insecticides. They constitute over 25% of the insecticide market (Georghiou, 1990).

Pyrethroids act as axonic poisons by binding to the voltage-gated sodium channel. This prevents the sodium channel 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).

#### 1.5.1.2 Organochlorines

Organochlorines are organic compounds containing at least one covalently bonded chlorine atom. They have wide structural and chemical variety which makes them a

candidate for a broad range of uses. Many pesticides in use are organochlorines. These include dichloro-diphenyl-trichloroethane (DDT), dicofol, endosulfan, and pentachlorophenol. Of all organochlorines DDT is well known because of its effectiveness.

DDT acts as a poison by destroying 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. This results in spontaneous firing of impulses causing the muscles to twitch followed by convulsions and death (Busvine, 1951).

#### 1.5.1.3 Organophosphates (OPs) and carbamates

OP's and carbamates target the cholinergic nerve junctions which are confined to the central nervous system (Hemingway, 1989). 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 (Bloomquist, 1996). 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), (Hemingway, 1989). 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.

## 1.6 Insecticide resistance in vector species

The greatest problem for vector control using insecticides is the development of insecticide resistance by vector species. 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). Resistance results from selection by an insecticide which allows insects with resistance genes to survive and pass their resistant trait to their offspring. The speed at which resistance develops depends on several factors, including how quickly the insects reproduce, the migration and host range of the insect, insecticide exposure rate of application and number of applications (Scott, 1995). 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.

Several years of intensive use of insecticides in public health and agriculture coupled with natural genetic mutations has led to a measurable shift in vector susceptibility to insecticides due to the specific selection of already preadapted individuals. The greatest increase in resistance occurred after the discovery of synthetic insecticides (Brown, 1986). The first record of resistance in African malaria vectors 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). Since then, the range of insecticide resistance covers all classes of compounds used for malaria vector control: organochlorides (including DDT and dieldrin),

organophosphates, carbamates and pyrethroids (Brown, 1986; WHO, 1992). Meanwhile the geographic distribution of resistance has continued to spread throughout Africa, ranging from the well known documented case of *An. gambiae s.s kdr* based resistance in West Africa (Martinez-Torres *et al.,* 1998; Pinto *et al.,* 2006) to malathion and fenitrothion resistance in *An. funestus* from Central and West Africa (Toure, 1982) and pyrethroid resistance in *An. funestus* in southern Africa (Hargreaves *et al.,* 2000; Brooke *et al.,* 2001).

In southern Africa the first case of resistance was recorded in the south-eastern lowveld of Zimbabwe in 1974 where a population of *An. arabiensis* resistant to benzene hexachloride (BHC) was discovered (Green, 1981). There are also reports of pyrethroid and carbamate resistant populations of *An. arabiensis* in South Africa and Mozambique (Hargreaves *et al*., 2003; Casimiro *et al.,* 2006b). For the malaria vector *An. funestus* there are records of deltamethrin resistance in South Africa (Hargreaves *et al.,* 2000), as well as carbamate and DDT resistance in Mozambique (Brooke *et al.,* 2001; Casimiro *et al.,* 2006a). Recently populations of *An. arabiensis* showed resistance to DDT in Gokwe in the midlands province of Zimbabwe (Masendu *et al.,* 2005).

The major concern of insecticide resistance is when it results in malaria control operational failures. Such scenarios are usually evident with spontaneous increase in malaria cases. Reports of IRS control failures due to insecticide resistance resulting in malaria epidemics were reported in Zimbabwe and South Africa (Green, 1981; Hargreaves *et al*., 2000).

## 1.7 Resistance mechanisms

Resistance to insecticides in mosquitoes is generally associated with behavioural, metabolic or physiological changes in insects, and results from three main types of mechanism: reduction in insecticide penetration, metabolism of the insecticide by enzymes preventing the insecticide reaching target site and or the modification of the insecticide target site (Hemingway *et al.,* 2004).

### 1.7.1 Behavioural resistance

Insecticide avoidance, also called "behavioural resistance", is the ability of some vectors to avoid contact with an insecticide (WHO, 1957). This is triggered by selective pressures exerted by the insecticide (Nauen, 2006). Behavioural resistance can be stimulus-dependent or stimulus-independent. Stimulus-dependent behavioural resistance is when an insect is able to detect an insecticide, and then avoid contact with 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. This phenomenon was first observed in mosquitoes exposed to DDT and was termed restlessness (Buxton, 1945). This behaviour has now been suggested to be insecticide dependent. Pyrethroids are known to elicit behavioural response in insects (Threlkeld, 1985), while DDT has been shown to be an irritant insecticide (Busvine, 1964). Behavioural resistance is important in malaria control as it can result in change in behaviour of targeted vectors. Indoor biting mosquitoes can change their biting behaviour to outdoor biting (Chareonviriyaphap *et al.,* 1997; Potikasikorn *et al.,* 2005). This will consequently compromise control measures if the strategy in use solely depends on targeting indoor resting mosquitoes.

### 1.7.2 Decreased insecticide penetration

An insecticide 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). The first case of decreased insecticide penetration was reported by Plapp and Hoyer (1968), who attributed DDT and dieldrin resistance by a strain of housefly (*Musca domestica)* to reduced penetration by these two insecticides. Farnham (1971, 1973) showed that modification of insect cuticle is controlled by a gene, *pen* for penetration. This mechanism was also attributed to resistance found in a permethrin-selected strain of housefly (DeVries and Georghiou, 1981).

In mosquitoes resistance due to decreased insecticide penetration is not well understood compared to other resistance mechanisms such as target-site and metabolic resistance. In a study carried out using *An. gambiae* populations from Nigeria, Tanzania and The Gambia, Anyanwu *et al*. (2000) showed that malathion-resistant strains from Nigeria had thicker cuticles compared to susceptible strains. More recently, studies have shown that cuticular genes are over expressed in pyrethroid resistant *An. gambiae* probably indicating the importance of decreased insecticide penetration as a resistance mechanism (Awolola *et al.,* 2008; Djouaka *et al.,* 2008).

### 1.7.3 Metabolic resistance

Metabolic detoxification is one of the most common mechanisms of insecticide resistance (Hemingway and Karunaratne, 1998). The enzymes responsible for detoxification of insecticides are transcribed by three members of large multigene enzyme systems: monooxygenases (P450's), non-specific esterases (NSE), and glutathione S-transferases (GST's), (Hemingway *et al.,* 2004). 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 results in production of enzymes which can efficiently detoxify the insecticide (Li *et al.,* 2007).

#### 1.7.3.1 Monooxygenase-based resistance

Monooxygenase-mediated metabolism is a common mechanism by which insects become resistant to insecticides (Feyereisen, 1999). Monooxygenases or cytochrome P450s are enzymes constituting a large complex superfamily of heme containing enzymes widely distributed in most organisms including insects (Scott *et al.,* 1998). Because of their genetic diversity, broad substrate specificity, and catalytic versatility, P450s are involved in a variety of reactions including the regulation of titers of endogenous compounds such as hormones, fatty acids and steroids (Reed *et al.,* 1994; Mansuy, 1998), and in catabolism and anabolism of xenobiotics such as drugs, pesticides (Elissa *et al.,* 1993;

Vulule *et al.,* 1994; Brooke *et al.,* 2001) and allelochemicals (Kulkarni and Hodgson, 1980).

In insects more than 660 P450 genes, found in CYP4, CYP6, CYP9, CYP12, CYP15A, CYP18A, CYP28A, CYP29A, CYP48, CYP49, CYP301- CYP318, CYP319A, CYP21A, CYP324, CYP325, CYP329, and CYP332-CYP343 families and subfamilies have been described (Li *et al.,* 2007). The cytochrome P450 enzymes confer insecticide resistance via increased levels of P450 activities resulting from elevated expression of P450 genes. This up-regulation has been recorded in 25 P450 genes, belonging to 4 families CYP4, CYP6, CYP9, and CYP12 (David *et al.,* 2005). In mosquitoes a few of these have thus far been linked to insecticide resistance. Over expression of CYP6Z1, CYP6Z2, CYP6M2, CYP6P3 and CYP325A3 have been associated with pyrethroid resistance in both laboratory and field populations of *An. gambiae* and *An. arabiensis* (Nikou *et al.,* 2003; David *et al.,* 2005; Muller *et al.,* 2007a; Muller *et al.,* 2007b; Djouaka *et al.,* 2008; Awolola *et al.,* 2008). In *An. funestus* CYP6P9 was found elevated in a pyrethroid-resistant laboratory strain (Amenya *et al.,* 2008). Although the up regulation of these genes was associated with resistance their potential to metabolize pyrethroids remains unclear. Overexpression of a gene does not necessary translates into increased insecticide metabolism. Recently, McLaughlin *et al*. (2008) showed that although CYP6Z2 has been implicated in pyrethroid resistance it is incapable of metabolizing both permethrin and cypermethrin. However, Muller *et al*. (2008) functionally characterized over expressed CYP6P3 in pyrethroid resistant field collected *An. gambiae* and showed that the resultant

enzyme is capable of metabolizing both permethrin and deltamethrin. This highlights the importance of functionally characterizing unregulated proteins (genes).

Interestingly, P450 gene expression has been found to be life stage dependent. CYP6Z1 is predominantly expressed in adults, whereas CYP6Z2 is expressed at high levels in the larvae and CYP6Z3 is predominantly expressed in larvae and pupae (Nikou *et al.,* 2003). This developmental expression has been partly attributed to substrate specificity and endogenous function (McLaughlin *et al.,* 2008), however, the true picture still need to be ascertained.

**1.7.3.2 Esterase based resistance**

Esterases are a group of hydrolase metabolic enzymes capable of hydrolyzing compounds containing ester bonds. In insects esterases are frequently implicated in organophosphate (OP), carbamate, and pyrethroid resistance. Esterases confer resistance by rapid binding and slow turnover of insecticides preventing them from reaching their target site. Stoichiometry of esterases and insecticides is known to be 1:1 (Paton *et al.,* 2000), and therefore for the reaction to be effective the amount of enzyme has to far outweigh that of the insecticide. The overproduction of esterases can be through gene amplification, altered gene expression or in some instance mutations can occur resulting in enzymes which can more efficiently metabolize insecticides.

Gene expression and protein studies have shown that esterases genes *estα2*[1] and *estβ2*[1] are co-amplified and highly associated with organophosphate resistance in *Culex*

*quinquefasciatus* mosquitoes (Paton *et al.,* 2000). In the major Afro-tropical malaria vectors, *An. gambiae* s.s, *An. funestus*, and *An. arabiensis,* various reports of increased levels of esterase activity associated with insecticide resistance have been reported (Vulule *et al.,* 1999; Casimiro *et al,* 2006a, b; Matambo *et al.,* 2007). However, in these studies there was no correlation between elevated esterase activity and corresponding bioassay mortality data. Further studies need to be carried out to verify whether this elevation is truly linked to insecticide resistance. This can be achieved by detailed metabolic studies of insecticide resistant mosquito strains.

**1.7.3.3 Glutathione S-transferases (GSTs) based resistance**

GSTs are a family of multi-functional enzymes involved in detoxification of endogenous and xenobiotic compounds (Tu and Akgul, 2005). Insect GSTs can be conveniently grouped into three classes (I, II and III). Class II GSTs constitute the most important metabolic enzymes. They can catalyse the conjugating of reduced glutathione with xenobiotics such as plant allelochemicals and insecticides, thereby converting them to less toxic water soluble forms (Singh *et al.,* 2001). GSTs are further divided into two groups, microsomal and cytosolic depending on their location within the cell (Hemingway *et al.,* 2004). Only four microsomal GSTs have been recorded in insects, one in *Drosophila melanogaster* and three in *An. gambiae* (Enayati *et al.,* 2005). These microsomal GSTs have never been implicated in insecticide resistance. The other group, cytosolic GSTs, constitute the most important group because of their role in insecticide metabolism. Insect cytosolic GSTs have been recently reclassified into six classes; Delta, Epsilon, Omega, Sigma, Theta and Zeta (Enayati *et al.,* 2005).

GSTs occur naturally in insects where they play various roles including detoxification of xenobiotics, intracellular and circulatory transport of endogenous lipophilic compounds, biosynthesis of hormones and cellular anti-oxidation (Lee, 1991; Enayati *et al.,* 2005). Elevated GSTs have been implicated in organophosphate, pyrethroid and DDT resistance (Wang et *al.,* 1991; Prapanthadara *et al.,* 1995; Vontas *et al.,* 2001; David *et al.,* 2005). Elevation of GST is monitored biochemically by comparison of total GST activity between suspected resistant strains and known susceptible baseline cohorts using model substrates such as 1-chloro-2, 4-dinitrobenzene (CDNB) and 1, 2-dichloronitrobezene (DCNB). However, variation in the levels of total GST between resistant and susceptible cohorts does not give clear insights on the mechanisms of resistance. Progress has been made in identifying genes associated with GST based resistance. Currently seven GST genes have been implicated in insecticide resistance by either gene amplification or overexpression (Li *et al.,* 2007). GST gene amplification associated with insecticide resistance has been recorded in two insect species: an organophosphate resistant housefly strain (Wang *et al.,* 1991) and a pyrethroid resistant plant hopper *N. lugens* (Vontas *et al.,* 2001).

In mosquitoes, the well studied detoxification of dehydrochlorination of DDT to the non-insecticidal metabolite dichlorodiphenyltrichloroethane (DDE) catalyzed by the cytosolic epsilon GST, GSTe2, conferring resistance to DDT is through gene overexpression (Ortelli *et al.,* 2003; David *et al.,* 2005). Over expression of a sigma class GST (GSTS1-2) has also been associated with pyrethroid resistance in *An. gambiae* (Muller *et al.,* 2007a; Awolola *et al.,* 2008). A further two delta class GSTs, GSTD1-6 and GSTD11

have been associated with permethrin resistance in a permethrin resistant *An. gambiae* population from southern Benin (Djouaka *et al.,* 2008). In southern Africa, elevation of GSTs has been associated with DDT resistance in *An. arabiensis* (Hargreaves *et al.,* 2003; Masendu *et al.,* 2004). However, the molecular mechanism of this resistance has not been studied in detail.

### 1.7.4 Target site resistance

Target site resistance results from a change in sensitivity of the target site to an insecticide. This insensitivity is usually correlated with a single amino acid substitution which sufficiently alters the dimensional structure of the site of action preventing the insecticide from binding effectively. The target sites involved in insects are the voltage-gated sodium channels from nerve membranes targeted by pyrethroids and DDT, acetylcholinesterase targeted by organophosphates and carbamates and GABA receptors targeted by cyclodienes.

#### 1.7.4.1 Sodium Channels

Sodium channels are glycoprotein spanning neuronal membranes and form the major sites for nerve action potential. Structurally the major subunit of the channel, α-subunit, is composed of four homologous domains (I to IV), each consisting of six transmembrane molecules (SI to S6) (Figure1.1), (Noda *et al.,* 1984).

Voltage-gated sodium channels are the target site for both pyrethroids and DDT (Hemingway *et al.,* 2004). These insecticides operate by altering the normal function of the channel preventing repolarization of action potential.

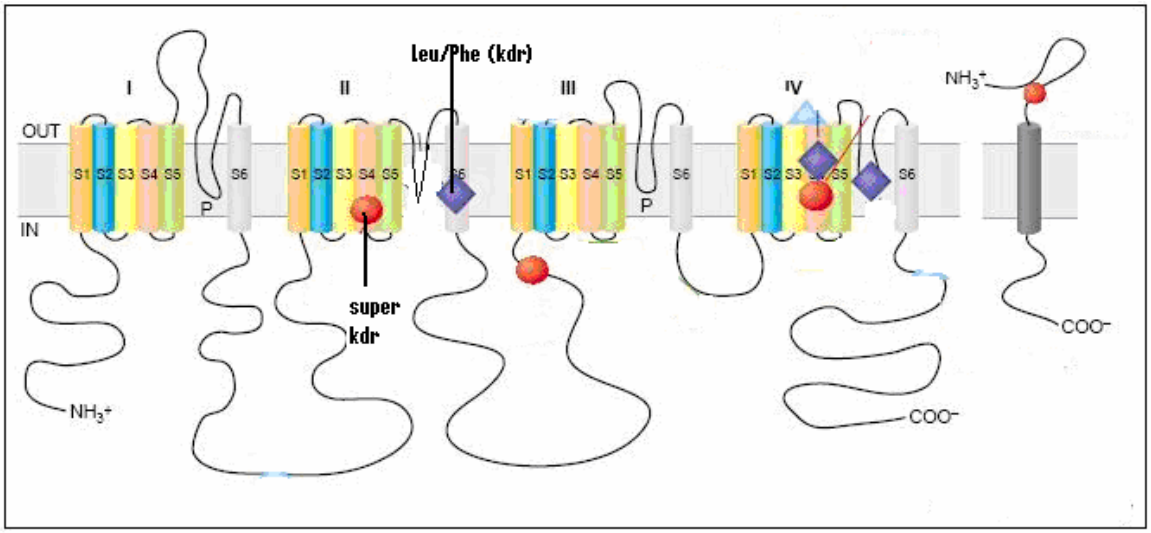



**Figure 1.1:** Diagrammatic representation of domain I-IV of the sodium channel showing the *kdr* and super *kdr* mutations on the domain II that have been implicated in conferring pyrethroid resistance in insect species (modified from Hemingway and Ranson, 2000).

Insensitivity to pyrethroids and DDT has been recorded in various insect species (Williamson *et al.,* 1996; Brengues *et al.,* 2003; Enayati *et al*., 2003). This insensitivity was first recorded in a pyrethroid resistant strain of the housefly, *Musca domestica*, and was termed knockdown resistance (*kdr*) (Busvine, 1951). Molecular cloning of the sodium channel gene from resistant insects has shown that resistance is due to specific mutations along the domain II of the sodium channel (Miyazaki *et al.,* 1996; Williamson *et al*., 1996; Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000).

For the malaria vector *An. gambiae* s.s, two mutations at the domain II of the voltage gated channel are associated with resistance to permethrin and DDT (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000; Pinto *et al.,* 2006). The first mutation consists of a leucine (TTA) to phenylalanine (TTT) transversion at residue 1014 (L1014F) 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 second mutation consists of a leucine (TTA) to serine (TCA) transition (L1014S) has been reported mainly in the S-molecular form (Santolamazza *et al.,* 2008) is found mainly in East Africa (Ranson *et al.,* 2000). Both these mutations have been observed in the sibling species *An. arabiensis* (Diabate *et al.,* 2004; Stump *et al.,* 2004; Verhaeghen *et al.,* 2006). Several reports of *kdr* in both *An. gambiae* s.s and *An. arabiensis* have shown a lack of correlation between the mutation and bioassay mortality data (Matambo *et al.,* 2007; Abdalla *et al.,* 2008; Moreno *et al.,* 2008). There are no records of *kdr* mutation in southern Africa despite these reports. The PCR based diagnosis for *kdr* mutation is allele specific and was developed for *An. gambiae* (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). It is likely that other mutations occurring on the target site are present but cannot be detected by the current methods. Therefore, further molecular studies of the *An. arabiensis para*-sodium channel are essential to elucidate whether cross resistance to DDT and pyrethroids exhibited by some mosquito populations is a result of other mutations on the sodium channel.

### 1.7.4.2 Acetylcholineesterase (AChE)

Acetylcholineesterase is a serine esterase in the alpha/beta hydrolase fold enzyme family, (Ollis *et al.,* 1992), Figure 1.2. AChE is encoded by genes designated as *ace* genes (Toutant, 1989). In most insects, including *Anopheles* mosquitoes, two genes (ace-1 and ace-2) are present (Weill *et al.,* 2002). AChE plays a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. It 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).

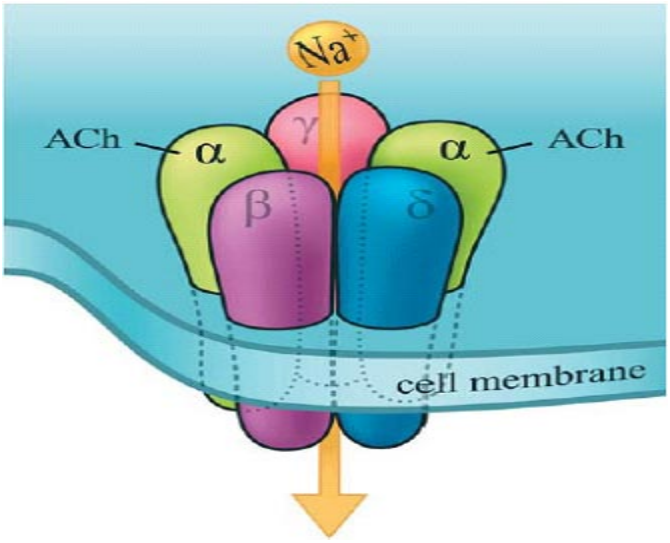



**Figure 1.2:** Schematic illustration of an Acetylcholine receptor with its binding sites (http://www.innovitaresearch.org/news/05012801.html)

AChE is the target for the largest group of insecticides, organophosphates and carbamates (Brogdon *et al.,* 1988). Intensive use of these insecticides has led to the development of resistance in many target species that are important in both agriculture and public health (Georghiou, 1990; Fournier and Mutero, 1994). Resistance to organophosphates and

carbamates involves changes in the AChE gene rendering it less sensitive to these insecticides (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). Mutant forms of AChE have been characterized biochemically and show a wide spectrum of insensitivity between species as well as a marked range of insensitivity to different compounds within species (Devonshire and Moores, 1984).

Mutations in the *ace*-2 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 *ace*-1 gene have been associated with resistance in *Culex pipiens* (Weill *et al.,* 2002), *An. albimanus* and *An. gambiae* (Weill *et al.,* 2004).

In southern Africa there are very few reported cases of altered AChE. This might be attributed to the chemicals used for vector control by the respective malaria control programmes. It is only in Mozambique where altered AChE was found at very low frequency in *An. funestus* populations from various localities (Casimiro *et al.,* 2006a) and recently at very high frequency in *An. arabiensis* (Coleman *et al.,* 2008). This is a cause of concern as organophosphates and carbamates are extensively used in agriculture and at times for targeted larval control. Sensitivity of the AChE of malaria vectors from southern Africa needs to be continuously monitored.

### 1.7.4.3 Gamma-aminobutyric ($GABA_A$) Receptors

The $GABA_A$ receptor is a heteromultimeric-gated chloride ion channel (Figure 1.2) in the insect's nervous system and neuromuscular junctions (Bermudez *et al.,* 1991). The insect $GABA_A$ receptor is the site of action of cyclodienes, ivermectins and phenylpyrazoles (Ffrench-Constant *et al.,* 2000).

Most cases of cyclodiene resistance appear to be due to decreased sensitivity of the $GABA_A$ 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 amino acid change resulting in resistance to cyclodienes.

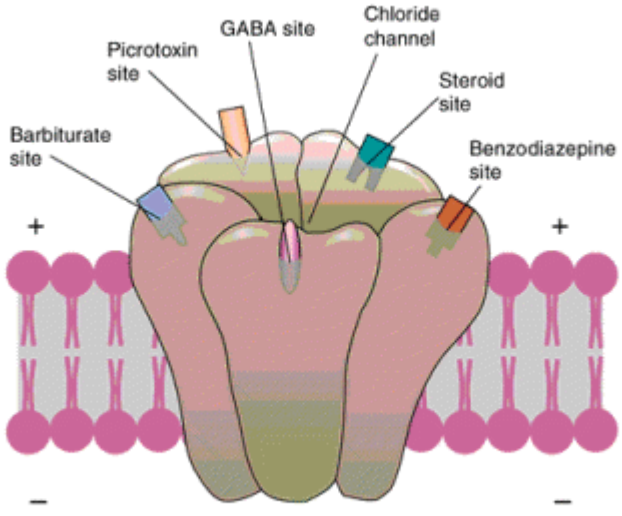



**Figure 1.3:** Schematic illustration of $GABA_A$ receptor with its binding sites (Adapted from Lackovic, 2003).

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 (Ala- Ser) 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).

In mosquitoes, dieldrin resistance has been reported in all the major African malaria vectors *An. funestus*, *An. gambiae s.s* and *An. arabiensis* (Coetzee, 2006, Brooke *et al.,* 2006). However, no detailed work has been done on the mechanisms of resistance in the mosquito $GABA_A$ receptor despite the prevalence of cyclodiene resistance which, together with their high mammalian toxicity, led to their withdrawal from agriculture in 1974 and in public health in 1987 (WHO, 2001b). Du *et al*. (2005) recently showed that a mutation conferring the substitution alanine 296 to glycine (A296G) 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 Ala296Ser is associated with dieldrin resistance in a laboratory strain of *An. arabiensis*.

## 1.8 Insecticide resistance management

Insecticide resistance management incorporates strategies to minimize resistance development and preserve the utility of the insecticide in use. In malaria control there are only 12 insecticides belonging to four chemical groups (organophosphates,

organochlorines, carbamates and pyrethroids) recommended for IRS and only pyrethroids approved for bednet impregnation (WHO, 2001b). Resistance management is thus a requirement. Unfortunately, most countries which rely on insecticide based control strategies do not have resistant management policies. Only 53 out of 109 malaria countries claim to have a strategy for resistance management (WHO, 2008).

There are three resistance management strategies that can be used: 1) the use of an insecticide until resistance begins to affect operations, 2) rotational use of insecticides and 3) simultaneous use of insecticides having different mode of action (Denholm and Rowland, 1992). Rotational use of insecticides is the favoured approach. This strategy is based on the adverse fitness cost associated with resistance genes (Bourguet *et al.,* 2007). In the absence of selection pressure resistant individuals are naturally eliminated from the population. Therefore, by rotating insecticides with different modes of action individuals carrying resistant genes for a particular insecticide are eliminated when a switch is made to another class of insecticide. Thus it might take a long time to select for resistance to any of the two insecticides being used. However, this is based on the assumption that mosquitoes carrying two different resistant alleles for two different insecticides are rare. This is also only possible if the resistant gene has a selective disadvantage during the absence of section pressure. Rotational use of insecticides has been shown to be effective in other vector control programmes. The African Programme for Onchocerciasis Control (APOC) in West Africa successfully managed resistance in *Simulium* vectors by switching between different non-residual larvicides in response to detection of resistance to the preferred compound, temephos (Hougard *et al.,* 1993).

Simultaneous using of insecticides can be divided into ‘mosaics’ which involves use of two insecticides separately: for example a bednet impregnated with more toxic insecticide such as a carbamate at the ceiling of the net and a pyrethroid at the bottom of the net (Guillet *et al.,* 2001). Another tactic for simultaneous use of insecticide involves spraying a cocktail of two or more insecticides (mixtures). The principle of simultaneous use of insecticides is based on the fact that resistance will evolve more slowly to individual insecticides. It is also highly unlikely that any population will contain individuals carrying resistant genes to all classes of insecticides being used. This type of resistance management strategy was successfully implemented in southern Mexico for controlling *An. albimanus* which had become resistant to a variety of insecticides. Implementation of resistant management strategies resulted in a marked decrease in resistance gene frequencies (Hemingway *et al.,* 1997).

## 1.9 Problem definition and justification

In southern Africa malaria control programmes heavily depend on insecticides for vector control. Such control strategies have a disadvantage of resistance developing in targeted populations if no resistance management is in place. As discussed earlier, effective resistance management programmes require prior information on the susceptibility status of the target vectors to insecticides available for their control. Information on insecticide resistance mechanisms operating within the vectors is also an important consideration when drawing up evidence based resistance management policies. Such information is crucial for the selection of appropriate insecticides to be used for a control programme.

In most southern African countries significant progress has been made in obtaining baseline information on the susceptibility status of malaria vectors. However, there is limited information on the resistance mechanisms involved. Insecticide resistance in this region was reported as far back as the 1970s when a population of *An. arabiensis* resistant to BHC was recorded in Zimbabwe (Green, 1981). Currently there are reports of DDT resistance in *An. arabiensis* in Kwazulu/Natal, South Africa and Gwave, Zimbabwe (Hargreaves *et al.,* 2003; Masendu *et al.,* 2005). In addition to these, a population of *An. arabiensis* resistant to permethrin (78% mortality) was collected in Mamfene, KZN, South Africa (Mouatcho *et al.,* 2009). In Mozambique low levels of pyrethroid and carbamate resistance have been reported in *An. arabiensis* (Casimiro *et al.,* 2006b). With this gradual increase in insecticide resistance, studies to understand the intricacies of resistance mechanisms involved needed to be carried out. Such studies will provide evidence based recommendations on resistance management strategies to prevent resistance impacting on malaria control operations.

Initial investigations of pyrethroid and DDT resistance in *An. arabiensis* from southern Africa using biochemical assays have implicated metabolic based resistance mechanisms (Hargreaves *et al.,* 2003; Casimiro *et al.,* 2006b). These results have shown DDT resistance to be associated with elevated GSTs (Hargreaves *et al.,* 2003; Masendu, 2004) while pyrethroid resistance is associated with elevated activity of monooxygenases (Casimiro *et al.,* 2006b). In these studies enzyme elevation was not significantly correlated with the corresponding bioassay mortality data. Therefore this information cannot be extrapolated and used for formulating resistance management strategies or help

in choosing alternative insecticides as it is inconclusive. This therefore justifies the need to carry out further studies to investigate the intricacies of resistance mechanisms in *An. arabiensis* from southern Africa.

## 1.10 Objectives

This study was conducted in two neighbouring countries South Africa and Zimbabwe. The overall aim was to obtain baseline information on the susceptibility status of *An. arabiensis* from a malaria endemic area in Zimbabwe. It also provides information on underlying resistance mechanisms in resistant wild populations by assaying for enzymes which are normally implicated in insecticide resistance. In addition this study investigated changes in insecticide resistance levels, detoxification enzymes and over expressed genes following artificial selection of laboratory colonies. This study provides the first step in identifying metabolic genes which can be used to develop assays to monitor metabolic resistance.

### 1.10.1 Specific objectives

**Zimbabwe**

1) To provide updated information on the composition, distribution and vector status of malaria vectors from a malaria endemic area in Zimbabwe.

2) To evaluate the susceptibility level of *An. arabiensis* to all classes of insecticides recommended for vector control.

3) To characterize resistance mechanisms in insecticide resistant wild populations.

**South Africa**

1) To artificially select for permethrin and bendiocarb resistance in *An. arabiensis* colonies.

2) To determine mechanisms conferring permethrin and bendiocarb resistance in *An. arabiensis* colonies artificially selected for resistance using biochemical techniques.

3) To determine the expression profile of CYP6Z1, CYP6Z2, CYP6Z3, CYP6M2, CYP6P3 and CYP4G16 in permethrin resistant *An. arabiensis* strains using quantitative PCR (qPCR).

# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Study area

Mosquito sampling was carried out in Gwave, (17º55'S; 28º41'E), a village located in Gokwe South District, Midland's Province of Zimbabwe (Figure 2.1). The study area lies at an altitude of 800m and falls under natural farming region V, which is characterized by a single rainy season from October/November to April, a cool dry season from mid May to mid August and hot dry period from mid August to mid November (Masendu, 2004). The major rainfall season is November-February averaging 400 to 1200 mm annually with the highest monthly rainfall in either January or February. The mean daily temperature during transmission season ranges from 26$^{o}$C to 30$^{o}$C with highest temperatures being recorded between October and December. A high relative humidity of 65-85% is encountered between November and February.

Gokwe has a population of 500 000 people of who entirely depends on subsistence farming for their survival. Cotton is the main cash crop grown in this area; however, several other crops are cultivated under small scale irrigation using water from artesian wells (Figure 2.2A) or shallow wells dug in sandy riverbed. These man made water bodies ensures mosquito breeding throughout the year. Other breeding sites include perennial swamps formed by water discharge from artesian wells (Figure 2.2B), small temporary rain puddles (Figure 2.2 C) and large semi-permanent ponds. These ponds are formed during the rainy seasons and can last up to 6 months.

Typical house in Gokwe consist of brick walls and grass thatch. Each household keep domestic animals very close to the homestead. These animals are kept in roofless wooden structures during the night.

Mosquitoes in this area are constantly under selection pressure from insecticides employed in agriculture and public health. The area falls within the annual house spraying program of the Zimbabwe Malaria Vector Control, plus large quantities of various insecticides covering all insecticide classes are applied for agricultural purposes.

Malaria transmission occurs throughout the year with 15.5% of the population are infected each year (NIHR, National Malaria Survey, 1991).

## 2.2 Mosquito collections

Mosquitoes were collected in February 2006 and January 2008. Adult anopheline mosquitoes were collected from the study area using man-bait tent trap catches (MBN) (as described by Mpofu and Masendu, 1986), (Figure 2.3A), exit window traps (Figure 2.3B) using the method described by Muirhead-Thomson (1948), indoor resting direct catches (Figure 2.3C), and cattle kraal collections. Larval collections (L.C) were done using the method described by Service, (1976).

The wild collected adult mosquitoes were identified morphologically as members of the *An. gambiae* complex or *An. funestus* group using hand lenses and taxonomic keys (Gillies and De Meillon, 1968 and Gillies and Coetzee, 1987). These specimens were

analysed as described in section 2.3. Adults emerging from larval collections were also identified morphologically and maintained with a 10% sugar solution under ambient conditions. While in the field, 2-3 day old adults from larval collections were used for field susceptibility assays. Wild collected adults were kept alive to produce $F_1$ progeny. Both live and dead specimens preserved over silica gel were transported to the Vector Control Reference Unit (VCRU), National Institute for Communicable Diseases (NICD), Johannesburg, South Africa for further analysis.

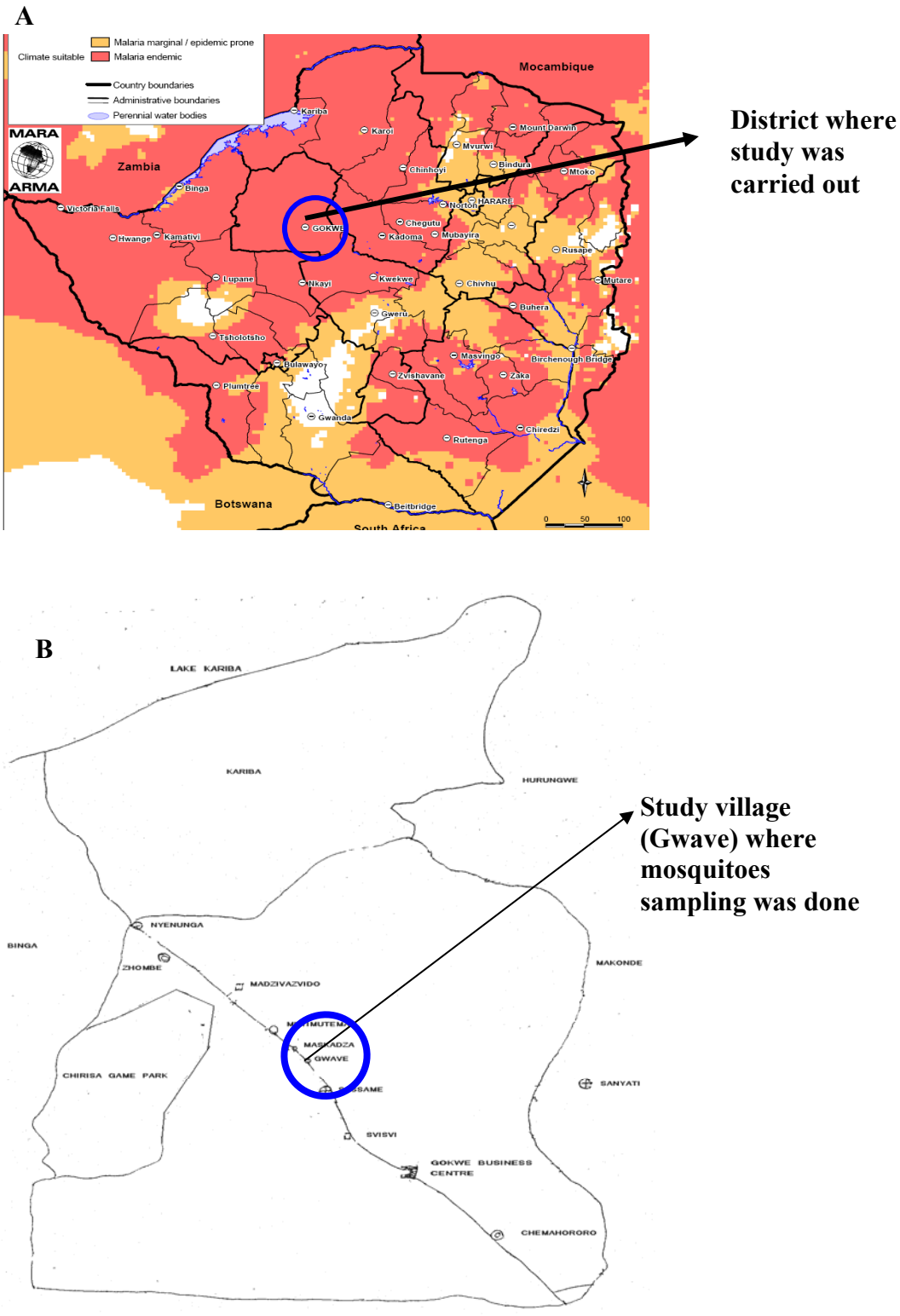



**Figure 2.1: A:** Map of Zimbabwe showing malaria distribution and location of the study site. **B**: localisation of the district where the study was carried out.




**Figure 2.2:** Mosquito breeding sites in study area. **A**: An artesian well in Gwave village which acts as a water source for villagers. **B:** Discharge of water from the well is continuous resulting in the formation of permanent swamps which act as breeding sites**.** C**:** Rain puddles formed around homesteads which act as breeding ground for *An. arabiensis*.




**Figure 2.3**: Mosquito collection methods used during field collections: **A**: man-baited tent trap, **B**: Window exit trap and **C**: Indoor house searching, D: cattle kraal

## 2.3 Mosquito Processing

A flow chart of the handling and processing of wild samples is given in figure 2.4. Preparation of chemicals and solutions is given in Appendix IA.

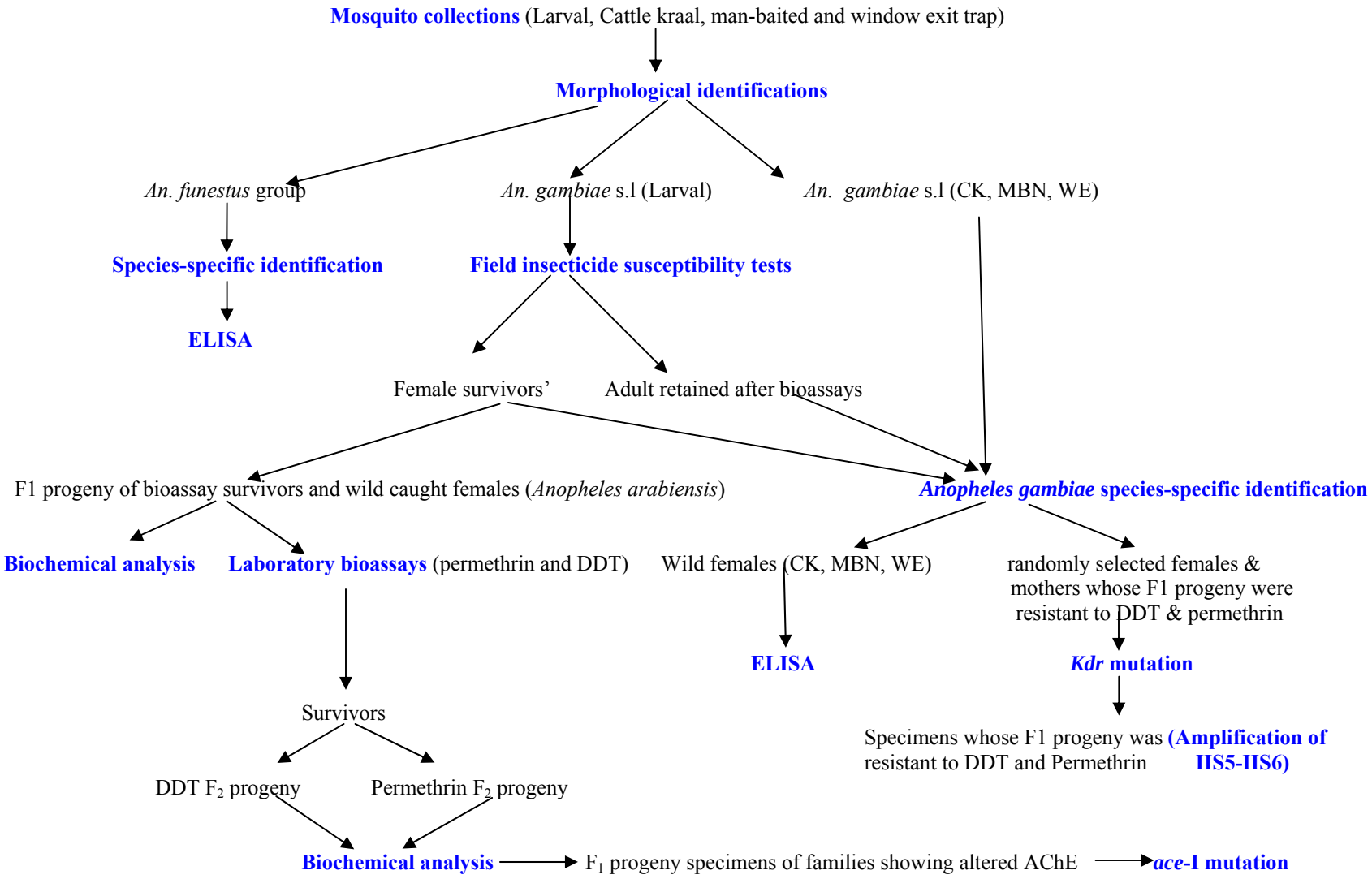



**Figure 2.4**: Summary of field collected mosquito processing (CK = cattle kraal; MBN = man-baited, WE = window exit).

### 2.3.1 Species-specific molecular identifications

Methods outlined in this section were used to identify wild-caught mosquitoes of the *An. gambiae* complex. Also included are methods for identification of *An. funestus* group and *An. longipalpis* on the basis that two specimens were unexpectedly collected during 2006.

#### 2.3.1.1 *Anopheles gambiae* complex

Wild caught *An. gambiae* complex specimens used for 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). The detailed methodology is described in Appendix IIA.

**Table 2.1**: Polymerase chain reaction (PCR) primers and sizes of the amplified products for species-specific diagnostic tests for the *An. gambiae* complex, the *An. funestus* group and the *An. longipalpis* group (bp = base pair).

| Species | primer | Primer sequence (5' to 3') | Fragment length |
|---|---|---|---|
| ***An. gambiae* complex species identification primers** | | | |
| *An. merus* | ME | TGA CCA ACC CAC TCC CTT GA | 464 bp |
| *An. gambiae s.s* | GA | CTG GTT TGG TCG GCA CGT TT | 390 bp |
| *An. arabiensis* | AR | AAG TGT CCT TCT CCA TCC TA | 315 bp |
| *An. quadriannulatus* | QD | CAG ACC AAG ATG GTT AGT AT | 153 bp |
| *An. gambiae* complex | UN | GTG TGC CCC TTC CTC GAT GT | |
| ***An. funestus* group species identification primers** | | | |
| *An. vaneedeni* | VAN | TGT CGA CTT GGT AGC CGA AC | 587 bp |
| *An. funestus s.s* | FUN | GCA TCG ATG GGT TAA TCA TG | 505 bp |
| *An. rivulorum* | RIV | CAA GCC GTT CGA CCC TGA TT | 411 bp |
| *An. parensis* | PAR | TGC GGT CCC AAG CTA GGT TC | 252 bp |
| *An. leesoni* | LEES | TAC ACG GGC GCC ATG TAG TT | 146 bp |
| *An. funestus* group | UN | TGT GAA CTG CAG GAC ACA T | |
| ***An. longipalpis* group species identification primers** | | | |
| *An. longipalpis* Type A | A3 | GCC GGG GTC TCA GAT CTT CA | 328 bp |
| *An. longipalpis* Type C | C1 | CCA AGC ACG TTG ATC CAG TAT TAC | 439 bp |
| *An. funestus* group | UN | TGT GAA CTG CAG GAC ACA T | |

#### 2.3.1.2 *Anopheles funestus* group

Two specimens morphologically identified as members of the *An. funestus* were processed using the polymerase chain reaction assay as described by Koekemoer *et al.* (2002) with slight modifications (Appendix IIB).

#### 2.3.1.3 *Anopheles longipalpis* group

Two specimens morphologically identified as *An. funestus* consistently gave hybrid amplicons (section 3.3.1 for details) using the multiple PCR of Koekemoer *et al.* (2002) suggesting that the samples might not belong to the *An. funestus* group. The *An. longipalpis* multiplex PCR assay (Koekemoer *et al.,* 2009) which is used to distinguish two molecular types of *An. longipalpis*, type A and C was then used to identify these two specimens. The detailed methodology is described in IIC.

## 2.4 Mosquito infectivity

A total of 530 female mosquitoes collected in 2006 and 36 females collected in 2008 were tested for *Plasmodium falciparum* infection using the ELISA procedure of Wirtz *et al.* (1987) as detailed in Appendix IID. All the ELISA solutions were prepared as described in the Appendix IB.

**Data analysis**

Results were first scored visually and then photometrically at 405nm using a microplate reader (Labsystems Multiskan RC, Genesis software version 3.03). Each positive sample was retested for confirmation. Samples were considered sporozoite positive if absorbance exceeded twice the mean value of seven negative controls (Beier *et al.,* 1988). Sporozoite

infections rates were expressed as the proportion of *P. faliciparum* positive of the total number of females tested and were calculated for each sampling method.

## 2.5 Insecticide susceptibility tests

These assays were conducted on wild caught mosquitoes and laboratory reared familial mosquitoes using WHO test kits following the standard WHO procedure (WHO, 1998).

Briefly, samples of wild collected *Anopheles gambiae s.l* reared from larval collections were exposed to 0.75% permethrin, 4% dieldrin, 4% DDT, 0.1% bendiocarb and 5% malathion. Twenty five to thirty sugar-fed female mosquitoes were used for each replicate. For each insecticide test, six cylinders, two serving as controls and four as treatments, were used. Control cylinders consisted of plain filter papers, while treatments consisted of filter paper impregnated with the respective insecticide at a standard concentration. All specimens used for field bioassays were then stored individually in eppendorf tubes with desiccant and transported to the NICD for laboratory processing. In order to verify whether insecticide susceptibility of wild caught mosquitoes were influenced by age, physiological state of the mosquitoes and pre-exposure to insecticides, bioassays were also carried out on $F_1$ progeny of wild-caught mosquitoes.

Once in the laboratory, live wild-caught females were blood fed on anesthetized guinea pigs and transferred into individual oviposition tubes to lay eggs. Families from each egg batch were reared separately through to 1-3 day old $F_1$ progeny and subdivided into two groups depending on the number of adults emerging. One group was split into two and

exposed to 4% DDT and 0.75% permethrin for one hour respectively and the other group was stored at -70ºC and subsequently used for biochemical analysis. For collections done in 2008 $F_1$ bioassay survivors were pooled. These resistant individuals were allowed to mate and their progeny, called $F_2$, were subsequently used for biochemical analyses.

All insecticide exposure papers were tested both prior to and after the exposures using an insecticide susceptible *An. arabiensis* colony (KGB) in order to confirm insecticidal activity.

**Data analysis**

Mortalities were analyzed based on the WHO susceptibility criteria. A resistant population is defined by mortality rates less than 80% after the 24 hour observation period while mortality rates greater than 98% are indicative of susceptible populations. Mortality rates between 80-98% suggest a possibility of resistance that requires confirmation. All data analysis was based on at least two replicates and appropriate controls.

## 2.6 Mosquito strains

Seven laboratory colonies maintained in the Botha De Meillon insectary at VCRU, South Africa and belonging to the *An. gambiae* complex were used in this study. For the sibling species *An. arabiensis* four laboratory colonies designated KGB, MBN, KWAG and MBN-DDT were used. KGB is an insecticide susceptible laboratory colony originating from Zimbabwe and has been maintained without insecticidal pressure since 1975. It was used for comparative purposes in biochemical assays and as a positive standard in *An.*

*gambiae* complex species-specific identifications. MBN and KWAG, both originating from Mamfene, Kwazulu/Natal, South Africa were used to characterize carbamate and pyrethroid resistance in this study. MBN was colonized in 2002 from wild populations which showed low levels of carbamate and DDT resistance during the time of collections. KWAG was colonized in 2005 using $F_1$ progeny from wild caught females showing low levels of permethrin resistance (78%). SENN-DDT was used as a positive control for the West African *kdr* mutation. This colony originated from Sudan, shows multiple insecticide resistance and has been under 4% DDT selection since 2004. One colony each for *An. quadriannulatus*, *An. merus* and *An. gambiae s.s* were also used during this study as positive controls in the *An. gambiae* complex species identifications.

## 2.7 Mosquito rearing and maintenance

Live adult mosquitoes collected from the field and colony mosquitoes used in all experiments were reared at 25$^{o}$C and 85% relative humidity in the Botha De Meillon insectary at NICD Johannesburg following the standard rearing procedure as described by Ford and Green (1972) and Hunt *et al.* (2005).

First instar larvae were reared in polythene plastic bowls (27cm x 16cm x 6.5cm) containing 250ml distilled water being fed on a mixture of brewers yeast (Vital Health Foods, South Africa) and finely ground dog biscuits (West's traditional crunching biscuits treats, Martin and Martin, South Africa) prepared at a ratio of 1:3. Larvae were kept at low density to avoid under feeding and maximise size. Fourth instar larvae were kept in plastic bowls containing distilled water covered with a netting material in which

they pupated and emerged as adults. Adults were transferred to 5-litre plastic cages with sleeves for easy access. These adults were maintained on 10% (w/v) sugar solution under standard insectary conditions described above and a photo period of 12:12 hour light/darkness, with a 45-min dawn and dusk light regimen. Female mosquitoes were offered a blood meal from anesthetized guinea pigs (ethical clearance 1993 047, Appendix III) four days post emergence. After three blood meals darkened Petri dishes acting as oviposition plates were placed into the plastic cages to facilitate egg oviposition. Eggs were washed once with 1% formalin and three times with distilled water and transferred into plastic bowls as described above.

## 2.8 Selection for resistance

Two colonies MBN and KWAG were selected with 0.1% bendiocarb and 0.75% permethrin respectively. Newly emerged males and virgin females were separated into different cages approximately six hours after eclosion to ensure that mating did not take place prior to insecticide exposure. Selection for resistance to each insecticide was initiated with exposure to 0.1% bendiocarb (carbamate) and 0.75% permethrin (pyrethroid), at an exposure time that caused approximately 50% mortality ($LT_{50}$). Male and female survivors 24 hour post-exposure were pooled into a single cage for each insecticide and left for four days to mate. Females were fed three times per week on guinea pig blood. An oviposition plate (darkened Petri dish with 20ml of distilled water) was provided after 3 blood meals and females were allowed to oviposit overnight. Egg plates were removed the following morning and the eggs washed with formalin and distilled water as previously described in section 2.4. Eggs were reared through to adults

to form the next generation. These were subjected to selection using 0.1% bendiocarb or 0.75% permethrin for each generation until mortalities below 50% were achieved. After every second generation, 60 individual mosquitoes (30 males and 30 females) of each progeny cohort were kept and stored at -70ºC and subsequently used to monitor changes in enzyme levels using biochemical techniques described in 2.10. Control colonies (MBN-Base and KWAG-Base) reared under the same conditions but not exposed to insecticide were tested randomly to monitor independent occurrence of resistance.

**Data analysis**

The number of mosquitoes exposed per generation and average % mortalities 24 hours post exposure were recorded. Observed differences in susceptibility between the baseline and selected generations were analyzed by comparing $LT_{50}$ values calculated from time-mortality regression analysis.

## 2.9 Synergists exposure assays

Synergists, piperonyl butoxide (PBO), diethyl maleate (DEM), and triphenyl phosphate (TPP) were used in experiments to synergize the bendiocarb and permethrin resistance phenotypes of *An. arabiensis* using methods described by Brogdon and McAllister (1997) and Brooke *et al.* (2006).

### 2.9.1 Synergist paper preparation

Synergists used were active ingredients; solutions were prepared by diluting with olive oil to desired concentrations of (4% for PBO, 8% for DEM and 1%, 4%, 8%, 10%, 16%, and 20% for TPP). To prepare a test paper 0.7ml of synergist solution was mixed with

1.2ml of acetone which acted as a carrier. The solution was evenly spread on a 12.5cm X 15cm Wattman number one filter paper. The treated paper was put on a bed of pins stuck in a cardboard mat and left for 24 hours to evaporate the acetone and obtain the desired concentration.

### 2.9.2 Synergist exposure

For each synergist assay three WHO holding tubes were set up, each with 25-30 mosquitoes. The initial step involved transferring mosquitoes from two of the holding tubes to tubes with filter papers impregnated with the respective synergist. The mosquitoes were allowed tarsal contact with the synergist for one hour. After one hour, mosquitoes from the two synergist tubes and the third control tube which were exposed to an insecticide of known concentration for one hour. The tube with mosquitoes exposed to synergist but not to insecticides acted as a control.

**Data analysis**

Mortality was scored after 24 hours post exposure. Following five replicates, mortalities between synergized and non-synergized samples were compared by replicate using Chi-square contingency tables.

## 2.10 Biochemical assays

The microtitre plate biochemical technique was carried out on samples of $F_1$ and $F_2$ progeny from wild caught females as well as $F_4$, $F_7$, $F_8$, and $F_{12}$ generations of the selected cohorts. 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) with modifications. The assays were designed to test for differences in enzyme activity between the samples and a known susceptible *An. arabiensis* colony. Therefore the use of standard curves as described by Penilla *et al.* (1998) was omitted. Detailed methodology and a brief outline of the principle of each enzyme assay are given in Appendix IIE.

**Data analysis**

Differences in enzyme levels/activity between the susceptible (KGB) and family/selected strains were analyzed using two sample *t*-test of means (Statistix 7.0, Analytical Software). All levels of significance were determined at $P < 0.05$.

## 2.11 Molecular assays

### 2.11.1 DNA extraction

DNA was extracted from single mosquitoes using the standard procedure described by Collins *et al.,* (1987), (Appendix II F).

### 2.11.2 Assay for knockdown resistance (*kdr*) (East and West Africa)

Mutations associated with knockdown resistance to pyrethroids and/or DDT were assayed in randomly selected bioassay survivors of wild caught females as well as those females where $F_1$ progeny showed resistance to both DDT and pyrethroids using the standard PCR assay described by Martinez-Torres *et al*. (1998) and Ranson *et al.* (2002) with modifications.

Three independent PCR assays were set up for each sample: the first contained the primers Agd2 (5'AGACAAGGATGATGAACC3') and Agd4 (5'

CTGTAGTGATAGGAAATTTA 3') which amplify a 137-bp product for the insecticide susceptible allele; the second contained primers Agd1 (5'ATAGATTCCCCGACCATG 3') and Agd3 (5'AATTTGCATTACTTACGACA3') for amplifying a 195-bp product associated with the West African resistant allele and the third contained primers Agd1 and Agd5 (5'TTTGCATTACTTACGACTG3') which amplify a 195-bp product associated with the East African resistant allele. PCR conditions were: initial denaturation at 95 °C for 1 minutes, 40 cycles each containing denaturation at 94 °C for 30 seconds, annealing at 50 °C for 30 seconds and extension at 72 °C for 30 seconds with final extension of the PCR products at 72 °C for 5 minutes. Soon after amplification amplicons were electrophoresed on a 2.5% (w/v) agarose gel with ethidium bromide. The documentation of the gel was carried out using an automated gel documentation system (Syngene G-box, sydr4/1152). Reference standards were as follows: the positive control consisted of a DNA template from mosquitoes with known West African (*kdr-w*) genotype i.e SENN-DDT (homozygous for the L1014F mutation), while KGB was used as homozygous susceptible, SS). A DNA-free reaction mixture was used as a blank in all cases. No colony with the *kdr-e* genotype was available to be used as positive control.

**Data analysis**

Amplicon sizes were compared to the resistant SENN-DDT (RR) and the susceptible KGB (SS) control against a 1000bp size marker (O'Gene ruler $^{TM}$, Fermentas Life Sciences. Cat no SM1153).

### 2.11.3 Sequence analysis of East and West African *kdr* mutations

In order to confirm *kdr* results obtained by PCR, sequencing of a 293-bp fragment flanking the *kdr* locus at domain II segment 6 of the para type sodium channel gene was done from genomic DNA of randomly selected samples which had showed presence of the *kdr* mutation using PCR as well as randomly selected samples which were susceptible for the mutation.

Amplification was done using primers AgD1 and AgD2 in 38 mosquitoes inclusive of six specimens which showed cross resistance to both DDT and permethrin exposure. Ten to fifty ng of DNA was added to a reaction mixture containing: 1 X reaction buffer, 0.238 mM of each dNTP, 1.91 mM $MgCl_2$, 0.188 mM each of primers AgD1 and AgD2 (Martinez-Torres *et al.,* 1998), and 0.02 U of *Taq* DNA polymerase. In all cases double distilled water was added to give a final volume of 25µl. Cycling conditions were as described in section 2.11.2. Five microlitres of the amplified fragments were analysed by electrophoresis on a 2.5% agarose gel and visualised under a UV transilluminator to confirm fragment size. The remaining 20µl of the amplified products were sequenced by Inqaba Biotechnical industries, South Africa.

**Data analysis**

Sequences were first submitted to the Basic Local Alignment Search Tool (BLAST) (http:www.ncbi.nlm.nih.gov/BLAST) to verify whether the *An. arabiensis* sodium channel gene has been amplified and only sequences which produced a significant alignment (>95% identity of the sequenced portion) with the *An. arabiensis* sodium channel gene (Accession number DQ263749) were aligned using the ClustalW

(http://www.ebi.ac.uk/cgi-bin/clustalw). Each sequence was compared to the resistant SENN-DDT (RR) and the susceptible KGB (SS) control using Lasergene software (DNASTAR version 7; SeqMan program, Inc, Madison, WI).

### 2.11.4 Sequencing of the IIS5-IIS6 domain of the sodium channel gene

Biochemical analysis and *kdr* diagnostic tests did not elucidate the resistance mechanism present in the eight field collected samples whose $F_1$ progeny showed resistance to both permethrin and DDT exposures. It was hypothesized that this could be attributed to an alternative mutation within the sodium channel gene. In order to test this hypothesis the presence of single nucleotide polymorphisms (SNPs) in a larger fragment of the sodium channel gene in these specimens were investigated. Sequencing of the IIS5-IIS6 domain was targeted as target site mutations conferring both DDT and pyrethroids resistance in other insect species are present in this region.

#### 2.11.4.1 Amplification of IIS5-IIS6 segment

Genomic DNA was extracted using the standard Collins *et al.* (1987) method as described in 2.11.1. Eight samples of *An. arabiensis* whose $F_1$ progeny was completely susceptible to both DDT and permethrin exposures were used for comparison purposes.

The genomic region spanning IIS5-IIS6 domains of the voltage sodium channel gene was PCR amplified using primer N4 (5' GTTGGTGCAGACAAGGATGA 3') as a common reverse primer, and either N1 (5' AACGATGGGTGCGTTAGGTA 3') or N3 (5' GTGCTATGCGGAGAATGGAT 3') as forward primers (Figure 2.5). Primers N1 and

N4 amplifies 1442 bp of the IIS5-IIS6 domain, while primers N3 and N4 amplifies 324bp of the same region (Okoye *et al.,* 2008).

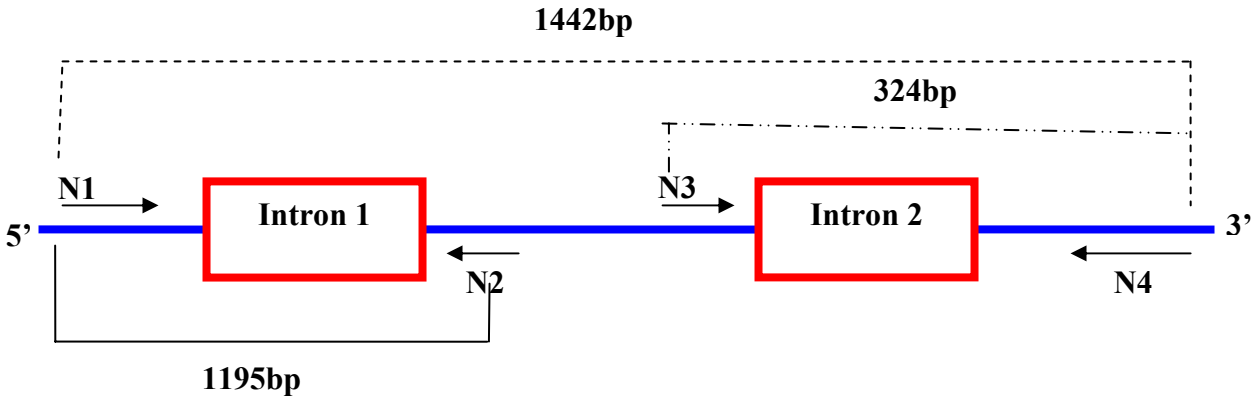



**Figure 2.5:** Illustration of the amplified sodium channel domain IIS5-IIS6 showing primer binding sites (bp = base pair).

Amplification was carried out using 10-50ng of DNA, 2.5µl of 10X buffer, one unit of *Taq* polymerase, 2mM $MgCl_2$ and 0.198µM of each primer were used in a 25µl total PCR volume. Thermocycling was performed for 40 cycles at 94 $^{o}$C for 1 min, 55$^{o}$C for 1 min and 72 $^{o}$C for 1 min with a final extension step at 72 $^{o}$C for 5 min. An initial denaturation step of 94 $^{o}$C was incorporated at the beginning of the cycling conditions. Five microlitres of the amplified fragments were electrophoresed on a 2.5% (w/v) agarose gel containing ethidium bromide. The gel was then documented in a gel documentation system (Syngene G:Box, Cambridge UK). The remainder of the PCR amplicons were sequenced by Inqaba Biotechnical industries, South Africa.

**Data analysis**

Blast search (http:www.ncbi.nlm.nih.gov/BLAST) was carried out to verify whether the right region of the sodium channel gene has been amplified. Only sequences with greater

than 95% identity with the either *An.gambiae* sodium channel gene, Intron 1 and partial sequence (AY615647), *An. gambiae* voltage-gated sodium channel gene, exons 2, 3 (Accession No. EU826538) were used for further analysis. Sequences were analysed using the DNASTAR software suite (DNASTAR Inc. Madison, WI, USA). Sequences were first aligned using MEGALIGN (DNASTAR) and ClustalW (http://www.ebi.ac.uk/cgi-bin/clustalw). Multiple sequence alignment of samples showing resistance to both DDT and permethrin and *An. arabiensis* susceptible samples were compared for any differences in nucleotide sequences.

### 2.11.5 *Ace*-$1^R$ mutation

A PCR diagnostic test was used to detect the presence of the G119S mutation in seven specimens which showed evidence of altered AChE during biochemical assays using the method described by Weill *et al.* (2004) with modifications. Briefly, the ace-$1^R$ gene was amplified by PCR with F (5' CCGGGCGCGACCATGTGGAA 3') and R (5' ACGATCACGTTCTCCTCCGA 3') oligonucleotide primers. The reaction was performed in 12.5µl volume containing 1.25µl of 10X buffer, 0.25mM dNTP, 1.5mM $MgCl_2$, 0.5U *Taq* polymerase, 3.3 µM of each primer and 1-10ng of extracted DNA. PCR cycling conditions included an initial denaturation step at 94 ºC for 2 mins, followed by 40 cycles of: denaturation at 94 ºC for 30 secs, annealing at 53 ºC for 30 secs and primer extension at 72 ºC for 1 min. These cycles were followed by a final auto extension at 72 ºC for 5 mins. Five microlitres of the amplicons were electrophoresed on a 2.5% agarose gel and visualized under an ultraviolet transilluminator to confirm whether the expected band size has been amplified while the remainder of each amplicon was sent to Inqaba

Biotechnical industries, South Africa for sequencing. Sequences were analyzed with Lasergene software as described in 2.11.3. Controls consisted of *An. arabiensis* individuals from families which did not show AChE activity during biochemical assays.

**Data analysis**

Sequences were first submitted to the Basic Local Alignment Search Tool (BLAST) and only those which produced a significant alignment with the *An. gambiae* AChE gene (Accession no. AGA488492) were aligned together using the DNASTAR, Lasergene 7 (Wisconsin, USA). The multiple sequence alignment was used to identify single nucleotide polymorphisms (SNPs).

## 2.12 Gene expression studies

### 2.12.1 RNA extraction

Total RNA was extracted from three day old adult mosquitoes for both the baseline and permethrin resistant selected cohorts. To minimise gene expression variations RNA was extracted from 10 mosquitoes per treatment for each of the three biological replicates. For each biological repeat, adult males and females from the baseline and permethrin selected cohorts were collected simultaneously and immediately used for RNA extraction. Briefly, mosquitoes were homogenized in 150µl TRI reagent (Ambion Inc. Austin, TX. Cat no. AM9738), using plastic pestles. After centrifugation at 12 000g for 10 min at 4 $^{o}$C, the TRI reagent containing RNA was transferred into new centrifuge tubes and homogenized for 15sec with chloroform and left at room temperature for 15 min. The mixture was centrifuged at 12 000g for 15 min at 4 $^{o}$C. The upper aqueous phase was transferred to another epperndorf tube and the RNA was precipitated using 200 µl isopropanol and

centrifuged at 12 000g for 10 min at 4 $^{o}$C. The isopropanol was discarded and the pellet washed in 200µl of 75% ethanol (v/v) by centrifuging at 7 500g for 5 minutes at 4 $^{o}$C. The ethanol was discarded and the pellet was vacuum dried for 5 min and suspended in 30µl of DEPC water. The RNA was treated with 0.4unit/ µl deoxyribonuclease (DNase1, (QIAGEN, Cat No. 79254)), for 15 min at 37 $^{o}$C to remove any contaminating DNA. The RNA was then re-extracted in 150 µl of TRI reagent as before and resuspended in DEPC treated water. After extraction, RNA quality and quantities were assessed using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Oxfordshire, UK) at wavelengths 230nm, 260nm and 280nm.

### 2.12.2 Synthesis of cDNA

Synthesis of cDNA was carried out on 2 µg of total RNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Forster City, CA, USA; Cat no. 4387406) following manufacturer's instructions. Briefly, 2 µg of RNA was added to 10µl of 2X RT buffer and 1µl of 20X enzyme mix, this was made to a final volume of 20µl by adding nuclease free water (QIAGEN Inc.). The reaction mixture was thermo cycled using iCycler Thermal Cycler system (Biorad, Hercules, CA, USA.) with the following conditions: Incubation at 37 $^{o}$C for 60 minutes followed heat inactivation at 95 $^{o}$C for 5 minutes. A negative control was performed as above, with water added in place of the RNA. Total cDNA was quantified using a Nanodrop spectrophotometer and stored at -20 $^{o}$C.

### 2.12.3 Primer design

The full length CYP6Z2, CYP6Z3 and CYP4G16 gene sequence of *An. gambiae* deposited on NCBI website were used to design the specific primer using the Beacon Designer 3.0 software (Biorad, Hercules, CA, USA). Specificity of the primers was confirmed by sequencing genomic DNA from *An. arabiensis* specimens from the selected cohorts. For CYP6Z1, CYP6M2, CYP6P3 and CYP307A1 the primer sequence designed for *An. gambiae s.s* were used (Nikou *et al.,* 2003; Muller *et al.,* 2007a). Specificity of primers was confirmed by sequencing PCR products obtained from amplification of genomic DNA from *An. arabiensis* from the selected cohorts. All primer pair sequences used for quantitative Real Time (RT)-PCR are summarised in Table 2.2.

### 2.12.4 Optimisation reactions

#### 2.12.4.1 Initial optimisation and sequencing

Initial real time optimisation reaction was carried out in an iCycler Thermal Cycler system (Biorad, Hercules, CA, USA.) using CYPZ1 primers. As a starting point annealing temperature, primers and $MgCl_2$ concentration described by Nikou *et al.* (2003) were used. These were then adjusted to improve specificity and yield of PCR products. Different $MgCl_2$ concentrations ranging from 1mM to 3mM and annealing temperatures ranging from 50 to 60 ºC were used. Other thermal cycling conditions were maintained as described by Nikou *et al.* (2003). The PCR products were electrophoresed on a 2.5% (w/v) agarose with ethidium bromide. After getting the cycling conditions and $MgCl_2$ giving high yield and the correct transcript size (Table 2.2), amplicons were sequenced by

Inqaba biochemical industry for sequencing to confirm whether the correct amplicon has been amplified.

**Table 2.2:** Sequences of oligonucleotide primers used for P450 gene quantification

| Gene | Accession No. | Primer | Sequence (5'TO 3') | Transcript length |
|---|---|---|---|---|
| CYP6Z1 | AF487535 | CYP6Z1_qF<br>CYP6Z1_qR | TTA CAT TCA CAC TGC ACG AG<br>CTT CAC GCA CAA ATC CAG AT | 146bp |
| CYP6Z2 | | CYP6Z2_F<br>CYP6Z3_R | ATC GCT TCG GTG TTC TTC<br>AAT CAA TTC AGG CTG GAG AG | 182bp |
| CYP6Z3 | | CYP6Z3_F<br>CYP6Z3_R | CAA CAA CCT GTA CCA CAA GTC<br>GGA TCG TGC TCT TCA TTG C | 162bp |
| CYP6M2 | AY193729 | CYP6M2_F<br>CYP6M2_R | GTA TGA TGC AGG CCC GTA TAG<br>GCC ATA ATG AAA CTC TCC TTC G | 112bp |
| CYP6P3 | | CYP6P3_F<br>CYP6P3_R | AGC TAA TTA ACG CGG TGC TG<br>AAG TGT GGA TTC GGA GCG TA | 121bp |
| CYP4G16 | | CYP4G16_F<br>CYP4G16_R | TAG AGC GGT GCC TTA TGG<br>CGA TTC CAA GCG GTG AAG | 158bp |
| 18S | | 18S_F<br>18S_R | TAC CTG GGC GTT CTA CTC<br>CTT TGA GCA CTC TAA TTT GTT C | 130bp |
| S7 ribosomal | AY380336 | S7_F<br>S7_R | GTG CCG GTG CCG AAA CAG AA<br>AGC ACA AAC ACT CCA ATA ATC AAG | 472bp |
| rpL8 | | rpL8_F<br>rpL8_R | CAT CAG CAC ATC GGT AAG<br>ACA GAG CAC TCA CTA CTC | 162bp |
| bactin | | bactin_F<br>bactin_R | ACC AAG AGC CTG AAG CAC<br>CGA GCA CGA CAC ACT ATA TAC | 123bp |
| tbp | | tbp_F<br>tbp_R | GAC ATC GTC ATC AAC AAC<br>CCG TAC AGG TAA TCT TCC | 181bp |
| gapdh | | gapdh_F<br>gapdh_R | GAC TGC CAC TCG TCC ATC<br>CCT TGG TCT GCA TGT ACT TG | 139bp |

### 2.12.4.2 Optimisation of real-time reaction

Real-time PCR was optimized using the following cycling conditions: Initial denaturation step at 95 ºC for 3 minutes, followed by 40 cycles of: denaturation at 94 ºC for 15 secs, annealing was varied from 55-60 ºC for 30 secs and primer extension at 72 ºC for 25secs and a final auto extension at 72 ºC for 5 minutes. A final step was incorporated to

continuously monitor fluorescence through the dissociation temperature of the PCR products at a temperature transition rate of 0.5 $^{o}$C/sec to generate a melt curve. Concurrently forward and reverse primers were optimised each with a concentration of 100, 200, 300, 400 and 500nM. After optimising both annealing temperature and primer pair concentrations as determined by production of an amplification plot sigmoid in shape and a melting curve giving a single peak, PCR products were collected and electrophoresed on a 2.5% (w/v) agarose gel to confirm product size. The remainder of the amplicons were sequenced by Inqaba biochemical industry, South Africa. These thermal cycling conditions were subsequently used in all quantitative PCR (qPCR) (Figure2.6).

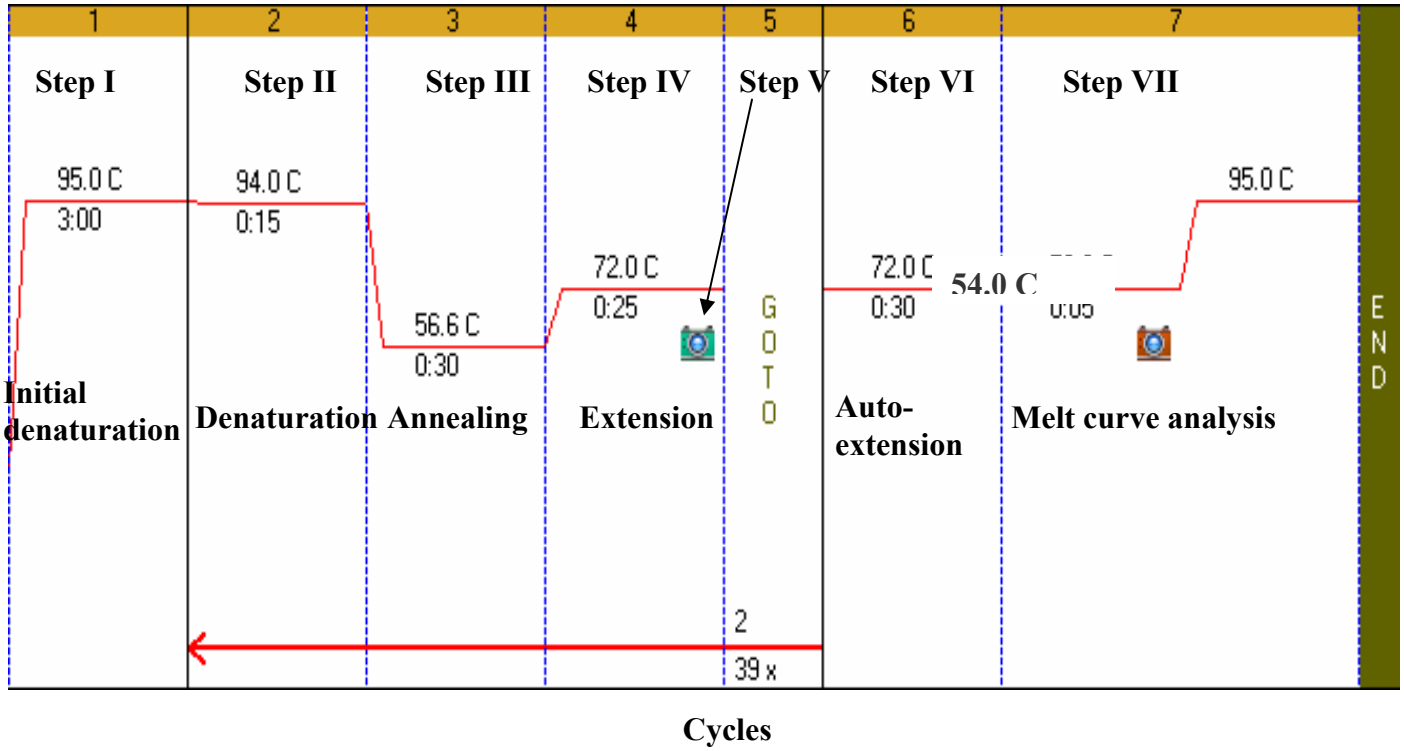



**Figure 2.6**: Diagrammatic representation of thermal cycling conditions used for CYP6Z1 gene quantification (For other genes cycling conditions differed only in the annealing temperatures; these were CYP6Z2 -53.9 º C ; CYP6Z3 -53.9 ºC; CYP6M2 -55.3 ºC; CYP6P3 -53.2 ºC; and CYP4G16 -53.9 ºC). Cycling temperatures are indicated above red cycling line. Duration of time at specific temperature point are indicated below the red cycling line (minutes:seconds)

#### 2.12.4.3 Selection of reference genes for gene expression studies

Six genes: Beta actin (bactin), 18S ribosomal RNA (18S), M2 ribosomal protein L8 (rpL8), tata box binding protein (tbp), glucose-6-phosphate dehydrogenase (gapdh) and ribosomal S7 were selected for assessment based on previous use as internal control for gene expression studies (Nishimura *et al.,* 2006; Muller *et al.,* 2008). Primers were designed based on the *An. gambiae* full-length gene sequence deposited on the NCBI website. Each gene was amplified in duplicate in 3 susceptible and 3 resistant samples using PCR conditions described for CYP6Z1 gene quantification (Figure 2.6). Five microlitres of the PCR amplicons were electrophoresed on a 2.5% agarose gel to verify amplicon size. The remainders of the amplicons were pooled and sent to Inqaba biochemical industry for sequencing to confirm whether the right gene had been amplified. Threshold values (Cq) were directly used to compare difference in expression of each gene between the susceptible and resistant samples.

### 2.12.5 Relative quantification

Quantification of expression levels of each gene (CYP6Z1, CYP6Z2, CYP6Z3, CYP6M2, CYP6P3 and CYP4G16) was performed in a CFX 96 real time PCR machine (Biorad, Hercules, CA, USA). 18S rRNA gene served as an internal control to account for differences in initial cDNA and reaction efficiency was amplified on the same plate and the same thermal profile for each quantification reaction. Concurrently a standard curve was generated for both the target gene and house keeping using a 2 fold dilution series from 80ng to 0.076ng. Each dilution concentration was run in duplicate, while

reactions for the target gene and 18S rRNA were performed in triplicate sets. All reactions were repeated three times.

All amplifications were carried out in a total volume of 25 µl containing 12.5 µl 2X iQ$^{TM}$SYBR$^{®}$ Green Supermix (Bio-Rad, Hercules, CA; Cat No. 170-882) containing (100mM KCl, 40mM Tri-HCl, pH 8.4, 0.4mM of each dNTP, 50 U/ml i*Taq* DNA polymerase, 6mM $MgCl_2$, SYBR Green I, 20nM fluoresein and stabilizers), 200mM of each specific primer pair specific for each gene and 100ng of cDNA template. The qPCR cycling conditions were as summarised in Figure 2.6. Acquisition of data was carried out at each cycle immediately after the extension step. A final auto extension step was incorporated at 72 $^{o}$C for 25 sec. After the cycling protocol, a final step was applied to all reactions by continuously monitoring fluorescence through the dissociation temperature of the PCR products at a temperature transition rate of 0.5 $^{o}$ C/sec to generate a melt curve. Melt curve and agarose gel analysis were conducted for each gene to ensure only a single amplicon was amplified for each reaction.

**Data analysis**

Relative expression levels of each gene were calculated using the comparative cycle threshold method described by Pfaffl, (2001). Briefly, amplification efficiencies for the target and housekeeping gene were automatically calculated by the CFX software manager (Bio-Rad, Hercules, CA, USA), with relative gene quantities normalized against the 18S ribosomal RNA (18S). Expression levels between the baseline (calibrator) and permethrin selected cohorts (sample) were statistically analysed using the CFX software manager (Biorad). Statistical difference in expression levels was analysed using REST 2008 statistical package (Corbett Life Sciences).

# CHAPTER 3

## MALARIA VECTORS STATUS AND INSECTICIDE SUSCEPTIBILITY STUDIES IN GWAVE VILLAGE, ZIMBABWE.

### 3.1 Introduction

Malaria cases in Zimbabwe are now comparatively higher than the pre 2000 era. Increase in malaria cases especially in 2003/4 season has been attributed to the socio-economic challenges facing the country resulting in difficulties in IRS coverage (Midzi *et al.,* 2004). However, despite a marked increase in IRS and ITN coverage between 2004 to date, malaria cases though declining are still comparatively high. Therefore, it is possible that other factors other than socio-economic challenges are playing a role.

The current malaria vector control initiatives in Zimbabwe are wholly chemical based. Insecticide based vector control strategies coupled with indiscriminate use of agricultural chemicals has been shown to select for resistance in malaria vectors (Diabate *et al.,* 2002). There is a growing concern that the current strategies for malaria vector control in Zimbabwe are probably under threat resulting in the current high malaria cases. This is complicated by lack of evidence based resistance management strategies.

For any vector control programme using insecticides as the corner stone for control activities there should be continuous monitoring of susceptibility to insecticides being used by the programme. There is also need to update the insecticide susceptibility status of malaria vectors and possible underlying resistance mechanisms. This information can

then be used to formulate evidence based resistance management policies. Inorder to achieve this, as discussed previously, comprehensive information on the malaria vector species composition forms the first step, followed by susceptibility to insecticides being used for their control and an understanding of the underlying resistance mechanisms.

In Zimbabwe the composition and distribution of malaria vectors is well documented (Masendu *et al.,* 2005). Information on the susceptibility status of malaria vectors to insecticides used by the Zimbabwean National Malaria Control Programme (ZNMCP) remains unclear; this is despite decades of pesticide usage in both agriculture and health. In 2002 a population of *An. arabiensis* resistant to DDT was discovered in Gwave gardens during a national anopheline survey (Masendu *et al*., 2005). Despite this evidence of the presence of resistance in the major malaria vector, very few attempts have been made to investigate the underlying resistance mechanisms involved. Monitoring has been confined to standard WHO bioassays conducted biannually by the National Institute of Health Research (NIHR).

This section details investigations of the vector status of the major malaria transmitter in Zimbabwe, *An. arabiensis* and it's susceptibility to insecticides recommended for IRS and ITNs. Underlying biochemical and molecular resistance mechanisms were also investigated.

## 3.2 Materials and Methods

Details of materials and methods used in this study are described in Chapter 2.

## 3.3 Results

Some of the results of this chapter have been accepted for publication in the *Malaria Journal* and a copy of the paper can be found in Appendix IV.

### 3.3.1 Mosquito collections and identifications

Total numbers of anopheline mosquitoes collected in the field during the study period are presented in Table 3.1. In total, 943 anophelines belonging to four different taxa were collected. The *An. gambiae* complex comprised 98.8% of the overall collection. Other taxa collected included *An. squamosus* group (0.9%), *An. coustani* (0.5%) and *An. longipalpis* group (0.3%). Cattle kraal collections contributed 73.9% of the overall mosquito catches and recorded the highest taxa diversity. Larval, man-baited net trap and window exit collections produced the lowest species richness, catching only members of the *An. gambiae* complex. Indoor resting catches were not productive in 2006; no adults were collected from searches done in 25 houses. However, in 2008 *An. gambiae* complex mosquitoes exiting houses were caught in eight out of 12 houses (66.7%) where window exit traps were set up, and 36 individuals were caught.

Using PCR based techniques mosquitoes were identified to species-specific level. A total of 932 *An. gambiae* complex mosquitoes were identified to species level. Of these 679 (72.0%) were *An. arabiensis*, 139 (14.7%) were *An. merus* and 114 (12.1%) were *An.*

*quadriannulatus* spp A. *Anopheles arabiensis* was the dominant member of the *An. gambiae* complex across all the collection periods and methods with a significant proportion (77%) being caught using cattle kraal collections. Two specimens collected in 2006 and morphologically identified as belonging to the *An. funestus* group consistently gave hybrid amplicons (Figure 3.1: lanes 7 and 8) using the standard *An. funestus* group species-specific PCR suggesting that they might have been morphologically misidentified (Kent *et al.,* 2006). They were subsequently identified as *An. longipalpis* Type C using the multiplex PCR of Koekemoer *et al.* (2009).

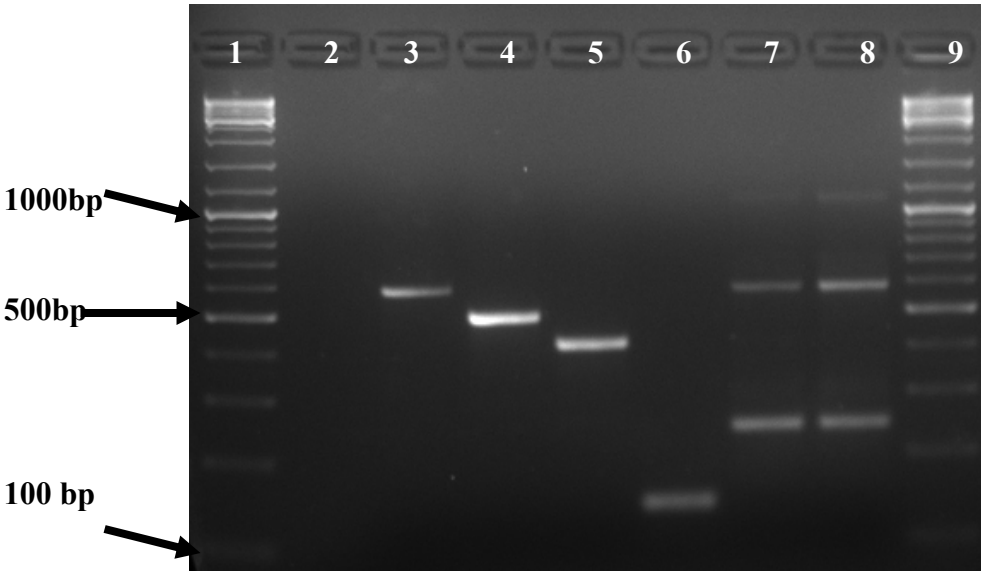



**Figure 3.1**: Amplified fragments using the *An. funestus* group species-specific PCR of Koekemoer *et al.* (2002). Lane 1 and 9: Hyperladder IV (100bp) molecular marker; Lane 2: negative control; Lane 3: *An. vaneedeni* positive control; Lane 4: *An. funestus* positive control Lane 5: *An. rivulorum* positive control; Lane 6: *An. leesoni* positive control; Lane 7 and 8: *An. longipalpis* Type C.

**Table 3.1:** Summary of anophelines caught and identified to species level in Gwave (MBN = "man-baited net trap" and N = Sample size)

| Collection period | Collection Method | N | *An. arabiensis* | *An. quadriannulatus* | *An. merus* | *An. longipalpis* | *An. squamosus* | *An. coustani* |
|---|---|---|---|---|---|---|---|---|
| February, 2006 | Cattle kraal | 511 | 422 | 66 | 12 | 2 | 6 | 3 |
| | Larval | 43 | 36 | 6 | 1 | 0 | 0 | 0 |
| | MBN | 28 | 12 | 7 | 9 | 0 | 0 | 0 |
| January, 2008 | Cattle kraal | 181 | 101 | 7 | 73 | 0 | 0 | 0 |
| | Larval | 144 | 85 | 25 | 34 | 0 | 0 | 0 |
| | Window exit | 36 | 23 | 3 | 10 | 0 | 0 | 0 |
| Totals (%) | | 943 | 679(72.0) | 114(12.1) | 139(14.7) | 2(0.2) | 6(0.6) | 3(0.3) |

### 3.3.2 Mosquito infectivity

Table 3.2 summarizes infectivity rates of *An. gambiae* complex mosquitoes tested for *P. falciparum* infection. A total of 530 and 217 mosquitoes were examined in 2006 and 2008 respectively. In 2006, two specimens (0.4%) were positive. These two specimens were identified as *An. arabiensis* giving an overall infection rate of 0.5% for this species. Analysis per collection method per sibling species gave an infection rate of 14.3% for *An. arabiensis* collected by MBN catches. In 2008, six specimens (2.8%) were positive and these were identified as *An. arabiensis* giving an overall infection of 4.8% (n = 124) for this species. Analysis per collection method per sibling species gave an infection rate of 26.1% for *An. arabiensis* collected by window exit traps. *Anopheles merus* and *An. quadriannulatus* were not infected during the two collection periods.

**Table 3.2:** Comparison of *P. falciparum* circumsporozoites antigen rates for the *An. gambiae* complex

| Collection Period | Collection method | *An. arabiensis* | | *An. merus* | | *An. quadriannulatus* | | Total | |
|---|---|---|---|---|---|---|---|---|---|
| | | total tested | +ve n (%) | total tested | +ve n (%) | total tested | +ve n (%) | total tested | +ve n (%) |
| February, 2006 | Cattle kraal | 422 | 0 (0) | 12 | 0 (0) | 66 | 0 (0) | 500 | 0 (0) |
| | Man-baited net | 12 | 2 (16.7) | 9 | 0 (0) | 7 | 0 (0) | 30 | 2 (6.7) |
| | **Total** | **434** | **2 (0.5)** | **21** | **0 (0)** | **73** | **0 (0)** | **530** | **2 (0.4)** |
| January, 2008 | Window exit | 23 | 6 (26.1) | 10 | 0 (0) | 3 | 0 (0) | 36 | 6 (16.7) |
| | Cattle kraal | 101 | 0 (0) | 73 | 0 (0) | 7 | 0 (0) | 181 | 0 (0) |
| | **Total** | **124** | **6 (4.8)** | **83** | **0 (0)** | **10** | **0 (0)** | **217** | **6 (2.8)** |

### 3.3.3 Bioassays

#### 3.3.3.1 Insecticide susceptibility tests

Susceptibility tests using field caught mosquitoes was conducted on the following insecticides: 4% DDT, 0.75% permethrin, 4% dieldrin, 5% malathion and 0.1% bendiocarb. After PCR identifications of mosquitoes from the field, results showed that all three sibling species of *An. gambiae* complex collected were susceptible to 4% dieldrin and 5% malathion (Table 3.3). In 2006 *An. arabiensis*, *An. merus* and *An. quadriannulatus* were resistant to both 4% DDT and 0.75% permethrin exposures. In 2008, susceptibility to 4% DDT changed; *An. arabiensis* showed reduced susceptibility (94.8% mortality) while *An. merus* and *An. quadriannulatus* showed complete susceptibility. Resistance to 0.75% permethrin was recorded in all three sibling species. Only *An. quadriannulatus* showed reduced susceptibility to 0.1% bendiocarb (92.9%).

**Table 3.3:** Insecticide susceptibility status of wild caught *An. gambiae* complex (* Resistant according to WHO criteria, ! resistance to be confirmed according to WHO criteria; - susceptibility was not determined and n = sample size).

| Collection period | Species | Insecticides | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Permethrin | | DDT | | dieldrin | | bendiocarb | | malathion | |
| | | Total (n) | (%) mort | Total (n) | (%) mort | Total (n) | (%) mort | Total (n) | (%) mort | Total (n) | (%) mort |
| 2006 | *An. arabiensis* | 87 | 47* | 110 | 68.4* | 37 | 100 | 40 | 100 | 52 | 100 |
| | *An. merus* | 12 | 66* | 5 | 40* | - | - | - | - | 3 | 100 |
| | *An. quadriannulatus* | 4 | 75* | 20 | 70* | 4 | 100 | 6 | 100 | 11 | 100 |
| 2008 | *An. arabiensis* | 49 | 69.4* | 58 | 94.8! | - | - | 69 | 100 | - | - |
| | *An. merus* | 10 | 60* | 12 | 100 | - | - | 27 | 100 | - | - |
| | *An. quadriannulatus* | 7 | 71.4* | 5 | 100 | - | - | 14 | 92.9 | - | - |

### 3.3.3.2 $F_1$ Progeny susceptibility tests

Mosquito batches which were kept alive in the field and brought to the laboratory were reared individually into $F_1$ families. Of these, a total of 73 and 51 *An. arabiensis* families were exposed to 4% DDT and 0.75% permethrin respectively during 2006 and 2008. In 2006, mortality to DDT ranged between 28.6% and 100% with evidence of resistance being shown in 16 out of 59 families (25.4%) exposed. For permethrin exposures done in 2006, 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%. The trend for DDT resistance changed in 2008. Of the 14 families exposed to 4% DDT two families (family 682 and family 771) were resistant, with an average mortality across family of 95.2% (n = 286). Results of exposures to permethrin in 2008 showed evidence of resistance in 11 out of 14 families (78.8%) with an average mortality across family of 47.9% (n = 286). Table 3.4 details insecticide susceptibility test results of families $F_1$ progeny reared from wild caught *An. arabiensis* exposed to both 0.75% permethrin and 4% DDT on. In total eight families (27, 64, 94, 118, 144, 156, 682 and 771) showed resistance to both DDT and permethrin exposures.

**Table 3.4:** Insecticide susceptibility test results on 1-3 day old $F_1$ *An. arabiensis* reared from females collected from Gwave village in 2006 and 2008 exposed to both 4% DDT and 0.75% permethrin. Other family data are not shown on this table. Results are expressed as % mortality 24hr post exposure. (* indicate families showing cross resistance to DDT and permethrin, Fam = Family, n = sample size, % mort = % mortality, families numbers in green indicate families resistant to both DDT and permethrin, while resistant families as defined by WHO criteria are indicated in red).

**February 2006**

| | 4% DDT | | 0.75% permethrin | | | 4% DDT | | 0.75% permethrin | | | 4% DDT | | 0.75% permethrin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fam. | (n) | % mort | (n) | % mort | Fam. | (n) | % mort | (n) | % mort | Fam. | (n) | % mort | (n) | % mort |
| 27* | 7 | 28.6 | 13 | 53.9 | 82 | 9 | 100 | 8 | 63.6 | 113 | 10 | 60 | 4 | 100 |
| 37 | 11 | 72.7 | 11 | 81.8 | 84 | 10 | 90 | 11 | 54.5 | 118* | 9 | 66.7 | 6 | 16.7 |
| 38 | 9 | 100 | 12 | 66.7 | 85 | 13 | 100 | 10 | 50 | 120 | 10 | 100 | 10 | 100 |
| 46 | 5 | 100 | 8 | 0 | 90 | 8 | 100 | 10 | 100 | 135 | 11 | 100 | 10 | 80 |
| 48 | 10 | 100 | 10 | 70 | 94* | 8 | 62.5 | 9 | 44.4 | 139 | 11 | 100 | 6 | 66.7 |
| 53 | 11 | 100 | 12 | 100 | 95 | 13 | 69.2 | 6 | 100 | 144* | 14 | 78.6 | 15 | 66.7 |
| 57 | 7 | 100 | 15 | 46.7 | 97 | 11 | 81.8 | 13 | 92.3 | 149 | 11 | 54.6 | 10 | 100 |
| 64* | 12 | 33.3 | 8 | 50 | 101 | 9 | 100 | 11 | 18.2 | 156* | 13 | 69.2 | 11 | 45.5 |
| 66 | 7 | 100 | 11 | 45.5 | 103 | 9 | 100 | 10 | 90 | 169 | 10 | 100 | 10 | 60 |
| 74 | 12 | 16.7 | 12 | 100 | 104 | 8 | 87.5 | 12 | 50 | 166 | 8 | 100 | 12 | 83.3 |
| 77 | 14 | 100 | 11 | 63.6 | 108 | 7 | 100 | 8 | 100 | 178 | 7 | 100 | 9 | 55.6 |
| 80 | 10 | 100 | 10 | 90 | 111 | 11 | 100 | 11 | 72.7 | 180 | 9 | 100 | 9 | 100 |

**January 2008**

| | 4% DDT | | 0.75% permethrin | | | 4% DDT | | 0.75% permethrin | | | 4% DDT | | 0.75% permethrin | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fam. | (n) | % mort | (n) | % mort | Fam. | (n) | % mort | (n) | % mort | Fam. | (n) | % mort | (n) | % mort |
| 654 | 11 | 100 | 15 | 93.33 | 740 | 16 | 100 | 18 | 72.2 | 821 | 12 | 100 | 15 | 53.3 |
| 681 | 14 | 100 | 13 | 61.54 | 755 | 13 | 100 | 20 | 55 | 854 | 15 | 100 | 15 | 100 |
| 682* | 25 | 74 | 19 | 68.23 | 769 | 23 | 100 | 21 | 52.4 | 856 | 10 | 100 | 8 | 50 |
| 712 | 12 | 100 | 15 | 40 | 771* | 10 | 78 | 14 | 14.3 | 858 | 15 | 100 | 15 | 100 |
| 738 | 11 | 100 | 10 | 20 | 808 | 20 | 100 | 17 | 35.3 | | | | | |

### 3.3.4 Biochemical assays

#### 3.3.4.1 Enzyme activity of $F_1$ progeny

**Glutathione-S- transferase (GST)**

Figure 3.2 shows the average level of GST activity/family of $F_1$ progeny of adult *An. arabiensis* females collected from Gwave in 2006 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. 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 corresponding control (KGB). Of the families with suppressed GST activities, families 94, 144 and 150 were resistant to DDT 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.

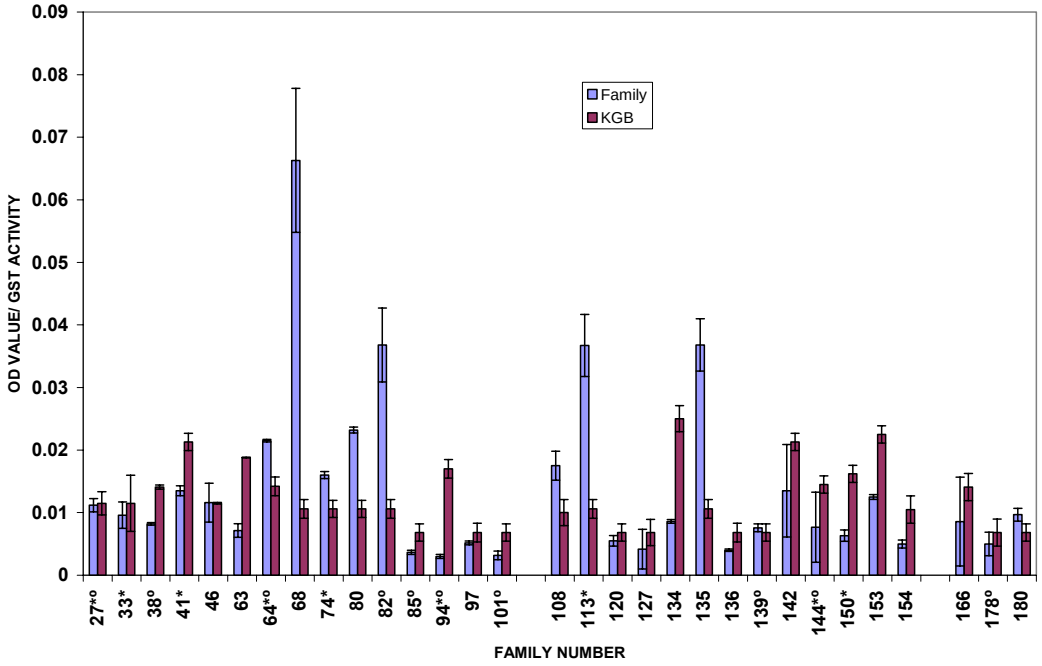



**Figure 3.2**: Average levels of glutathione-S-transferase (GST) activity in *An. arabiensis* $F_1$ progeny, by family, and corresponding GST activity means for susceptible *An. arabiensis* (KGB) samples assayed simultaneously (***** Family resistant to DDT exposures º Family resistant to permethrin exposure).

**General esterase activity**

Figures 3.3 and 3.4 show the average levels of non specific esterase activity monitored using alpha and beta-naphthyl acetate as substrates in *An. arabiensis* $F_1$ 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 = 27.3%), ($P < 0.05$). Families 38, 41, 103, 139, and 156 gave activities significantly lower than the reference colony ($P < 0.05$). All the other families showed no significant differences in activity compared to their corresponding KGB ($P > 0.05$) strain.

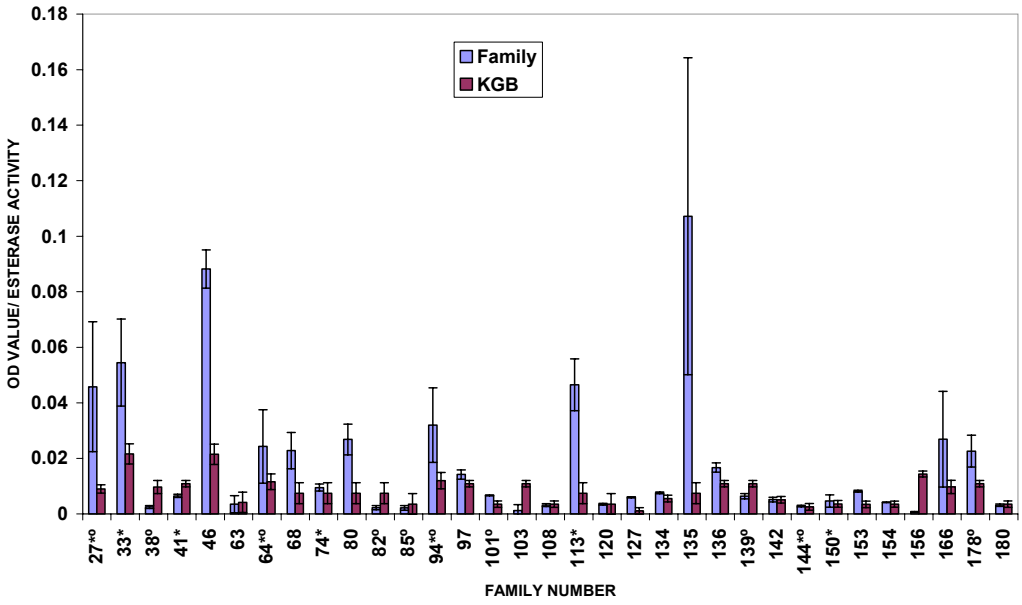



**Figure 3.3:** Average levels of non-specific esterase using alpha-naphthyl acetate as a substrate in $F_1$ progeny of wild caught *An. arabiensis*, by family, and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) samples assayed simultaneously (* Family resistant to DDT exposures, º Family resistant to permethrin exposure).

Non-specific esterase activity based on beta-naphthyl acetate as a substrate (Fig. 3.4) showed that only two families (i.e. 2/31 = 6.5%) gave significant increase in esterase activity ($P < 0.05$), while 14/31 (45.2%) showed activities significantly lower than the reference strain (KGB) ($P < 0.05$). 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.

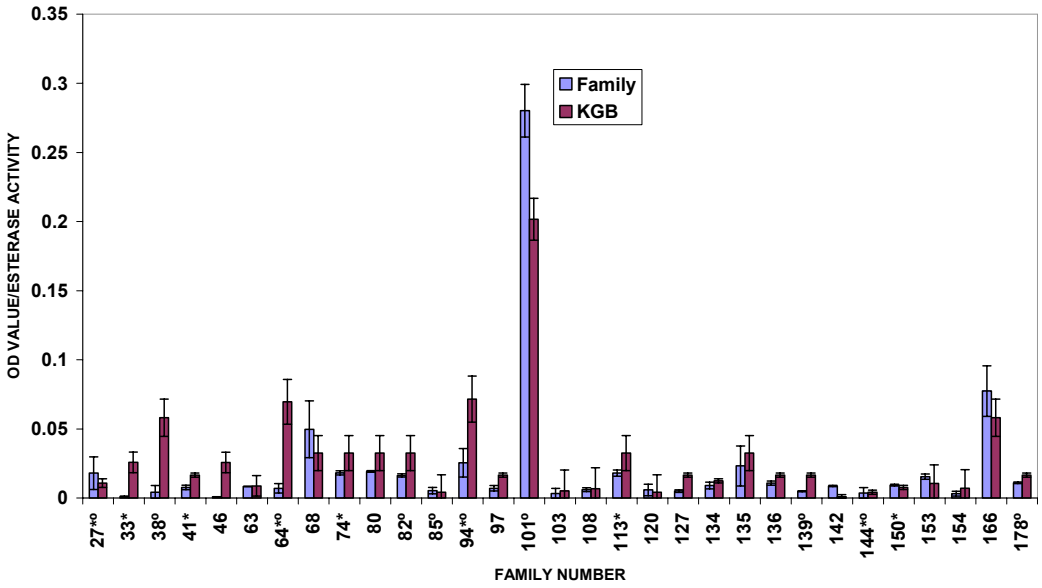



**Figure 3.4:** Average levels of non-specific esterase using beta-naphthyl acetate as a substrate in $F_1$ progeny of wild caught *An. arabiensis*, by family, and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) assayed simultaneously (* Family resistant to DDT exposures, º Family resistant to permethrin exposure).

**Monooxygenase assay**

Figure 3.5 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 = 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 correlation between monooxygenase activity and bioassay mortality data across

families. 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.

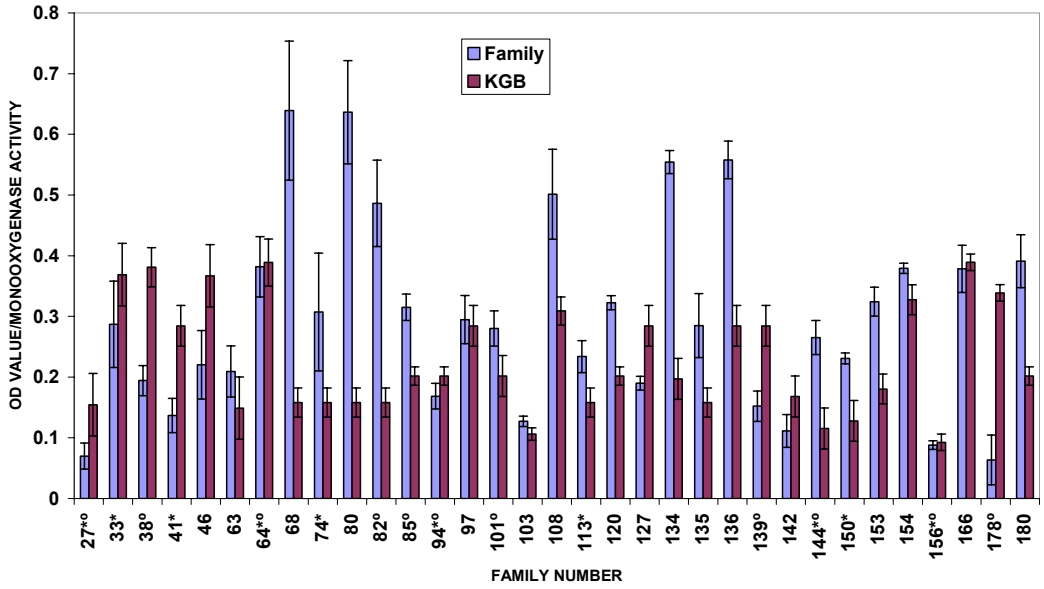



**Figure 3.5:** Average levels of monooxygenase activity in $F_1$ progeny of wild caught *An. arabiensis*, by family line, and corresponding means for the *An. arabiensis* strain (KGB) assayed simultaneously (***** Family resistant to DDT exposures, º Family resistant to permethrin exposure).

**Insensitive Acetylcholinesterase assay**

The mean percentage propoxur inhibitions of AChE for the familial $F_1$ progeny of wild caught *An. arabiensis* are given in Fig. 3.6. 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) showed evidence of insensitive AChE despite absence of carbamate and organophosphates resistance in the bioassays.

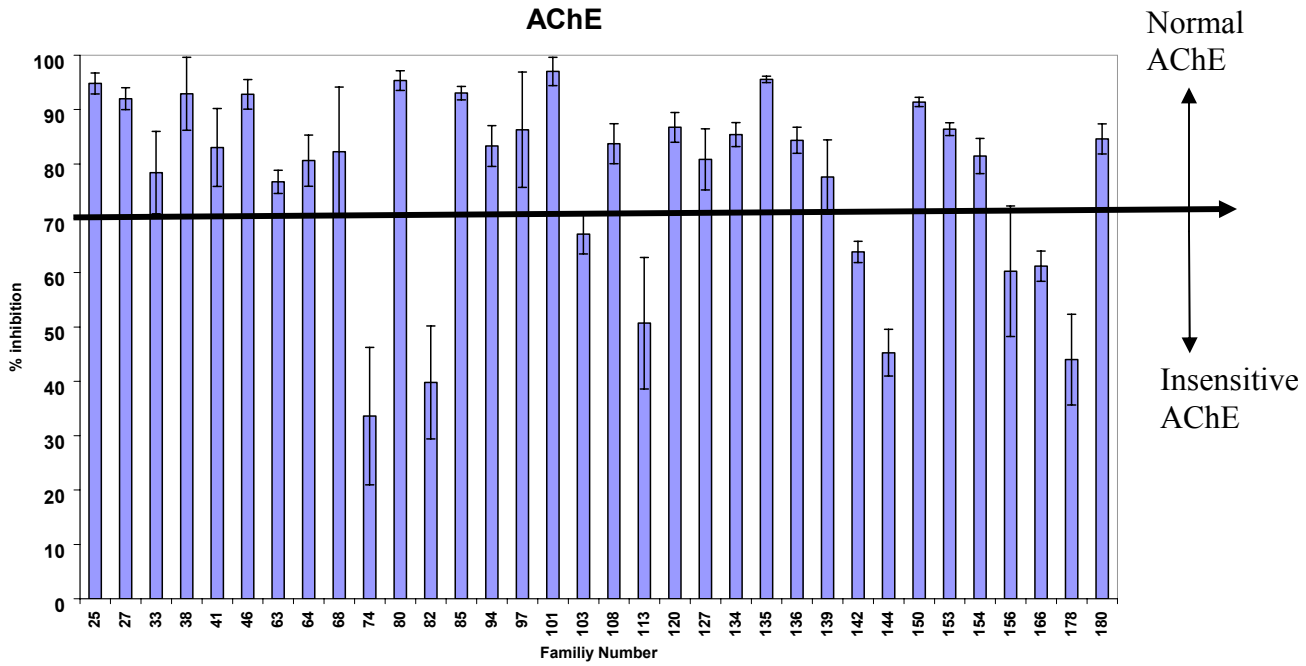



**Figure 3.6:** Mean acetylcholinesterase percentage inhibition by propoxur in $F_1$ progeny of *An. arabiensis* reared from 34 wild-caught females.

### 3.3.4.2 Enzyme activity of $F_2$ progeny

**Permethrin screened $F_2$ progeny**

In 2006, there was lack of correlation between bioassay mortality data and enzyme activity in the $F_1$ progeny assayed. We hypothesised that this was probably due to the presence of some susceptible individuals in the samples tested. This might have a masking effect on the true enzyme activity in the resistant individuals. In 2008, $F_1$ adults showing survival to either 4% DDT or 0.75% permethrin were pooled and separated into

two groups. This procedure was done in the hope of eliminating as many susceptible individuals. One group was stored at -70$^{o}$C for biochemical assays. The remaining resistant individuals were allowed to mate and their progeny reared into $F_2$ generation and used for biochemical analyses. Corresponding bioassays could not be carried out due to limited number of adults which emerged.

Figure 3.7-3.10 summarizes comparison of enzyme activities between $F_1$ and $F_2$ progenies of mosquitoes reared from permethrin resistant families. GST activity was significantly elevated ($P < 0.05$, $n = 16$) in the $F_1$ progeny compared to the reference colony KGB. The GST activity however decreased in the $F_2$ progeny reared from survivors of $F_1$ progeny exposed to 0.75% permethrin. There was no significant difference ($P > 0.05$) in GST activity between the $F_1$ and $F_2$ progeny (Fig. 3.7).

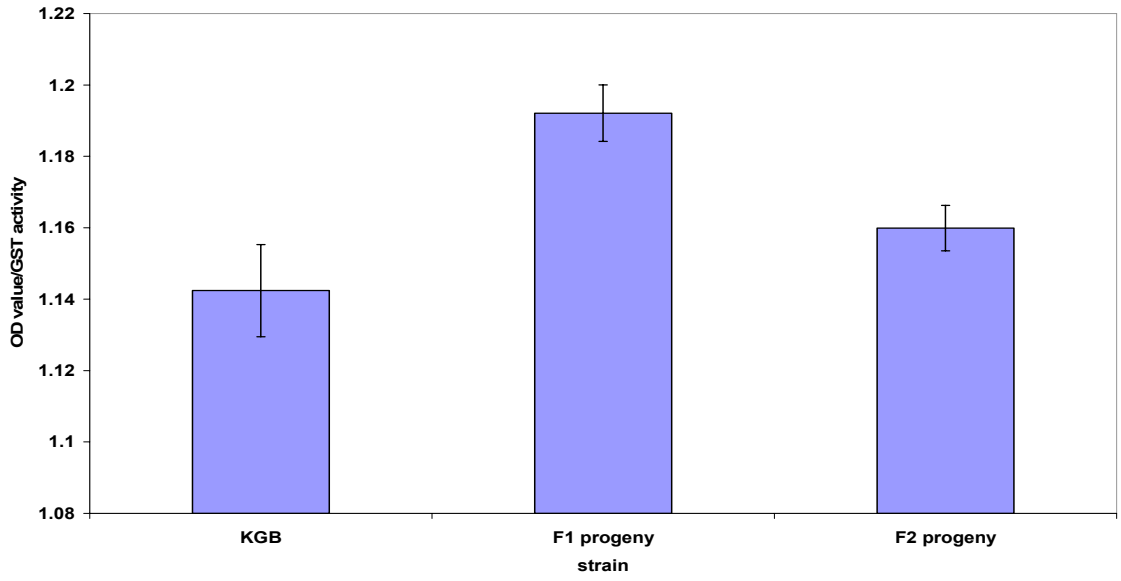



**Figure 3.7:** Average levels of glutathione-S-transferase (GST) activity in *An. arabiensis* $F_1$ and $F_2$ progeny screened for permethrin resistance, and corresponding GST activity means for susceptible *An. arabiensis* (KGB) samples assayed simultaneously.

Non-specific esterase activity monitored using alpha-naphthyl acetate as a substrate showed that there was no significant difference between the $F_1$ progeny compared to the reference colony ($P > 0.05$). However, after exposing the $F_1$ progeny to permethrin there was an increase in esterase activity in the $F_2$ progeny (Fig. 3.8) but this increase was not statistically significant ($P > 0.05$).

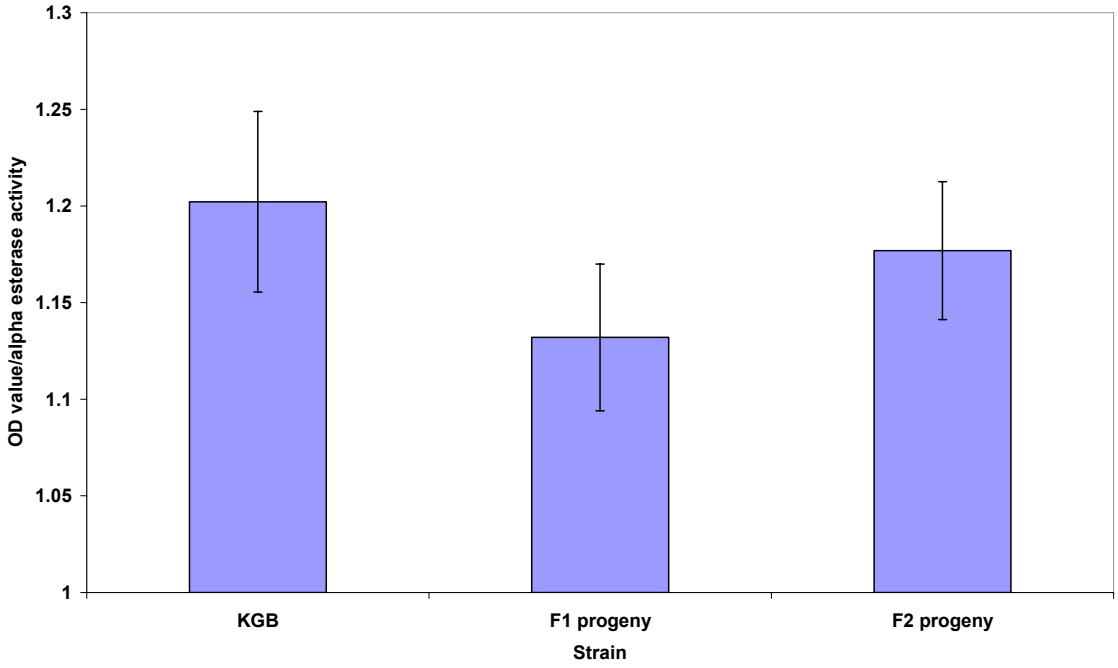



**Figure 3.8:** Average levels of non-specific esterase using alpha-naphthyl acetate as a substrate in $F_1$ and $F_2$ progeny of wild caught *An. arabiensis* and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) samples assayed simultaneously.

Non-specific esterase activity based on beta-naphthyl acetate as a substrate showed that the reference colony had significantly higher general esterase activity compared to both $F_1$ and $F_2$ progeny ($P > 0.05$). There was no change in esterase activity after screening for permethrin resistance (Fig. 3.9).

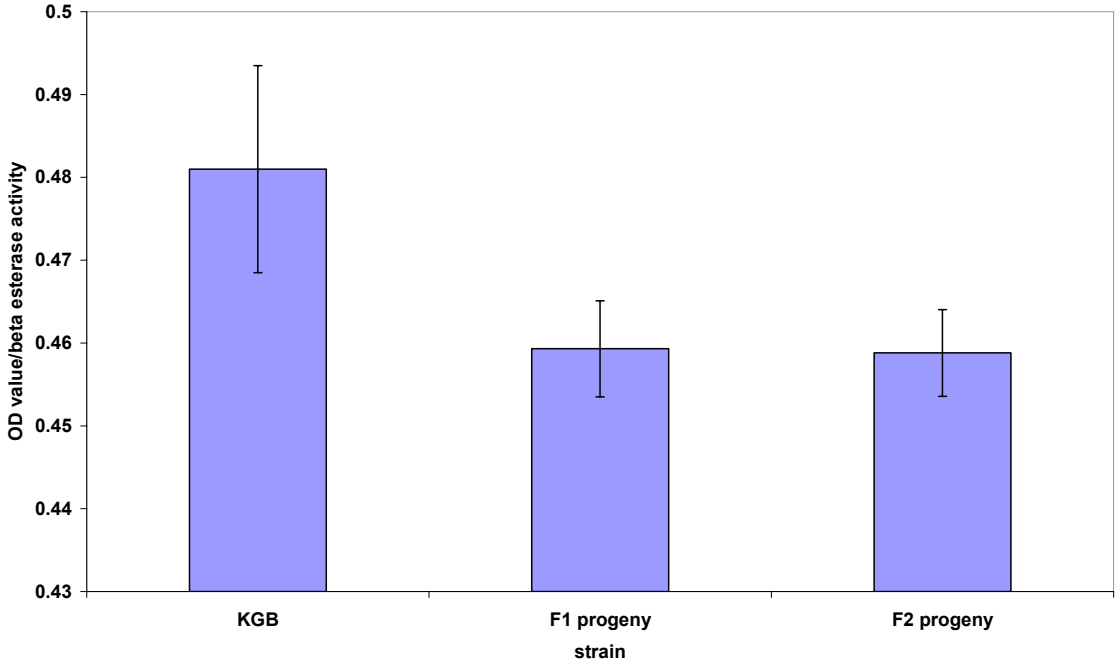



**Figure 3.9:** Average levels of non-specific esterase using beta-naphthyl acetate as a substrate in $F_1$ and $F_2$ progeny of wild caught *An. arabiensis* and corresponding esterase activity for the susceptible *An. arabiensis* (KGB) assayed simultaneously.

Figure 3.10 shows the average level of monooxygenase activity of $F_1$ and $F_2$ progeny of adult females collected from Gwave compared with the standard susceptible KGB strain assayed on the same plate. Both the $F_1$ and $F_2$ progeny showed significantly higher levels of monooxygenase activity ($P < 0.05$) than the corresponding reference control (KGB). There was a significant increase in monooxygenase activity in the $F_2$ progeny compared to the $F_1$ progeny ($P = 0.041$).

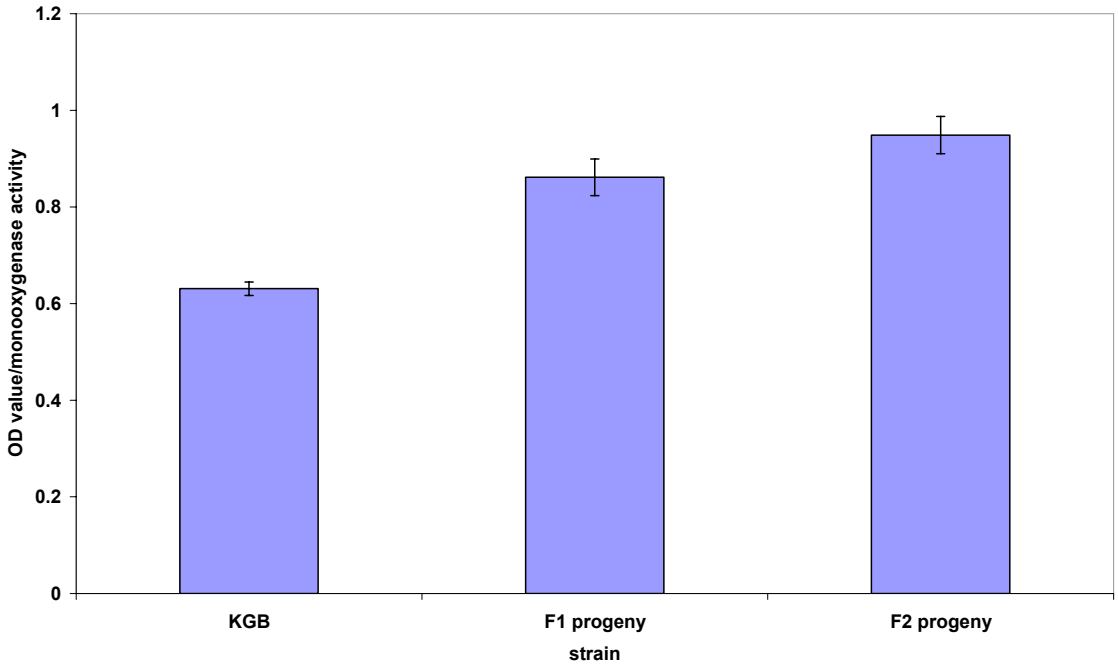



**Figure 3.10:** Average levels of monooxygenase activity in $F_1$ and $F_2$ progeny of wild caught *An. arabiensis* and corresponding means for the *An. arabiensis* strain (KGB) assayed simultaneously.

**DDT screened $F_2$ progeny**

No biochemical analysis could be carried out on the DDT $F_2$ cohorts because there were not enough survivors to obtain $F_2$ progeny (refer to Table 3.6).

## 3.3.5 Molecular assay for *kdr*

### 3.3.5.1 PCR assay

A total of 57 specimens, 22 individual mosquitoes resistant to DDT and 27 resistant to permethrin and 8 specimens whose $F_1$ progeny showed resistance to both DDT and permethrin were genotyped by PCR for the L1014S and L1014F *kdr* mutation. Genotyping for the L1014S *kdr* mutations showed complete absence of this mutation in

all 57 specimens assayed. Figure 3.11 summarises L1014F *kdr* genotyping carried out on samples collected in 2006. Results showed that *kdr* mutation was absent (SS) in 53.6% of the DDT survivors assayed, while 32.1% of the samples assayed were heterozygote (RS) and 14.3% were resistant homozygote (RR). PCR assays for permethrin resistant samples showed that the *kdr* mutation was absent in 63.6% of those assayed while resistant homozygotes (RR) and heterozygotes (RS) were 15.2% and 21.2% respectively. Analysis of families showing cross resistance to DDT and permethrin showed that four of these families were heterozygous resistant (RS) while two families were homozygous resistant (RR). In 2008, only two females whose $F_1$ progeny showed evidence of resistance to both DDT and permethrin were assayed for the L1014S and L1014F *kdr* mutation. These two specimens were both homozygous susceptible (SS).

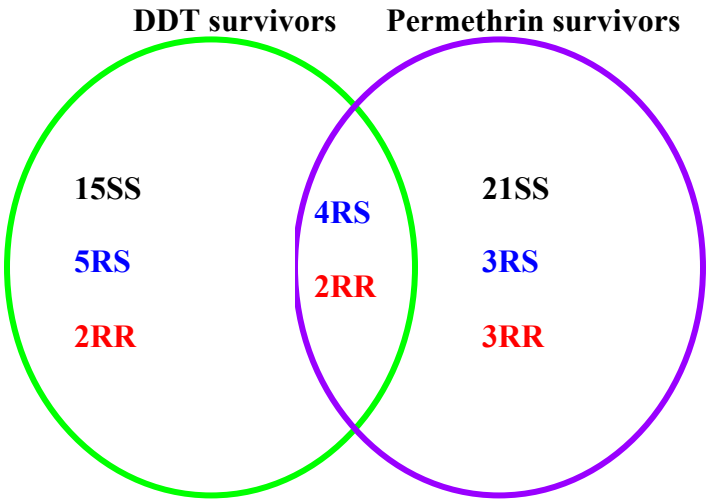



**Figure 3.11:** Summary of *kdr* genotype from *An. arabiensis* samples collected in 2006 obtained by diagnostic PCR of Martinez-Torres *et al.* (1998), (-DDT survivors, -Permethrin survivors, RR homozygous resistant, **RS** heterozygous resistant, SS homozygous susceptible).

### 3.3.5.2 Sequence confirmation of PCR genotypes

Due to the fact that the PCR is not reliable (Matambo *et al.,* 2007; Abdalla *et al.,* 2008) samples were also sequenced to confirm results. The 293 bp regions containing the *kdr* mutation was amplified by PCR in 38 specimens. Of these specimens, 12 specimens had been PCR genotyped as RS, seven had been genotyped as RR while the rest were genotyped as SS. The resultant PCR products were sequenced directly. Using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/Genbank), all individual sequences showed a 98.5% to 100% sequence identity to the *An. arabiensis* para-type sodium channel gene with a query coverage above 95% sequence (accession number DQ2637491). Contrary to PCR results alignment of individual consensus sequences (Fig. 3.12) and searching for the codon associated with "Leu-Phe" and "Leu-Ser" *kdr* mutations showed absence of the mutation in all mosquitoes screened.

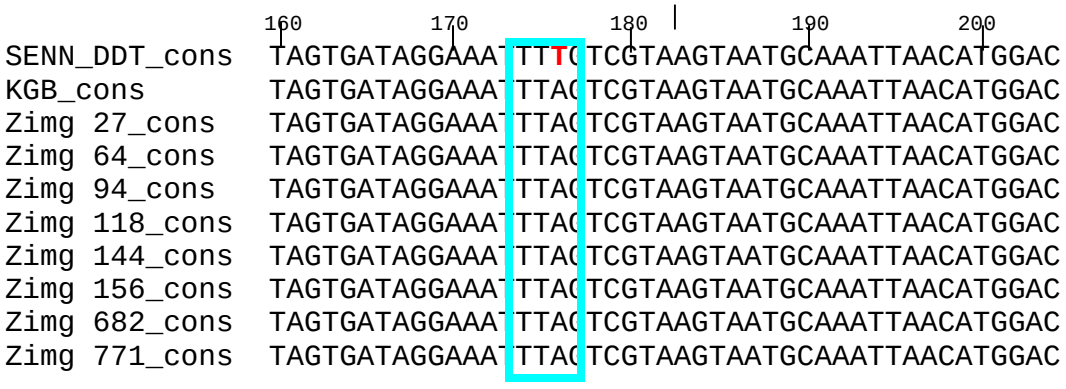



**Figure 3.12**: Truncated alignment of partial consensus sequences (cons) of *An. arabiensis* samples showing cross resistance to DDT and permethrin collected from Gwave with the control sequences of SENN-DDT (resistant, known RR genotype) and KGB (susceptible, known SS genotype) highlights the key location where *kdr* polymorphism occurs. The nucleotide in red indicates the site of West African (L1014F) *kdr* polymorphism.

### 3.3.5.3 Sequence analysis of the IIS5-IIS6 domain

DNA fragments corresponding to the domain IIS5-IIS6 region were PCR amplified using three sets of primer pairs from field collected female specimens whose $F_1$ progeny showed resistance to both DDT and permethrin. Internal primer pair N3: N4 yielded an approximately 238 bp product, while internal primers N2: N4 produced approximately 1182 bp PCR product. Primer pair N1: N4 which flank domain IIS5-IIS6 yielded a 1425 bp product. The amplified PCR products were sent to Inqaba biotechnical industries, South Africa for sequencing. The sequencing was done in both directions using the same primers (N1, N2, N3 and N4). BLAST analysis of the sequences against the NCBI database (http://www.ncbi.nlm.nih.gov/Genbank) confirmed that the *An. arabiensis* sodium channel fragment had been amplified. All sequenced samples showed a nucleotide identity greater than 99% and query coverage above 95% with either *An. arabiensis* or *An. gambiae* sodium channel genes deposited on the NCBI database (accession numbers DQ263749, AY615647 and GU248312). Alignment of field collected female specimens whose $F_1$ progeny showed resistance to both DDT and permethrin against field collected females whose $F_1$ progeny was susceptible to both insecticides showed that there was a 98.5% identity between the two insecticide phenotypes. Figure 3.18 shows the truncated aligned nucleotide sequences of the IIS5-IIS6 region of the voltage-gated sodium channel in the samples where polymorphisms were detected. There was substitution of **G** with **T** at position 568 in sample 144. At the same position Zimg 64 and Zimg 771 were heterozygous. This position corresponds to position 99 of *An. gambiae* sodium channel gene, Intron 1 deposited on NCB1 gene bank (AY615647). There was insertion of a **T** at position 1049 in two samples, Zimg 94 and

Zimg 682. This position corresponds to position 71 of *An. arabiensis* sodium channel protein partial sequence deposited on the NCBI gene bank (GU248312).

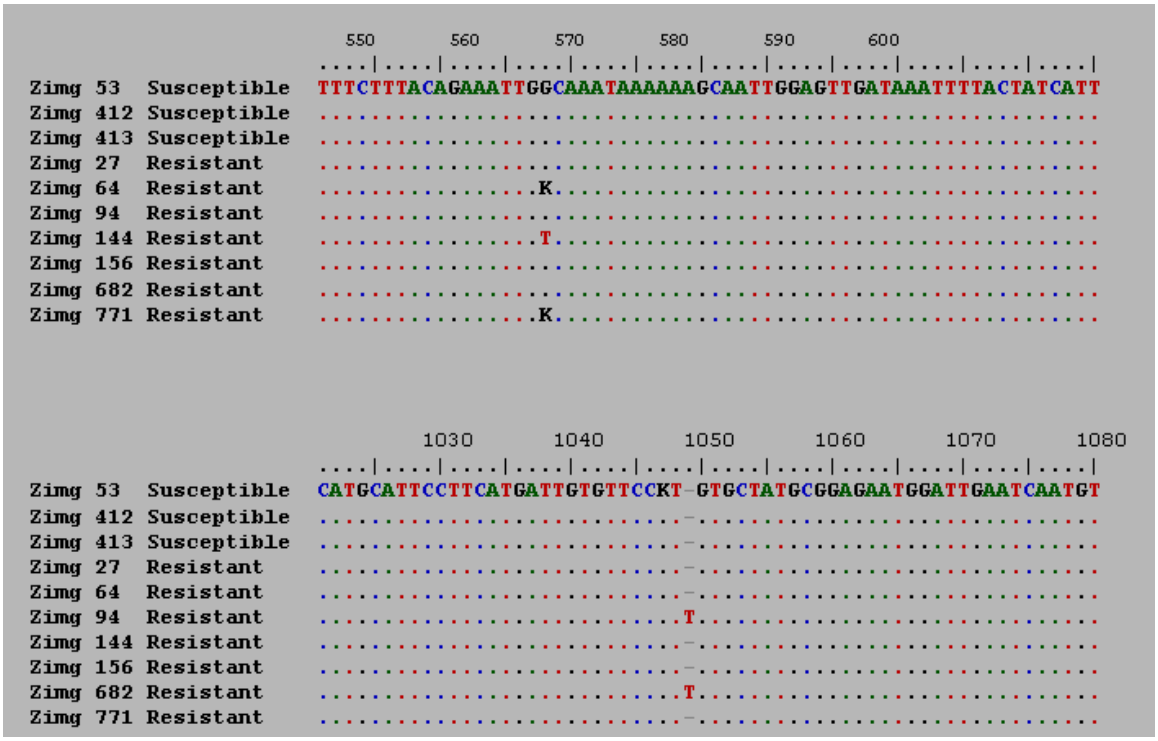



**Figure 3.13**: Comparison of nucleotide sequence of the sodium channel domain IIS5-IIS6 showing regions with nucleotide differences in *An. arabiensis* samples collected in Gwave village showing resistance to both DDT and pyrethroids and the corresponding nucleotide sequence of samples which were fully susceptible to DDT and pyrethroids. Identical positions are indicated (.) and (-) indicate missing nucleotides and K is IUPAC nucleotide code for G or T. Multiple sequence alignment was generated using the ClustalW (http://www.ebi.ac.uk/cgi-bin/clustalw).

### 3.3.6 *Ace-1*$^{R}$ mutation

To confirm presence of *ace-1*$^{R}$ mutation, 9 specimens which showed evidence of altered AChE activity during biochemical assays were sequenced for SNPs detection. A 194 bp fragment of *ace*-1 gene flanking the region where *ace*-1$^{R}$ mutation has been described was PCR amplified in these samples and corresponding controls. Direct sequencing of these PCR products yielded sequences which showed 89% homology (132/152 nucleotides and 0/152 gaps) and query coverage of 96% with the *An. gambiae ace*-1 gene deposited on the NCBI GENBANK (Accession XM321792.1). Searches of the G119S mutation associated with insensitive acetylcholinesterase did not show any substitution on this codon (Fig. 3.14). Alignment of these specimens using ClustalW did not show any alternative single nucleotide polymorphisms (SNPs) along this region.

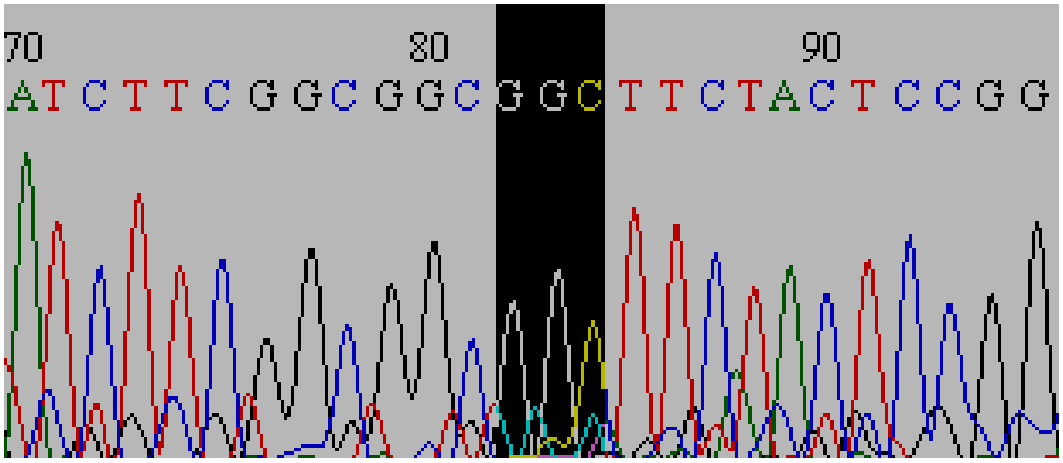



**Figure 3.14:** Cut-down chromatogram obtained by sequencing PCR products of samples from Gwave village showing the key codon where base substitution (G-A) normally occurs resulting in *ace*-1 mutation.

## 3.4 Discussion

### 3.4.1 Mosquito collections

The distribution of anopheline mosquitoes in Zimbabwe was investigated by Masendu *et al.* (2005). There are approximately 43 anopheline taxa distributed across the country, of which seven are known to occur within Gokwe district (Crees and Mhlanga, 1985; Masendu *et al*., 2005). Members of the *An. gambiae* complex are known to be widely distributed across all the low lying areas of Zimbabwe with *An. arabiensis* being the most widely distributed species (Mpofu, 1985; Manokore *et al*., 2000; Masendu *et al.,* 2005). In Gokwe three members of the *An. gambiae* complex, *An. arabiensis*, *An. quadriannulatus* and *An. merus* are known to occur in sympatry (Masendu *et al.,* 2005).

During this study four anopheline taxa; *An gambiae* complex, *An. longipalpis*, *An. coustani* and *An. squamosus* groups were collected. This taxa diversity is consistent with species ranges previously recorded in this area (Manokore *et al*., 2000; Masendu *et al.,* 2005). 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 behaviour. The exophilic behaviour of *An. coustani* and members of the *An. gambiae* complex are well documented (Gillies and Coetzee, 1987). Cattle kraal collections also resulted in relatively more mosquitoes than the other collection methods indicating that it is a much better method of sampling to obtain a large number of mosquitoes in this particular area. However, its main disadvantage is that it mainly collects zoophilic mosquitoes and these samples provide little or no information on their preference to human feeding. The total number of mosquitoes collected was below expectations. In previous studies mosquito collections were very productive yielding figures as high as

1200 (Manokore *et al.,* 2000). The low catches can be attributed to two main reasons. In 2006, mosquito sampling coincided with the National Malaria spraying programme. During the collection period 72% of the village had been covered by the NMCP spraying team. All homesteads visited had been sprayed with Icon® 10% Wettable Powder. 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. In 2008, houses had not been sprayed when mosquito sampling was done. However, there was heavy rainfall for the greater part of the sampling period and this could have significantly affected the number of mosquito collected.

The proportion of *An. arabiensis* caught from cattle kraals compared with other collection methods such as window exit and MBN traps was interesting. The latter two methods contributed only 5.2% of the total *An. arabiensis* catches. This can be explained from two fronts. It could be possible that *An. arabiensis* populations in this area exist as two distinct populations one which has endophilic tendencies and the other with exophilic behaviour. Indoor residual spraying practised yearly in this area might be playing a role in reducing the density of the indoor resting population. Although during 2008 no spraying had been done the low numbers of indoor catches can be explained by personal vector control initiatives such as the use of mosquito coils or some low levels of insecticidal or repellent effect from the previous round of spraying. It could also be possible that years of IRS using DDT and pyrethroids resulted in a change of feeding behaviour. *Anopheles arabiensis* population from this area might have adapted to feeding outdoors where there is a minimal chance of encountering insecticides. Exophilic

tendencies may be selected through long-term use of excito-repellant insecticides such as DDT and pyrethroids (Ameneshewa and Service, 1996). However, historical data has shown that this phenomenon might not be true. This warrants further investigations as it has serious implication for malaria vector control which is predominantly IRS based. Another contributing factor might be the fact that almost every household in the study area has a cattle kraal proximal to the homestead. This might be acting as a form of zooprophylaxis whereby mosquitoes are being diverted to feed on cattle rather than humans.

### 3.4.2 Species-specific identification

Transmission of malaria is complicated by the number of vector species involved. Studies carried out on the African continent indicate that at least two of the main vector species often occur in sympatry with non-vectors (Gillies and Coetzee, 1987; Coluzzi, 1984). Most of these vectors also occur as members of cryptic or sibling species complexes that include both vector and non-vector species (Coetzee *et al.,* 2000). 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* species A occur in various sympatric combinations. *Anopheles quadriannulatus* species A can be found in sympatry with one or both of the malaria vectors (Taylor *et al*., 1986; Coetzee *et al.,* 1993; Crees, 1996; Masendu, 1996; Masendu *et al*., 2005).

During this study 98.8% of the total catches constituted members of the *An. gambiae* complex. This was expected as collections were mainly targeting 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* species A) were collected. These three species were also found in sympatry by Manokore *et al*. (2000) and Masendu *et al*. (2005). Analysis of distribution of the three species showed that *An. arabiensis* predominates over the other species contributing 72.5% of the total collections. This concurs with similar studies carried out in this area which showed that *An. arabiensis* is the predominant sibling species in Gokwe district (Manokore *et al.,* 2000; Govere, 2003; Masendu *et al.,* 2005). Cattle kraal collection constituted 77% of *An. arabiensis* collected. This is expected as it has been shown that a high proportion of *An*. *arabiensis* will readily feed on animal hosts (Gillies and Coetzee, 1987). The set up of a standard homestead in the study area consist of sleeping houses which are proximal to cattle pens. This can divert mosquitoes to feed on cattle instead of humans (Lyimo and Ferguson, 2009), but would need further investigations.

The presence of *An. quadriannulatus* species A*,* a zoophilic species in man baited net and window exit trap collections is not a new phenomenon and has been recorded by Hunt and Mahon, (1986). In their study Hunt and Mahon, (1986) collected *An*. *quadriannulatus* species A inside 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 collected by Sharp and Quicke, (1984) in South Africa.

The presence of *An. merus* was not surprising. Although this salt water breeder is found mainly in coastal areas of east/ southern Africa, it has been recorded previously in this locality (Masendu, 2004). The species is known to survive in salt pans (Gillies and Coetzee., 1987), and the area where mosquitoes were collected is characterized by high fluoride content which can be considered saline. The occurrence of *An. merus* in collection methods such as window exit trap might indicate that this species might be feeding on humans therefore has potential to transmit malaria. Unfortunately, source of blood meal in blood fed specimens was not determined. Studies conducted by Masendu (2004) also revealed that *An. merus* is predominant in some areas of Gokwe accounting for 89.6% and 72.3% of human landing collections indoors and outdoors respectively. *Anopheles merus* has been implicated in transmitting malaria in coastal areas such as Tanzania (Temu *et al.,* 1998) and its role in transmission warrant further investigations. The sympatry of *An. merus* with the other two sibling species in larval habitats confirms the tolerance of this species to freshwater as reported by Coetzee *et al*. (1993).

Absence of *An. gambiae s.s* in our collections probably points to the once effective indoor residual house spraying campaign. Before the country faced its current economic challenges, indoor residual spraying was done yearly across all malaria endemic areas. *Anopheles gambiae s.s* is highly anthropophagic and exhibits endophilic behaviour and is thus highly susceptible to indoor residual spraying (Gillies and Coetzee, 1987). This species was last recorded in Zimbabwe in 1992 where it was confined to the northern fringes of the country (Masendu *et al.,* 2005).

The molecular identification of *An. longipalpis* Type C which had been morphologically identified as *An. funestus* highlights the importance of thoroughly identifying mosquito vectors to species level. While *An. longipalpis* Type C is not known to transmit malaria, *An. funestus* is a very efficient malaria vector and its occurrence has very serious implication on malaria transmission. This emphasises the importance of species identification to prevent misconceptions in vector dynamics.

### 3.4.3 Mosquito Infectivity

One of the most important entomological factors 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 and Graves, 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 (Beier *et al.,* 1988). The sporozoite rate, i.e. the proportion of mosquitoes with sporozoites in their salivary glands, indicates whether a vector can transmit malaria and is therefore an important factor used in studying the dynamics of malaria. 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.

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

During this study, an overall infectivity rate of 0.5% and 2.8% for *An. arabiensis* was recorded in 2006 and 2008 respectively. The 2006 infectivity rate is comparable with previous studies done by Mpofu in 1985. However, the 2008 infectivity rate is exceptionally high. The last incident of high *Plasmodium* infection rate in *An. arabiensis* was recorded in the early 1990's by Freeman during a malaria outbreak in the Midlands province (Freeman, 1995). Analyzing sporozoite rate per species and per collection method showed abnormally high figures of 16.7% for MBN in 2006 and 26.1% for window exit trap collections in 2008 compared to those from cattle kraal collections. This can be attributed to a number of factors. Firstly the sample sizes (n= 14 and n = 26) were too small to make definite conclusions. Another contributing factor is probably due to bias in sampling. Mosquitoes were collected from a single village with a history of malaria outbreaks. In fact during the 2008 collection period, two children from this village were seriously ill and malaria was positively confirmed as the cause of their illness using microscopy at the local district hospital. 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.

ELISA detects circumsporozoite proteins which can also be present in the developing oocyst dissolved in haemolymph. Not all oocysts are able to circumvent the various immunological challenges exerted on them before they develop into infective stages. There are various studies which have shown that ELISA overestimates the number of infected mosquitoes with some studies recording an overestimation of 1.5 times more

(Beier *et al*., 1990). In research done in Cameroon to compare the sensitivity and the specificity of the ELISA to the gold standard of microscopic examination, Fontenille *et al.* (2001) showed that ELISA overestimates the salivary gland infection rate by as much as 12%. Due to time and financial constraints an alternative method was not used in this study. It would be interesting to repeat this study covering a wider geographical area. The absence of infections in the other species suggests that they might not be playing any role in malaria transmission in Gwave.

Results of this study showed a conspicuous trend in mosquito's infectivity which warrants further investigations. Only mosquitoes collected indoors i.e. MBN and window exit traps were infected by *P. falciparum*. This leads to speculation that *An. arabiensis* from this area exists as two distinct populations; one population being zoophilic and the other anthropophilic.

### 3.4.4 Susceptibility of *An. gambiae* complex to insecticides

Several insecticide susceptibility surveys have been carried out in Gokwe (Murahwa, 1995; Manokore *et al*., 2000; Govere, 2003). All screening tests done to date indicate susceptibility of *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 the present study all the tested wild *An. arabiensis* populations and $F_1$ progeny were fully susceptible to 0.1% bendiocarb, 4% dieldrin and 5% malathion. This may be due to the sample area having no history of use of these classes of insecticides and therefore resistant individuals have not yet been selected to a detectable level. After PCR identifications were carried out, DDT and pyrethroid resistance was confirmed in *An. arabiensis* collected in 2006. Permethrin resistance was also confirmed in *An. arabiensis* in 2008. Although *An. quadriannulatus* and *An. merus* results showed resistance to permethrin over the two sampling periods, sample size was too small to make definite conclusions. It will be essential to make follow-up studies to ascertain the true picture on the insecticide susceptibility of these two species.

In this study, resistance to DDT was variable. In 2006 populations from this area were resistant to DDT but two years later there was only reduced susceptibility to the same insecticide. This might be attributed to two factors. Firstly, there is a significant difference in sample sizes between the two collection periods. In 2008, 14 families were used for susceptibility studies compared to 59 families in 2006. Secondly the variation might be explained by the resistance management strategy being practised by the National Malaria Control Programme (NMCP). DDT is used interchangeably with pyrethroid insecticides (Icon®). During the 2007 malaria season, the area had been sprayed with Icon and this might have reduced the selective pressure imposed by DDT resulting in an increase in DDT susceptible individuals in the specimens collected during 2008. However, DDT resistance genes are still circulating within the population as can be seen by the presence of 14.3% DDT resistant families in the 2008 collections.

Selection for DDT resistant individuals might have started as far back as the 1950's to 1970's when DDT and BHC were the insecticides of choice during the first malaria control initiatives of the country (Green, 1981). DDT was used in the 1970's to control tsetse flies (Matthiessen, 1985). In agriculture, organochlorines 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 indoors. *Anopheles quadriannulatus* was resistant to pyrethroids and DDT in 2006, while in 2008 it showed resistance to permethrin and reduced susceptibility to bendiocarb. Resistance to permethrin in the wild population of *An. gambiae* complex and the *An. arabiensis* of $F_1$ progeny of wild collected adults was recorded consistently. Such resistance levels were not expected as all previous studies had discounted pyrethroid resistance in this area. 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 0.025% deltamethrin and 0.1% lambdacyhalothrin and concluded that the resident mosquitoes were still susceptible to these pyrethroid insecticides.

The discovery of populations which are resistant to pyrethroids has serious implications for malaria control strategies. Pyrethroids are the main insecticides used by the NMCP and are 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 the 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 favouring the emergence of pyrethroid resistance (Lacey and Lacey, 1990; Ellisa *et al.,* 1993; Martinez-Torres *et al.,* 1998; Diabate *et al.,* 2002; Nwane *et al.,* 2009).

### 3.4.5 Biochemical Analysis

Insect populations have evolved a variety of mechanisms to counter the effect of exposure to insecticides. Metabolic detoxification of insecticides using specialized enzyme systems is one of the common ways in which insects respond to insecticide exposures. Increased enzyme activities of metabolic enzymes such as 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 $F_1$ 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 $F_1$ progeny of the wild-caught *An. arabiensis* assayed (48.5% of the families had elevated monooxygenase activity, 27.3% and 6.5% for alpha and beta-naphthyl acetate esterases respectively). This suggests that monooxygenases and general esterases may account for the pyrethroid resistance

observed during bioassays. Though there was no statistical correlation between bioassay mortality data and monooxygenase activity in 2006, possible involvement of this enzyme system was supported by biochemical assays carried out on $F_2$ progeny reared from permethrin resistant $F_1$ families. This selection for permethrin resistance in the $F_1$ progeny resulted in an increase in monooxygenase activity. The involvement of monooxygenases in pyrethroid resistance is not peculiar to this study. Several reports have shown that elevated levels of monooxygenases are associated with pyrethroid resistance in *An. gambiae, An. arabiensis* and *An. funestus* (Brogdon *et al.,* 1988, Vulule, 1999; Hargreaves *et al.,* 2000; Brooke *et al.,* 2001; Enayati *et al*., 2003; Casimiro *et al.,* 2006a; b; Amenya *et al.,* 2008). It is also highly likely that esterases are also playing a significant role in pyrethroid detoxification in *An. arabiensis*. Results from this study showed correlation between elevated esterase activity and resistance to pyrethroids. After further selecting $F_1$ progeny with permethrin in mosquitoes collected in 2008, there was a gradual increase in general esterase activity suggesting their role in permethrin resistance. The role of esterases in insecticide resistance is well documented; they have been implicated in resistance of insects to all classes of insecticides (Li *et al.,* 2007). The combined effect of elevated monooxygenase and esterase activity in permethrin resistance observed in this study has been previously reported in *Anopheles* mosquitoes (Vulule *et al.,* 1999). Future studies are needed where the role of these enzymes in resistance can be established using synergist assays.

DDT resistance observed in 2006 might be attributed to an increased level of GST activity found in some families assayed. Probable involvement of GST is further

strengthened by absence of elevated GST in 2008 collections which were susceptible to DDT. Resistance to DDT in mosquitoes has been frequently attributed to increased production of GST enzymes which catalyze DDT detoxification reactions (Clark and Shamaan, 1984; Brooke *et al*., 2001).

Data from insensitive acetylcholinesterase assays suggest the absence of altered AChE resistance genes in the population. The presence of some families which showed altered acetylcholinesterase using the biochemical method described by Penilla *et al.* (1998) highlights the disadvantages of relying on a single assay for confirming resistance mechanisms. Use of biochemical assays to detect altered AChE, though, accurate can be affected by homogenate preparation. Yellow chromophores present in insect homogenate if not properly ground have been shown to affect the precision of biochemical assays for insensitive AChE (Moores *et al.,* 1988). The use of organophosphates and carbamates is relatively prevalent in Gokwe with the spraying of cotton with Rogor® (Dimethoate), carbaryl and benfuracarb being widespread (Masendu, 2004). Therefore, any changes to carbamates by the NMCP should be done after careful consideration.

### 3.4.6 Molecular assay for knockdown resistance

Knockdown resistance (*kdr*) is one of the two major mechanisms in which insects become resistant to DDT and pyrethroids (Hemingway *et al.,* 2004). *Kdr* has been associated with mutations in the sodium channel gene in many insect species (Miyazaki *et al.,* 1996; Park and Taylor, 1997; Martinez-Torres *et al.,* 1998). In *An. gambiae* s.s two point mutations in the voltage-gated sodium channel gene, a Leu-Phe substitution at

position 1014 (L1014F) and a Leu-Ser substitution (L1014S), confer *kdr* resistance to DDT and pyrethroids (Martinez-Torres *et al.,* 1998; Ranson *et al.,* 2000). The Leu- Phe substitution mutation has now been recorded in *An. arabiensis* (Diabete *et al*., 2004; Matambo *et al.,* 2007; Himeidan *et al.,* 2007; Abdalla *et al.,* 2008).

In this study, the Leu-Phe mutation in the sodium channel gene was detected by allele-specific polymerase chain reaction. Confirmation tests using sequence analysis showed complete absence of the *kdr* allele. This confirmed 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; Abdalla *et al.,* 2008). 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) and the FRET/Melt Curve analysis have been developed (Lynd *et al.,* 2005; Verhaeghen *et al*., 2006). More recently, pyrosequencing as an alternative method was investigated and results showed that it is more reliable compared to the conventional PCR method (Vezenegho, 2008).

Complete absence of *kdr* mutation from the samples assayed was unexpected. Evidence of a target site resistance mechanism in this population 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 $F_1$ progeny of wild caught *An. arabiensis* showed 8 families carrying cross-resistance to permethrin and DDT. This is a

strong indication of resistance being conferred by a common mechanism in these families. DDT and pyrethroids as reviewed in Chapter 1 share a similar target site, the voltage-gated sodium channel and we had anticipated that insensitivity of this common target site might help to explain this cross-resistance. Biochemical assays done on these families did not demonstrate any common metabolic enzymes involved which could be attributed to DDT and permethrin resistance. To further analyse whether target site insensitivity was involved, domain IIS5-IIS6 of the voltage-gated channel was sequenced in resistant and susceptible samples to search for alternative polymorphisms. This region was chosen on the basis that mutations along this region confer resistance to DDT and pyrethroids in other insect species. Our results showed that although mutations were detected along these regions these were not systematic and could not be attributed to the resistance to both DDT and permethrin observed in our sample. This, however, does not mean that target site insensitivity can be discounted as an insecticide resistance mechanism in *An. arabiensis* from Zimbabwe. It is likely that other mutations might be occurring along other segments of the sodium channel gene that we did not investigate.

## 3.5 Manuscripts published from this Chapter

**Munhenga, G., Masendu, R.H., Brooke, B.D., Hunt, R.H., and Koekemoer, L.L.** (2008) Pyrethroid resistance in the major malaria vector *Anopheles arabiensis* from Gwave, a malaria-endemic area in Zimbabwe. *Malaria Journal* **7**: 247.

# CHAPTER 4

# CHARACTERIZATION OF PYRETHROID AND CARBAMATE RESISTANCE IN *ANOPHELES ARABIENSIS*

## 4.1 Introduction

Information on pyrethroid and carbamate resistance mechanisms in *An. arabiensis* in southern Africa is limited. In addition, insecticide resistance research on *An. arabiensis* has mainly been carried out in field-selected strains after resistance has already developed (Hargreaves *et al.,* 2003; Casimiro *et al.,* 2006b; Coleman *et al.,* 2008). In most instances the processes leading to this resistance is masked by various other selection pressures imposed on the mosquitoes in the field. This often results in speculation on the true involvement of several mechanisms in resistance to specific insecticides. Laboratory controlled studies normally provide a clear picture of resistance development (Scharf *et al.,* 1997).

In this section pyrethroid and carbamate resistance was characterized in two *An. arabiensis* laboratory colonies using a selection-based approach. Synergism and biochemical assays were used to determine possible underlying resistance mechanisms in the strains studied.

## 4.2 Material and Methods

Details of materials and methods used in this section are described in Chapter 2.

### 4.2.1 Selection for permethrin resistance

A laboratory colony of *An. arabiensis* (KWAG) from Kwazulu/Natal South Africa was colonized in 2005 using $F_1$ progeny from wild caught females. This population showed permethrin resistance (78% mortality) at the time of collection. The resistance levels had been lost by the time selections were initiated (98% mortality). The colony was placed under selection pressure using permethrin at an exposure time causing approximately 50% morality as described in section 2.8. The progress of selection was monitored using the WHO insecticide susceptibility assays (WHO, 1998). The final difference in susceptibility levels to permethrin between the selected and baseline cohorts was compared using $LT_{50}$ means of the cohorts.

### 4.2.2 Selection for bendiocarb resistance

A susceptible strain of *An. arabiensis* (MBN) from Kwazulu Natal South Africa was colonized in 2002 from wild populations which had low levels of carbamate resistance during the time of collections. MBN was placed under bendiocarb selections for 12 generations as described in section 2.8. The progress of selection was monitored using the WHO insecticide susceptibility assays (WHO, 1998). The final susceptibility level to 0.1% bendiocarb was monitored by determining the $LT_{50}$ values of the baseline colony, F8 and $F_{12}$ generations.

### 4.2.3 Biochemical assays

Homogenates from 20 individuals from selected generations were screened for elevated non-specific esterases, glutathione-S-transferases, monooxygenases and the presence of altered acetylcholinesterase as detailed in section 2.10.

## 4.3 Results

Some of the results of this chapter have been published in the *South African Journal of Science* (Appendix V).

### 4.3.1 Selection for pyrethroid resistance (KWAG-Perm)

Selections started with exposures of *An. arabiensis* baseline colony to 0.75% permethrin at four different exposure times (20, 30, 40 and 60 minutes). Mean percentage mortalities 24 hr post exposure for the respective exposure times are summarised in Fig. 4.1. Average mortalities were 68%, 77%, 98% and 99% respectively. An exposure period of 20 minutes was subsequently used to start the selections because it had enough survivors to obtain the next generation.

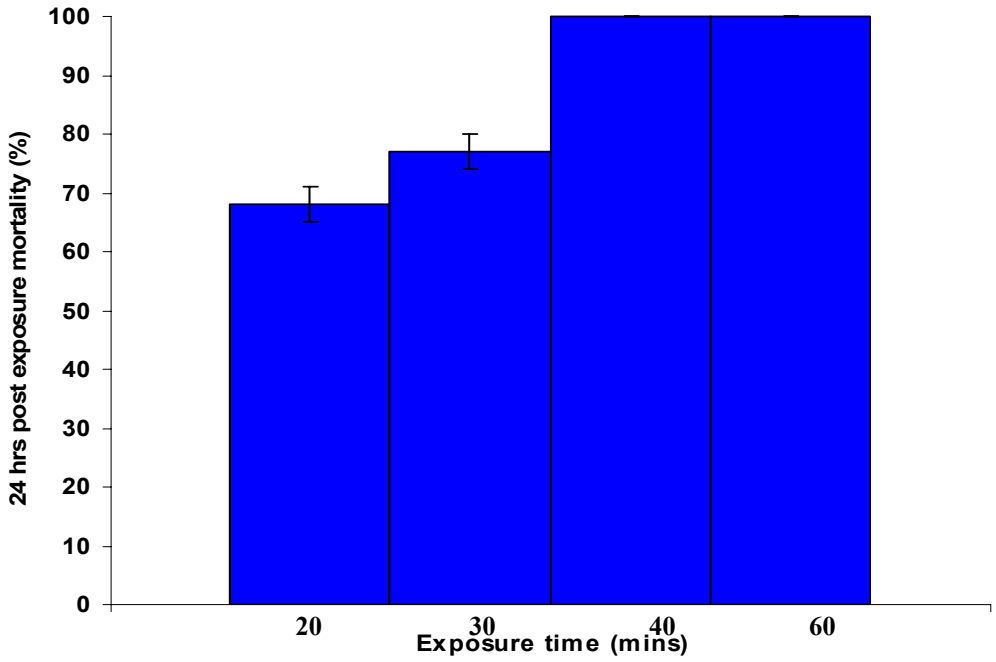



**Figure 4.1:** Insecticide bioassay results using 0.75% permethrin exposures at 4 different exposure times against the $F_0$ generation of the KWAG *An. arabiensis* colony.

Permethrin susceptibility test results by generation ($F_0$-$F_{12}$), based on selection for pyrethroid resistance using 0.75% permethrin are summarised in Table 4.1. Tolerance to permethrin rapidly increased over successive generations. Within six generations, resistance as defined by WHO criteria (WHO, 1998) was achieved. Survivorship increased from the 6$^{th}$ to the 8$^{th}$ generation and stabilized thereafter. The WHO bioassays of unselected baseline colony (KWAG) carried out simultaneously with selected cohorts showed susceptibility to permethrin as evidenced by 98% mortality (results not shown).

**Table 4.1:** Summary of average 24 hour post exposure mortality for 12 generations of *An. arabiensis* (KWAG-Perm) selected on 0.75% permethrin.

| Sample | No. tested | Exp. Time | % mortality | SE |
|---|---|---|---|---|
| $F_0$ | 1223 | 20 | 68.1 | 2.89 |
| $F_2$ | 632 | 30 | 55.9 | 2.94 |
| $F_4$ | 284 | 40 | 64.7 | 7.45 |
| $F_6$ | 885 | 60 | 57.9 | 4.43 |
| $F_8$ | 1084 | 60 | 46.7 | 3.91 |
| $F_{10}$ | 664 | 60 | 45.1 | 5.31 |
| $F_{12}$ | 732 | 60 | 44.9 | 4.13 |

Figure 4.2 presents a time mortality curve based on 0.75% permethrin exposures comparing three generations: baseline colony (KWAG-base), $F_7$ and $F_{12}$ selected cohorts. After fitting a linear regression model using the mortality curve and comparing exposure time causing 50% mortality ($LT_{50}$) between the baseline colony ($F_0$) and the selected generations ($F_7$ and $F_{12}$) results showed an increase in resistance in the selected cohorts. The predicted ($LT_{50}$) was 15.1 minutes for the baseline colony and 55.7 and 58.2 minutes for the $F_7$ and $F_{12}$ selected cohorts respectively showing an approximately 3.8 fold increase in permethrin tolerance in the selected cohorts.

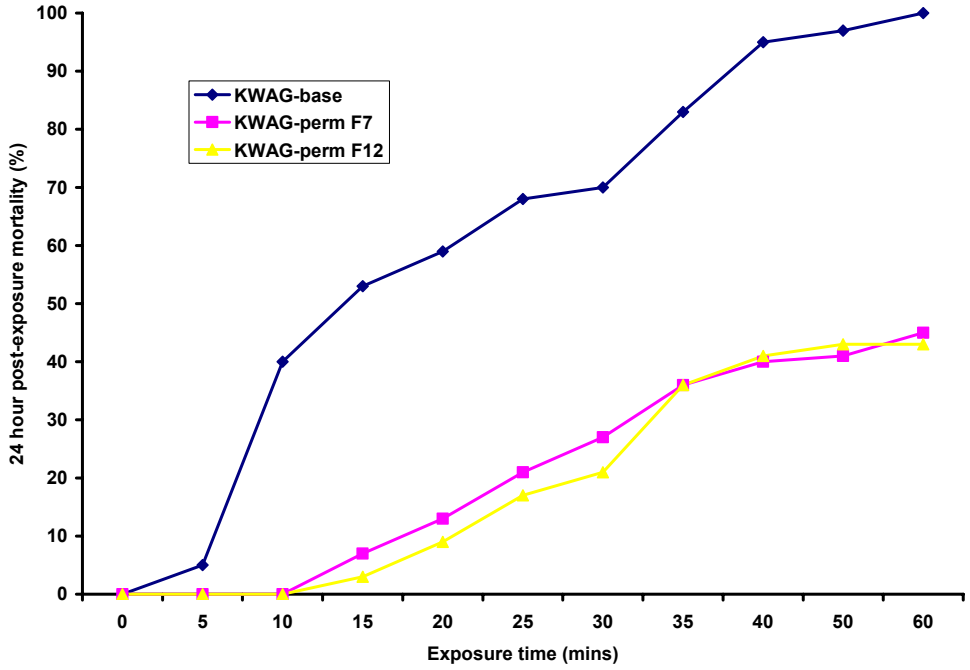



**Figure 4.2 :** Time-mortality curve based on 0.75% permethrin exposure comparing permethrin resistant selected cohorts $F_7$ and $F_{12}$ against KWAG-base.

### 4.3.2 Biochemical analysis on permethrin selected line (KWAG-Perm)

Table 4.2 presents a summary of enzyme activity monitored per generation by quantification of metabolic enzymes in selected cohorts compared with the baseline colony. Each generation were analysed separately as that generation emerged in the insectary e.g. F0 was analysed and approximately 2 months later the F4 generation etc. The results obtained were compared (Table 4.2) at the end of the 12$^{th}$ generation. Results indicate that there was a consistent increase in monooxygenases activity as selection progressed. Monooxygenase activity increased 2.6 fold in the $F_{12}$ cohorts compared to the parental cohorts ($F_0$). Activity of non-specific esterases monitored using alpha-naphthyl acetate as a substrate showed a gradual increase in activity up to the 4$^{th}$ generation. There was a sharp increase in the 7$^{th}$ generation and a steady increase thereafter. General

esterases monitored using beta-naphthyl acetate was lower in the selected cohorts compared with the baseline cohorts up to the 4th generation and showed a gradual increase in the resistant 7th and 12th generations. GST activity varied between generation and there was no systematic increase.

**Table 4.2:** Comparison of specific activities of α and β non-specific esterases, monooxygenases and GST from *An. arabiensis* susceptible control and three permethrin selected cohorts (15 individual mosquitoes from each generation were assayed for each enzyme system).

| **Test population Generation time permethrin exposure** | **α esterases activity (Mean OD value) ± SE** | **β esterases activity (Mean OD value) ± SE** | **monooxygenase activity (Mean OD value)± SE** | **GST activity (Mean OD value) ± SE** |
|---|---|---|---|---|
| $F_0$ | 0.114 ± 0.006 | 0.058 ± 0.001 | 0.778 ± 0.029 | 1.392 ± 0.011 |
| $F_4$ | 0.117 ± 0.006 | 0.055 ± 0.001 | 0.809 ± 0.047 | 1.401 ± 0.004 |
| $F_7$ | 0.283 ± 0.005 | 0.062 ± 0.001 | 0.932 ± 0.024 | 1.397 ± 0.033 |
| $F_{12}$ | 0.287 ± 0.005 | 0.067 ± 0.001 | 1.987 ± 0.031 | 1.395 ± 0.034 |

To ensure that the above mentioned differences (Table 4.2) were not due to technical variation of conducting analysis over a period of time, ,three selected generations were simultaneously monitored for enzyme activity by freezing a sub-sample of each generation. These samples were analyzed simultaneously and these results are presented in Figure 4.3. This shows direct comparison of GSTs, monooxygenases and general esterases activities monitored simultaneously between baselines ($F_0$), $F_7$ and $F_{12}$ generations. Results showed significant difference in monooxygenase activity between the three cohorts (ANOVA: DF = 3; P = 0.002). There was significant difference in monooxygenase activity from each generation assayed (student *t*-test: $P < 0.05$). The

highest monooxygenases activity was recorded in the $F_{12}$ cohorts. Activity of non-specific esterases monitored using α-naphthyl as a substrate showed significant difference between the three cohorts (ANOVA: DF = 3; P = 0.037). However, there was no significant difference in activity between the $F_7$ and $F_{12}$ generations (student *t*-test: P = 0.052). GST activity and non-specific esterases monitored using β-naphthyl acetate as a substrate showed no statistical difference between the baseline and selected cohorts (ANOVA: DF = 3; P > 0.05).

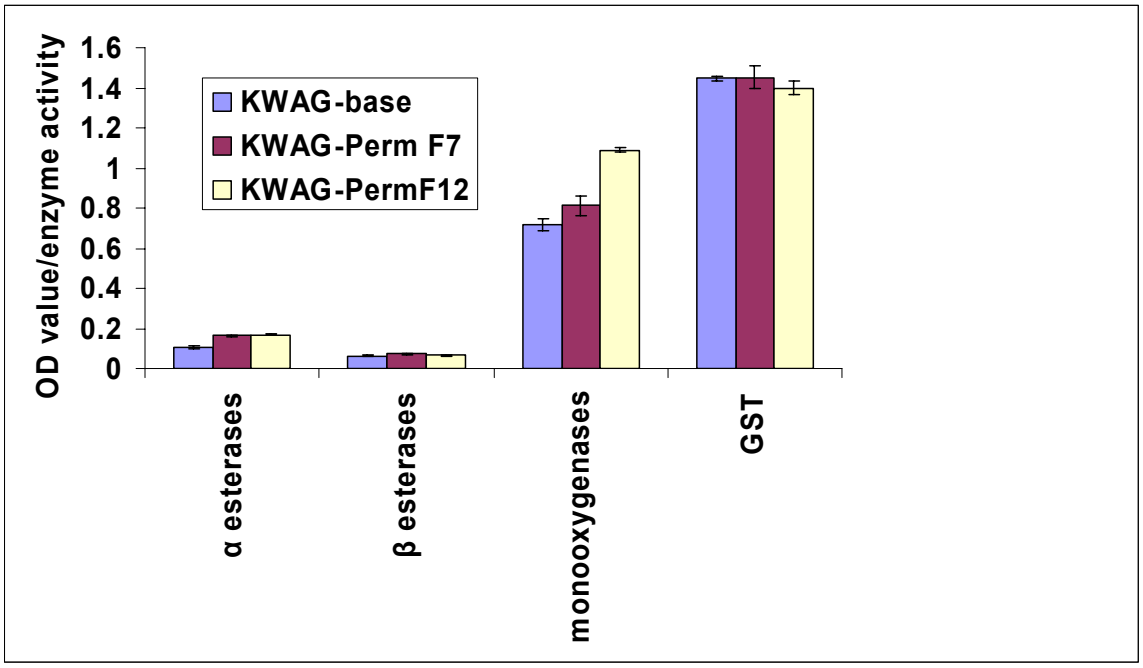



**Figure 4.3:** Comparison of α and β non-specific esterases, monooxygenases and GST levels from *An. arabiensis* KWAG baseline and the KWAG-Perm ($F_7$ and $F_{12}$) selected cohorts assayed on the same plate.

### 4.3.3 Synergist studies on permethrin selected line (KWAG-Perm)

Table 4.3 details the synergist results on the pyrethroid selected lines. Susceptibility to permethrin was restored by synergizing with PBO, while DEM had no effect on the level

of resistance to permethrin. The differences in mortality 24 hr post exposure between synergized and unsynergized samples using PBO was statistically significant ($\chi^2$=0.4, DF = 4, P < 0.05). There was no significant difference in mortality post exposure between sub-samples synergised with DEM and unsynergized samples ($\chi^2$=0.12, DF = 4, P > 0.05).

**Table 4.3 :** Comparison of synergized (PBO and DEM) and unsynergized mean % mortality 24 hr post exposure for samples of *An. Arabiensis* KWAG- Perm $F_{12}$ selected for resistance with 0.75% permethrin.

| **Treatment** | **n** | **% mortality ± SD (24 hr post-exposure)** |
|---|---|---|
| 4% PBO+ 0.75% permethrin | 200 | 98.3 ± 1.6 |
| 0.75% permethrin only | 200 | 41.8 ± 3.4 |
| 4% PBO only | 200 | 0.0 |
| 8% DEM + 0.75% permethrin | 200 | 43.9 ± 9.8 |
| 0.75% permethrin only | 200 | 41.7 ± 13.6 |
| 8% DEM only | 200 | 0.0 |

Table 4.4 shows synergists studies carried out on KWAG-Perm $F_7$ cohorts using TPP an esterase synergist. Tests were carried out on $F_7$ cohorts because there were not enough adult mosquitoes in the $F_{12}$ cohorts to carry out the same synergist bioassays. Results showed that TPP did not have any synergistic effect on the resistant phenotype. There was no difference in 24 hour post exposure mortality between synergised and unsynergized samples (P > 0.05).

**Table 4.4 :** Comparison of TPP synergized and unsynergized mean % mortality 24 hr post exposure for samples of *An. Arabiensis* KWAG- Perm $F_7$ selected for resistance with 0.75% permethrin.

| **Treatment** | **n** | **% mortality ± SD (24 hr post-exposure)** |
|---|---|---|
| 1% TPP + 0.75% permethrin | 113 | 43.6 ± 1.6 |
| 0.75% permethrin only | 121 | 40.0 ± 2.1 |
| 1% TPP only | 150 | 0.0 |
| 6% TPP + 0.75% permethrin | 145 | 40.0 ± 2.0 |
| 0.75% permethrin only | 200 | 42.3 ± 2.9 |
| 6% TPP only | 200 | 0.0 |

### 4.3.4 Selection for carbamate resistance (MBN-Carb)

*Anopheles arabiensis* originating from Kwazulu-Natal (MBN) was exposed to the diagnostic dosage of 0.1% bendiocarb using the WHO procedure at four different exposure periods (5mins, 10mins, 15mins and 20mins) in order to obtain an exposure time which gives sufficient insecticide contact time and enough survivors to select for a carbamate resistant line. After exposing the MBN baseline colony ($F_0$) to 0.1% bendiocarb for 5, 10, 15, and 20 minutes, mean mortalities 24 hour post exposure were recorded as 0.8%, 12.5%, 68.8% and 80.9% respectively (Fig. 4.4). Based on these results an exposure period of 15 minutes was chosen to select for a carbamate resistant line.

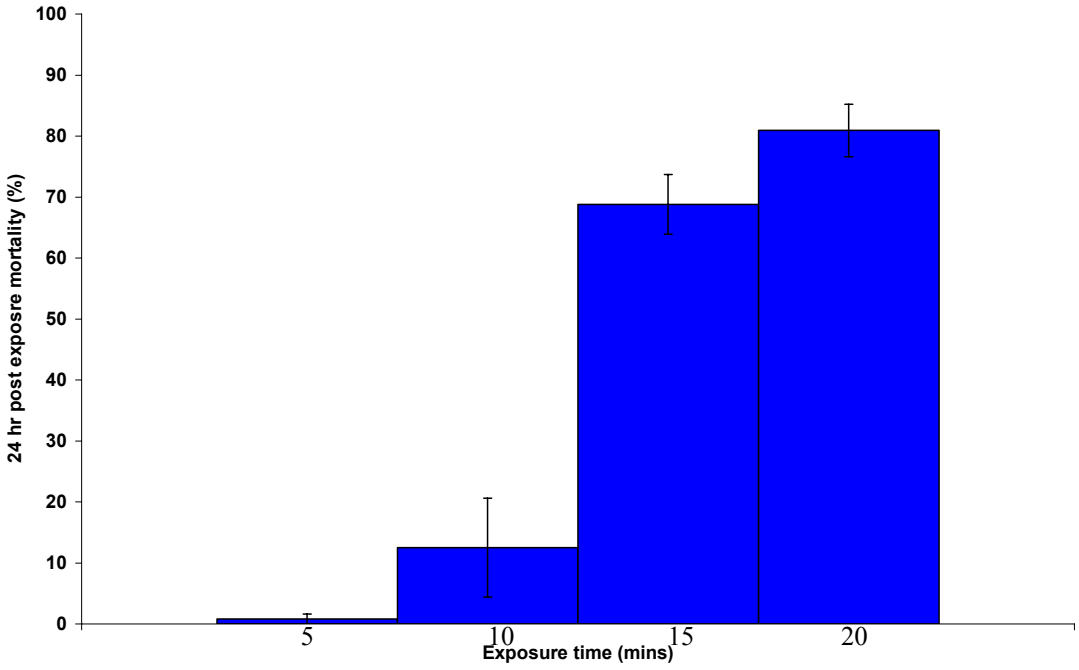



**Figure 4.4:** Insecticide susceptibility results of 0.1% bendiocarb exposures against the MBN baseline colony at different exposure times.

Results of susceptibility tests with 0.1% bendiocarb for the different generations under selection pressure are presented in Table 4.5. There was a slight increase in tolerance to 0.1% bendiocarb exposure from $F_1$ to $F_2$ generation. After increasing the 0.1% bendiocarb exposure time to 30 minutes in the $F_3$ generation, 24 hour post exposure mortality of 57.7% was recorded. In the $F_4$ generation exposure of mosquitoes to bendiocarb for 30 minutes continuously resulted in complete susceptibility. Consequently the exposure period was reduced to 20 minutes. Successive selections from the $5^{th}$ generation resulted in gradual increase in tolerance up to the $12^{th}$ generation.

**Table 4.5:** Summary of average 24 hour post exposure mortality for 12 generations of *An. arabiensis* (MBN-Carb) selected on 0.1% bendiocarb (SD = standard deviation).

| **Sample** | **No. tested** | **Exp. Time** | **Mean % mortality** | **SD** |
|---|---|---|---|---|
| $F_0$ | 235 | 15 | 67.2 | 7.9 |
| $F_1$ | 234 | 20 | 72.4 | 9.7 |
| $F_2$ | 184 | 20 | 68.5 | 10.1 |
| $F_3$ | 358 | 30 | 57.7 | 8.61 |
| $F_4$ | 320 | 30 | 100 | 0 |
| $F_4$ | 475 | 20 | 62.5 | 6.23 |
| $F_5$ | 496 | 20 | 60.6 | 5.49 |
| $F_6$ | 625 | 30 | 54.1 | 3.21 |
| $F_8$ | 607 | 40 | 68.1 | 2.72 |
| $F_{10}$ | 521 | 40 | 60.2 | 3.21 |
| $F_{12}$ | 484 | 40 | 54.7 | 2.2 |

A comparison of bendiocarb tolerance was assayed between the selected $F_8$, $F_{12}$ and the parental baseline cohorts ($F_0$). The $LT_{50}$ was 13 minutes for the baseline colony and 36.5 and 37 minutes for the $F_8$ and $F_{12}$ bendiocarb selected samples representing a 2.8 fold increase in bendiocarb tolerance in the selected cohorts. Statistical analysis showed a significant difference between the $LT_{50}$ of the baseline colony compared to the $F_{12}$ cohorts ($P = 0.0001$). However, following a 60 minute exposure to 0.1% bendiocarb of the $F_{12}$ cohorts, an average mortality of 87.5% was obtained (Fig. 4.5).

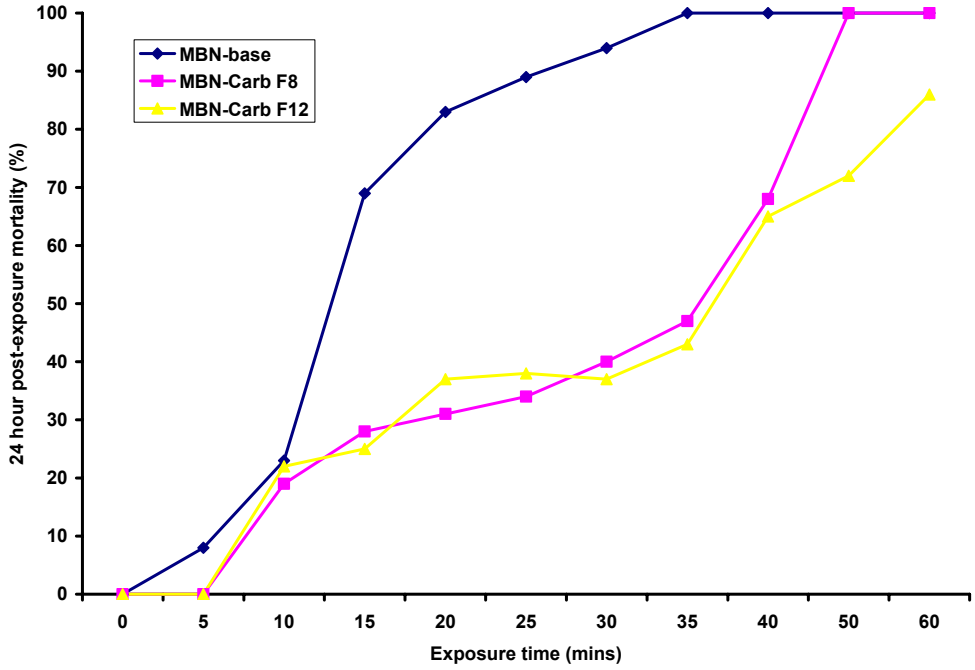



**Figure 4.5 :** Time-mortality curve based on 0.1% bendiocarb exposure comparing bendiocarb tolerant cohorts $F_8$ and $F_{12}$ against the parental colony (MBN-base).

### 4.3.5 Biochemical analysis on bendiocarb tolerant line (MBN-Carb)

Table 4.6 shows non-specific esterases, monooxygenase and GST assays performed separately on the baseline colony and bendiocarb selected cohorts. GST activity consistently increased throughout the selection period. General esterases using α-naphthyl remained constant in the first 4 generations and increased as the selections progressed. There was a 1.2 fold increase in activity in the $F_{12}$ generation compared to the baseline cohorts. General esterases monitored using β-naphthyl acetate fluctuated between the selected generations. Monooxygenases levels remained consistent throughout the selection period. There was no record of insensitive AChE in all the selected cohorts

**Table 4.6:** Comparison of specific activities of α and β non-specific esterases, monooxygenases and GST from *An. arabiensis* susceptible control and three bendiocarb selected cohorts (OD = optical density; SE = standard error).

| **Test population Generation time permethrin exposure** | **α esterases activity (Mean OD value) ± SE** | **β esterases activity (Mean OD value) ± SE** | **monooxygenase activity (Mean OD value)± SE** | **GST activity (Mean OD value) ± SE** |
|---|---|---|---|---|
| $F_0$ | 0.287 ± 0.002 | 0.142 ± 0.001 | 2.191 ± 0.017 | 1.716 ± 0.071 |
| $F_4$ | 0.273 ± 0.005 | 0.024 ± 0.000 | 2.212 ± 0.1012 | 1.958 ± 0.082 |
| $F_8$ | 0.301 ± 0.005 | 0.012 ± 0.001 | 2.081 ± 0.075 | 2.404 ± 0.084 |
| $F_{12}$ | 0.347 ± 0.002 | 0.100 ± 0.002 | 2.204 ± 0.086 | 3.965 ± 0.073 |

To account for variations of assays done on different days and times enzyme assays were done simultaneously for the three different generations. Figure 4.6 details non-specific esterases, monooxygenase and GST assays performed simultaneously on the baseline colony, $F_4$, $F_8$ and $F_{12}$ selected cohorts. Comparison of GST activity between generations showed significant increases in GST activities between the four cohorts (ANOVA: DF = 3, $P < 0.05$). Non-specific esterase activity monitored using α-naphthyl acetate showed significant difference between the four cohorts (DF = 3, $P < 0.05$). However, pair-wise comparison between the baseline and $F_8$ cohorts showed no significant difference in esterase activity (student *t*-test, $P > 0.05$). While a significant difference in activity was observed between the $F_0$ and $F_{12}$ cohorts. ANOVA analysis of monooxygenases and non-specific esterases monitored using β-naphthyl acetate found no significant differences in activities between the parental colony and selected cohorts ($P > 0.05$ in both cases). There was no change in the sensitivity of acetylcholinesterase to inhibition by propoxur, in either the selected strains compared to the baseline cohorts.

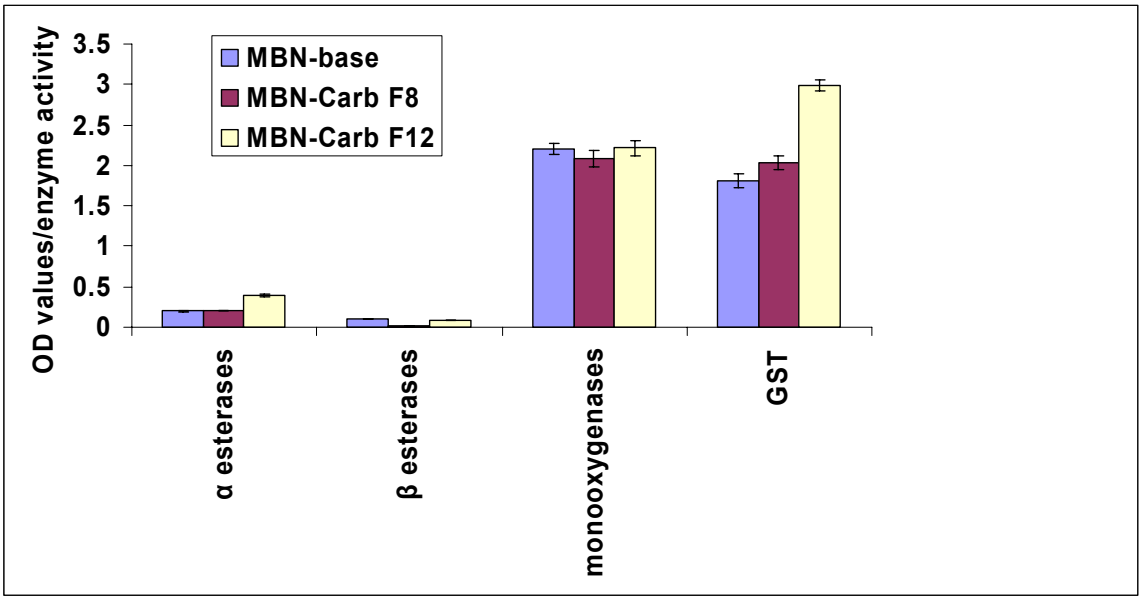



**Figure 4.6:** Comparison of α and β non-specific esterases, monooxygenases and GST levels from *An. arabiensis* MBN- baseline and the MBN-Carb ($F_8$ and $F_{12}$) selected cohorts assayed on the same plate.

### 4.3.6 Synergist studies on bendiocarb tolerant line (MBN-Carb)

The effects of PBO, DEM, and TPP synergist on 0.1% bendiocarb selected line are detailed in Table 4.7. Samples that were exposed for 40 mins to bendiocarb after 8% DEM exposure showed higher mortalities averaging 93.2% compared to the mean mortality of 72.6% in samples exposed to bendiocarb alone ($\chi^2$ = 12.71, P = 0.0027). Exposures of samples to 4% PBO followed by 40 minutes exposure to 0.1% bendiocarb (synergized) gave 76.1% mortality while the unsynergized sample gave a mean mortality of 74.1%. The difference in mortality between the two sub-samples was not significant ($\chi^2$ = 14.13, P = 0.063). The differences in mortality 24 hours post exposure between synergized and unsynergized samples using 8% TPP and 16% TPP was not statistically

significant. However, there were slight changes in susceptibility between the synergized and unsynergized samples.

**Table 4.7 :** Comparison of synergized (PBO, DEM and TPP) and unsynergized mean % mortality 24 hour post exposure for samples of *An. Arabiensis* MBN-Carb $F_{12}$ selected for resistance with 0.1% bendiocarb.

| **Treatment** | **n** | **% mortality ± SD (24 h post-exposure)** |
|---|---|---|
| 4% PBO+ 0.1% bendiocarb | 200 | 76.1 ± 5.7 |
| 0.1% bendiocarb only | 200 | 74.1 ± 7.2 |
| 4% PBO only | 200 | 0.0 |
| 8% DEM + 0.1% bendiocarb | 200 | 93.2 ± 2.1 |
| 0.1% bendiocarb only | 200 | 72.6 ± 2.8 |
| 8% DEM only | 200 | 0.0 |
| 16 % TPP + 0.1% bendiocarb | 200 | 79.8 ± 3.4 |
| 0.1 % bendiocarb only | 200 | 73.4 ± 3.1 |
| 16 % TPP only | 200 | 0.00 |
| 8 % TPP + 0.1 % bendiocarb | 200 | 77.8 ± 2.9 |
| 0.1 % bendiocarb only | 200 | 74.0 ± 1.9 |
| 8 % TPP only | 200 | 0.00 |

**NB** : Exposure time was 40 mins not 60 mins as recommended by WHO.

## 4.4 Discussion

### 4.4.1 Toxicological responses to permethrin selections

Resistance is defined by the WHO as the acquired ability of an insect population to tolerate doses of insecticide which will kill the majority of individuals in a normal population of the same species (WHO, 1992). *Anopheles arabiensis* from South Africa showed that physiological resistance to permethrin can be achieved in the laboratory after artificial selection.

Permethrin resistance was obtained after only six generation of permethrin selections. WHO defines susceptibility as follows: a mortality rate of 98%-100% indicates that a mosquito population is susceptible to insecticides, 80%–97% mortality indicates a possibility of resistance and confirmation is required, while mortality of less than 80% shows that resistance is present in the mosquito population (WHO, 1998). After the $8^{th}$ generation, levels of permethrin resistance in *An. arabiensis* remained constant despite continual insecticidal selective pressure. Increase in resistance was supported by an increase in $LT_{50}$ estimate. Following 12 generations of permethrin selection the $LT_{50}$ increased 3.8 times suggesting that insecticide selections increased the proportion of resistant individuals. This observation is consistent with the principle of insecticide resistance development in a population. Resistance development by selection with an insecticide 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 (Scott, 1995). The rapid development of permethrin resistance observed

in the study could have been potentiated by the presence of resistance genes in the parental colony used for selections. The parental colony had low levels of permethrin resistance (78% mortality) when it was established in 2005 (Mouatcho *et al.,* 2009). The presence of resistance in the natural population is probably due to the history of pyrethroid usage in this area. Deltamethrin replaced DDT for malaria vector control in KZN in 1996 (Maharaj *et al.,* 2005). Currently deltamethrin is being used to spray westernised houses only (Maharaj *et al.,* 2005). Usage of pyrethroids in agriculture and at household level is also prevalent in this area (Naidoo *et al.,* 2008). It is worrying that resistance levels will increase over generations as demonstrated by laboratory selection experiments.

This laboratory study provides important information for the South African malaria control programme in respect to pyrethroid usage. It shows that pyrethroid resistance genes are present in *An. arabiensis* from Kwazulu/Natal. This was confirmed independently by an insecticide susceptibility survey done in the area. *Anopheles arabiensis* from this area showed reduced susceptibility to deltamethrin and resistance to permethrin (Mouatcho *et al.,* 2009). In order to sustain the current pesticide regime which includes pyrethroids, a number of steps should be followed. Firstly, regular monitoring of changes in insecticide susceptibility levels of mosquitoes should form part of the National Malaria Control Programme. Underlying resistance mechanisms should be clearly elucidated so that alternative insecticides can be used for the control programme. Lastly pyrethroids should be used in rotation with other insecticide classes with different modes of action such as carbamates. DDT should remain as an alternative since there is no

evidence of target site insensitivity in this population. Such resistance management programme of using different types of insecticides in time and space are known to be very effective. Penilla *et al,* (1998) showed that frequency of carbamate resistance was reduced significantly by implementation of rotational use of insecticides.

This study showed that there is a potential of resistance developing in *An. arabiensis* population from KZN. However, this might take some time because in nature development of resistance is not as quickly as observed in this laboratory study. How quickly resistance develops depends on several factors, including insect reproductive rate, migration, frequency of the resistance genes in a population and number of genes interacting to produce the resistant phenotype, type of insecticide, rate of application and number of applications (WHO, 2003). However, this laboratory study showed that selection for pyrethroid resistance is greatly enhanced when a population is put under selective pressure using the same insecticide as is widely the case in agriculture, indoor residual spraying and larviciding.

### 4.4.2 Selection for permethrin resistance mechanisms

Major mechanisms of pyrethroid resistance in *Anopheles* mosquitoes involve either mutation within the insecticide target site (Martinez –Torres *et al.,* 1998; Ranson *et al.,* 2000) or an alteration in the rate of insecticide detoxification (Hemingway and Ranson 2000). Both types of resistance mechanisms have been detected in *An. arabiensis* (Hargreaves *et al.,* 2003; Casimiro *et al.,* 2006b; Abdalla *et al.,* 2008). Enzymes from the two members of large multigene enzyme systems: cytochrome P450 monooxygenases

and non-specific esterase have been implicated in pyrethroid resistance in mosquitoes (Hemingway and Ranson, 2000), and GST involvement in pyrethroid resistance has been demonstrated in the plant hopper *Nilaparvata lugens* (Vontas *et al.*, 2001).

In this study, increase in specific enzyme activity was correlated with an increase in survival after insecticide exposure. Our results indicated an increase in monooxygenase activities with a 2.5 fold increase in activity in the $F_{12}$ strains over the baseline cohorts. The significant difference in monooxygenase titers recorded in different pyrethroid selected cohorts compared with corresponding unselected cohorts strongly suggests that this enzyme group is associated with pyrethroid resistance. This was confirmed by synergist studies using PBO, a synergist that specifically inhibits monooxygenase activity. Permethrin exposures carried out after synergizing resistant $F_{12}$ samples reversed the resistance phenotype from a mortality level of 42% to 98%. The incomplete synergism indicates that other resistance mechanisms besides monooxygenases might be playing a role. However, the WHO definition of resistance will label the 98% result as being susceptible. This study confirms other reports which have associated monooxygenases with pyrethroid resistance in mosquitoes (Vulule *et al.,* 1999; Hemingway and Ranson 2000; Brooke *et al.,* 2001).

There was evidence of increased activity of general esterases in the selected resistant cohorts. General esterases monitored using an alpha-naphthyl acetate substrate increased significantly in the resistant phenotype. In this study there is no direct evidence to show

that esterases are involved in pyrethroid resistance. TPP synergist studies did not give clear answers.

### 4.4.3 Toxicological responses to bendiocarb selections

Selection for carbamate resistance was unsuccessful. Susceptibility to bendiocarb fluctuated greatly between the first five generations and stabilised thereafter. The reason for this fluctuation is unknown. Although predicted exposure time causing 50% mortality increased 2.8 fold in the $F_{12}$ bendiocarb selected samples, exposures of the $F_{12}$ generation for 60 minutes as recommended by WHO showed that mosquitoes are only tolerant but not resistant to bendiocarb. The apparent lack of resistance after bendiocarb selection for 12 generations may be explained on two fronts. Firstly bendiocarb selections are known to be very difficult (Georghiou, 1980). Secondly there is no history of rampant carbamate usage in the area where the parental colony used for these selections were collected. Most villagers in this area favour the use of pyrethroids for both agriculture and domestic purposes (Naidoo *et al.,* 2008). As a result, carbamate resistance genes in this population have not been selected to a high level. It is also possible that bendiocarb resistance genes are associated with fitness cost which may explain why it was extremely difficult to move beyond the levels observed in the $F_3$ generation. Another possible reason is that a huge amount of genetic variation is lost during mosquito colonisation (Vernick *et al.,* 2005) and it is possible that the carbamate resistance genes were lost during colonisation and thus the resistant genotype could not be artificially brought back.

### 4.4.4 Selection for bendiocarb resistance mechanisms

Several resistance mechanisms have been demonstrated in carbamate resistant arthropods. Detoxification by metabolic enzymes and choline esterases insensitive to carbamates are the dominating mechanism of resistance in insects exposed to carbamates (Hemingway, 1981). In carbamate resistant houseflies, reduced cuticle penetration by carbamates has also been implicated as a resistance mechanism. In African malaria vectors organophosphate and or carbamate resistance has been attributed to two main mechanisms: Mutation of the target site for these classes of insecticides, the (*Ace* gene), and esterase based resistance mechanisms (Karunaratne *et al.,* 1993).

During this study, bendiocarb selections resulted in a corresponding increase in GSTs and general esterases assayed using alpha-naphthyl acetate. Biochemical assays of general esterases in the $F_4$ selected cohorts showed no significant difference in esterase activity between the parental and selected cohorts. This correlated with bioassay results which showed that $F_4$ cohorts were still susceptible to bendiocarb exposures. Further selections resulted in increased general esterase activity monitored using α-naphthyl acetate. $F_{12}$ selected cohorts showed a 1.2 fold increase in activity compared to the parental baseline colony. This result suggests that a quantitative increase in esterases monitored using naphthyl acetate might be playing a role in bendiocarb tolerance. This was supported by synergist studies using TPP. TPP an esterase inhibitor partially reversed tolerance of $F_{12}$ cohorts to bendiocarb exposures from 74% in the unsynergized samples to 79% in the synergized samples regardless of the concentration of TPP used. The moderate effect of esterases as resistance mechanisms can be explained by the way esterases confer

insecticide resistance. Esterases normally confer resistance by rapidly binding the insecticide and slowly turning it over thus preventing it from reaching its target site (Karunaratne *et al.,* 1993). This type of resistance mechanism requires the presence of large amounts of the esterases to be effective. Results of this study, however only show a 1.2 fold increase in esterase activity in the selected cohorts. Other studies show 150 times increased esterase activity in organophosphate resistant *Culex quinquefasciatus* laboratory colony (Raymond *et al.,* 1989). In this study selection with bendiocarb also resulted in a significant increase in GST levels. A 1.9 fold increase in GST activity was measured in the $F_{12}$ bendiocarb selected cohorts. Involvement of GSTs was confirmed by synergist studies using DEM. Higher mortality rates (93.2%) occurred when the selected strains were synergized with DEM before exposure to 0.1% bendiocarb. Incomplete synergism by DEM indicates that involvement of other resistance mechanisms cannot be ruled out. However, suppression of GST activity by DEM might indicate a role for GSTs in carbamate tolerance in the tested strain. Involvement of GSTs in carbamate resistance has never been recorded in mosquitoes, with these enzymes better known to mediate resistance to organophosphates, organochlorines and pyrethroids (Li *et al.,* 2007). In southern Africa, bendiocarb resistance in *An. arabiensis* has been associated with insensitive AChE (Casimiro *et al.,* 2006b). Further investigations need to be carried out to ascertain the role of elevated GSTs in response to bendiocarb selections in this population.

Based on results of this study it can be concluded that *An. arabiensis* from southern Africa developed resistance to permethrin and tolerance to bendiocarb. This knowledge

regarding the level and mechanisms of resistance can be used to decide effective vector control methods and resistance management strategies. For example use of carbamates offers an alternative strategy for malaria vector resistance management. The absence of altered Ace genes also favours the use of organophosphates. The current practice of the usage of DDT to spray traditional houses should be maintained as it also helps for the elimination of *An. funestus,* a vector which has been shown to be resistant to pyrethroids.

This study also showed that presence of insecticide resistant in *An. arabiensis* did not result in the failure of the malaria control programme as in 1996-2001. This can be due to a number of reasons. Firstly, presence of other interventions such as effective case management to reduce parasitemia levels might be reducing transmission. Furthermore, *An. arabiensis* is not as an effective malaria vector in South Africa as *An. funestus*. This latter species is highly anthropophagic and insecticide resistance in this species resulted in a major malaria outbreak in South Africa during 1996-2001(Hargreaves *et al.,* 2000). The impact insecticide resistance in South African *An. arabiensis* on malaria transmission is minimal as this species are both anthropophagic (human feeding) as well as zoophagic (animal feeding) and will rest both indoors as well as outdoors.(endo- and exophylic).

## 4.5 Manuscripts published from this Chapter

**Mouatcho, J. C., Munhenga, G., Coetzee, M., Brooke, B. D., Hargreaves, K., and Koekemoer, L. L.** (2009) Pyrethroid resistance in a major African malaria vector, *Anopheles arabiensis*, from Mamfene, northern KwaZulu/Natal, South Africa**.**

# CHAPTER 5

# P450 GENE EXPRESSION STUDIES

## 5.1 Introduction

Biochemical and synergists studies have shed some light on metabolic based resistance mechanisms in Anophelines (Penilla *et al.,* 1998; Brooke *et al.,* 2001; Casimiro *et al.,* 2006b). These two techniques have been used to identify the main detoxification enzyme(s) families responsible for metabolic based insecticide resistance. Understanding specific roles of individual genes in resistance is vital. Technological advances have enabled the understanding of metabolic resistance at a molecular level. Microarray analysis and qPCR has shown that pyrethroid resistance in most mosquito species is mediated by up-regulation of one or more P450 genes (Li *et al*., 2007). In *An. gambiae* a number of CYP genes belonging to four different families have been associated with pyrethroid resistance (Nikou *et al.,* 2003; David *et al.,* 2005; Muller *et al.,* 2007a, b; Djouaka *et al.,* 2008). Although monooxygenases are considered to be an important metabolic mechanism conferring pyrethroid resistance in the *An. arabiensis,* there are limited studies which have been done at molecular level (Muller *et al.,* 2007b).

This section details comparison of six cytochrome P450s previously implicated in pyrethroid resistance in *An. gambiae* and *An. arabiensis*. The primary objective was to identify P450 genes over expressed in an *An. arabiensis* colony from South Africa

artificially selected for permethrin resistance. Proteins encoded by these genes could be promising candidates for permethrin-metabolism.

## 5.2 Material and Methods

Permethrin susceptibility of KWAG-Perm $F_{12}$ and KWAG-base colonies were first confirmed by exposing 100 females mosquitoes of each cohort to 0.75% permethrin using the standard WHO protocol as outlined in Appendix IIA. Quantification of expression levels for each of the six P450 genes was in a CFX 96 real time PCR machine as detailed in section 2.12.5. The 18S ribosomal RNA served as an internal control to account for differences in initial cDNA and reaction efficiency. All reactions were performed three times in triplicate sets (10 mosquitoes per triplicate). The cycling conditions used are detailed in Table 2.3. Fold over expression was calculated using CFX programme (Biorad) which is based on the comparative cycle threshold method described by Pfaffl (Pfaffl, 2001). Statistical difference in expression levels was analysed using REST 2008 (Corbett Life Sciences). During optimisation reactions it became evident that the ribosomal gene S7, commonly used for normalisation, varied in expression between KWAG-Perm $F_{12}$ and KWAG-base making it unsuitable as a reference gene. To address this problem six candidate genes commonly used as reference genes were assessed for their potential use as internal controls to normalise for expression of P450 genes of interest in permethrin selected and unselected cohorts using the procedure outlined in section 2.12.4.3.

## 5.3 Results

### 5.3.1 Level of permethrin susceptibility

WHO susceptibility tests carried out on cohorts of adult females of baseline and permethrin selected cohorts (KWAG-Perm $F_{12}$) are presented in Figure 5.1. Mortality after 1 hour exposure to 0.75% permethrin showed that the selected cohorts (KWAG-Perm $F_{12}$) were resistant to permethrin (42% mortality, n = 100) while the baseline colony showed an average mortality of 97.8% (n = 100).

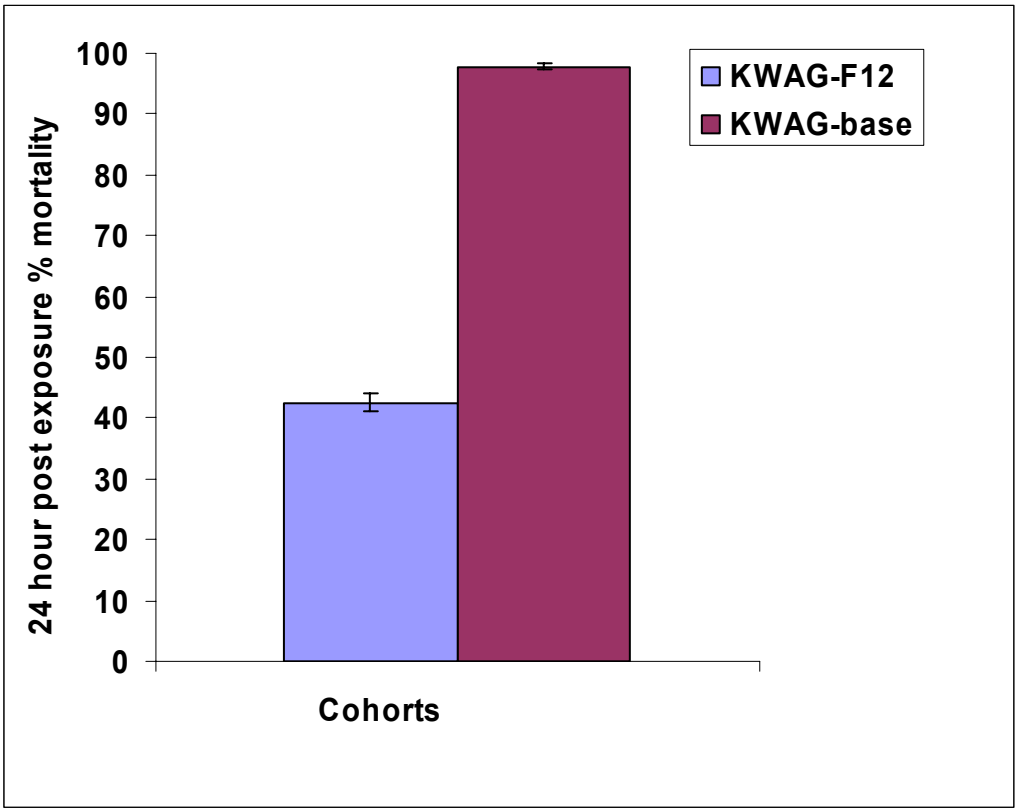



**Figure 5.1:** WHO susceptibility results for *An. arabiensis* cohorts analysed for gene expression studies.

### 5.3.2 Optimization of real time reactions

Quantification of genes was dependent on the accurate optimisation of each reaction. Accurate gene quantification was also dependent on the production of a standard curve with a slope between -3.3 to -3.8 reaction efficiency of between 90% and 105% (http://www.Qiagen.com). In all cases melt curve analysis was done to avoid detection of non-specific products such as primer dimers which have direct effect on the accuracy of the quantification procedure. Figure 5.2 shows an example of a well optimised reaction for CYP6Z1 gene quantification. The slope for the standard curves for CYP6Z1 and 18S was -3.37 and -3.369 while the efficiency was 98% and 98.1% respectively. The melting curve analysis of the CYP6Z1 PCR as well as the 18S PCR revealed only one specific product (Figure 5.3). To evaluate the correct reference genes to be used general expression levels of each candidate gene were calculated directly using mean real-time PCR threshold cycle (Cq) values in both selected and baseline colonies. When the Cq values of the candidate reference gene were statistically different between the selected and baseline colonies they were discarded (Table 5.1). Of all the genes tested 18S was the most suitable housekeeping gene. There was no statistical difference in general expression level between baseline and selected colonies.

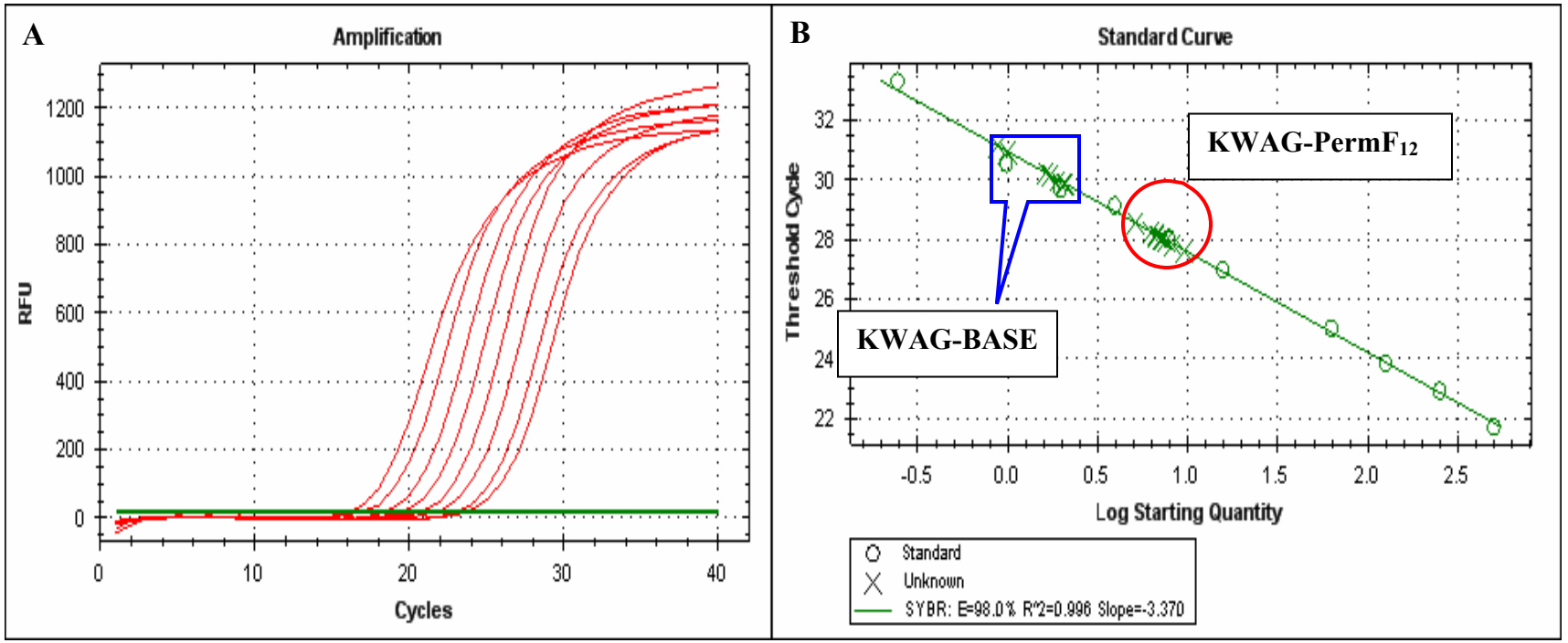



**Figure 5.2**: **A**) raw acquisition of fluorescence data profile for serial dilutions of cDNA amplified with CYP6Z1 primer set, **B**) standard curve based on serial dilution of cDNA amplified with CYP6Z1 primers showing clear distinct difference in CT values between resistant (KWAG-Perm $F_{12}$) and susceptible (KWAG-base) samples (RFU = relative florescence units, standard = cDNA sample serially diluted from 80ng to 0.076ng, unknown = target cDNA samples to be quantified).

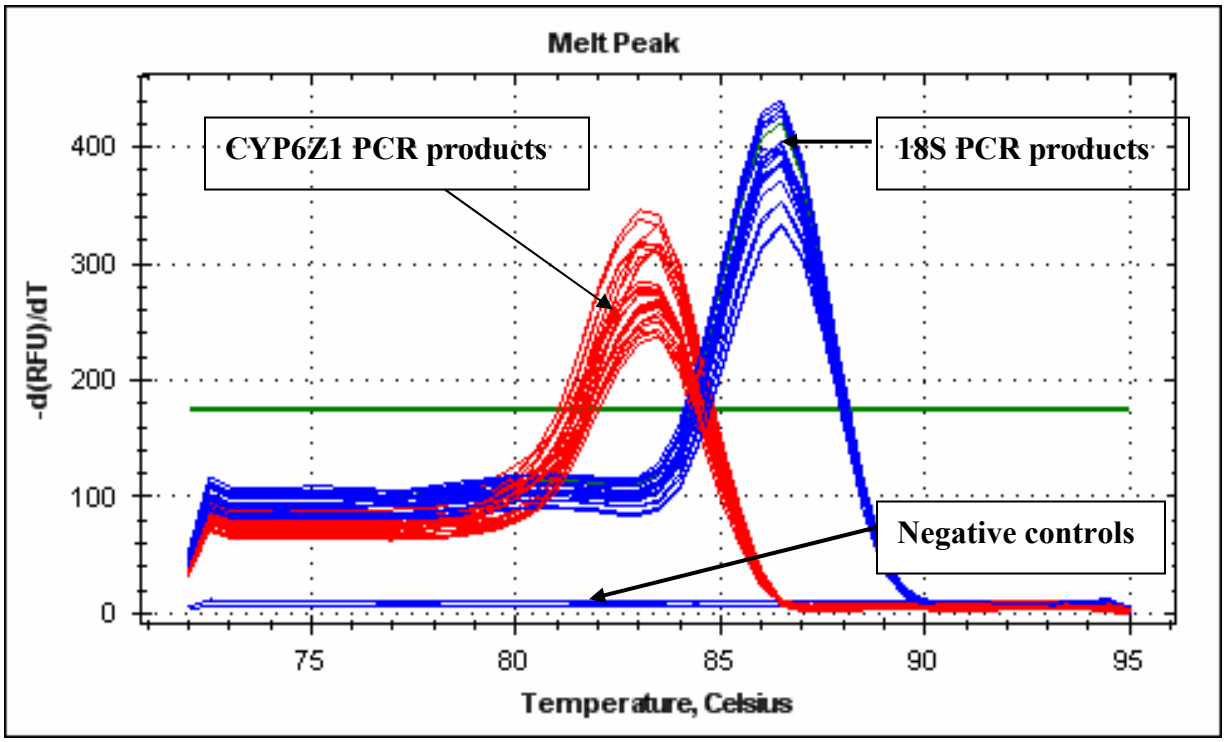



**Figure 5.3:** Melting curve analysis for CYP6Z1 gene expression. Fluorescence data were converted to derivative melting curves by plotting the negative derivative of the fluorescence with respect to temperature vs. temperature [-(dF/dT)vs.T] showing two distinct PCR peaks at 83.5ºC and 86.5 ºC visible for the target (CYP6Z1) and reference gene (18S) respectively. The negative control shows no visible PCR product.

**Table 5.1**: General expression levels of candidate reference genes in *An. arabiensis* KWAG-Perm $F_{12}$ and KWAG-base colonies.

| Candidate reference gene | KWAG-Perm $F_{12}$ [Cq (mean ± SE)] | KWAG-base [Cq (mean ± SE)] | P value |
|---|---|---|---|
| Bactin | 29.7 ± 0.368 | 23.9 ± 0.133 | 0.000 |
| 18S rRNA | 11.8 ± 0.111 | 12.0 ± 0.121 | 0.052 |
| tbp | 36.6 ± 0.485 | 32.9 ± 0.121 | 0.000 |
| gadph | 25.1 ± 0.352 | 18.4 ± 0.182 | 0.000 |
| S7 | 25.6 ± 0.225 | 18.1 ± 0.086 | 0.000 |

### 5.3.3 P450 expression profile

Figure 5.4 shows the qPCR analysis comparing constitutive expression of six P450 genes in the permethrin selected strain (KWAG-Perm $F_{12}$) with the parental baseline cohorts (KWAG-base). Results showed that four P450 genes, CYP6Z1, CYP6Z2, CYP6M2 and CYP4G16 were over-expressed in the selected cohorts. CYP6Z1 was up-regulated in the selected colonies with a relative fold expression of 4.667 and there was a statistically significant difference in the expression level between the parental base and permethrin selected colonies (P = 0.000). CYP6Z2 was up-regulated in the selected colonies with a relative fold-over expression of 1.718, P = 0.002. There was no statistical difference (P = 0.372), in the expression levels of CYP6Z3 between the selected and parental baseline colonies. CYP6M2 was up-regulated in the selected colonies by a fold over of 2.244, P = 0.000. CYP4G16 was up-regulated in the selected colonies by a fold over of 1.388, P = 0.05. Expression levels of CYP6P3 in the selected and baseline colonies were not statistically different P = 0.798.

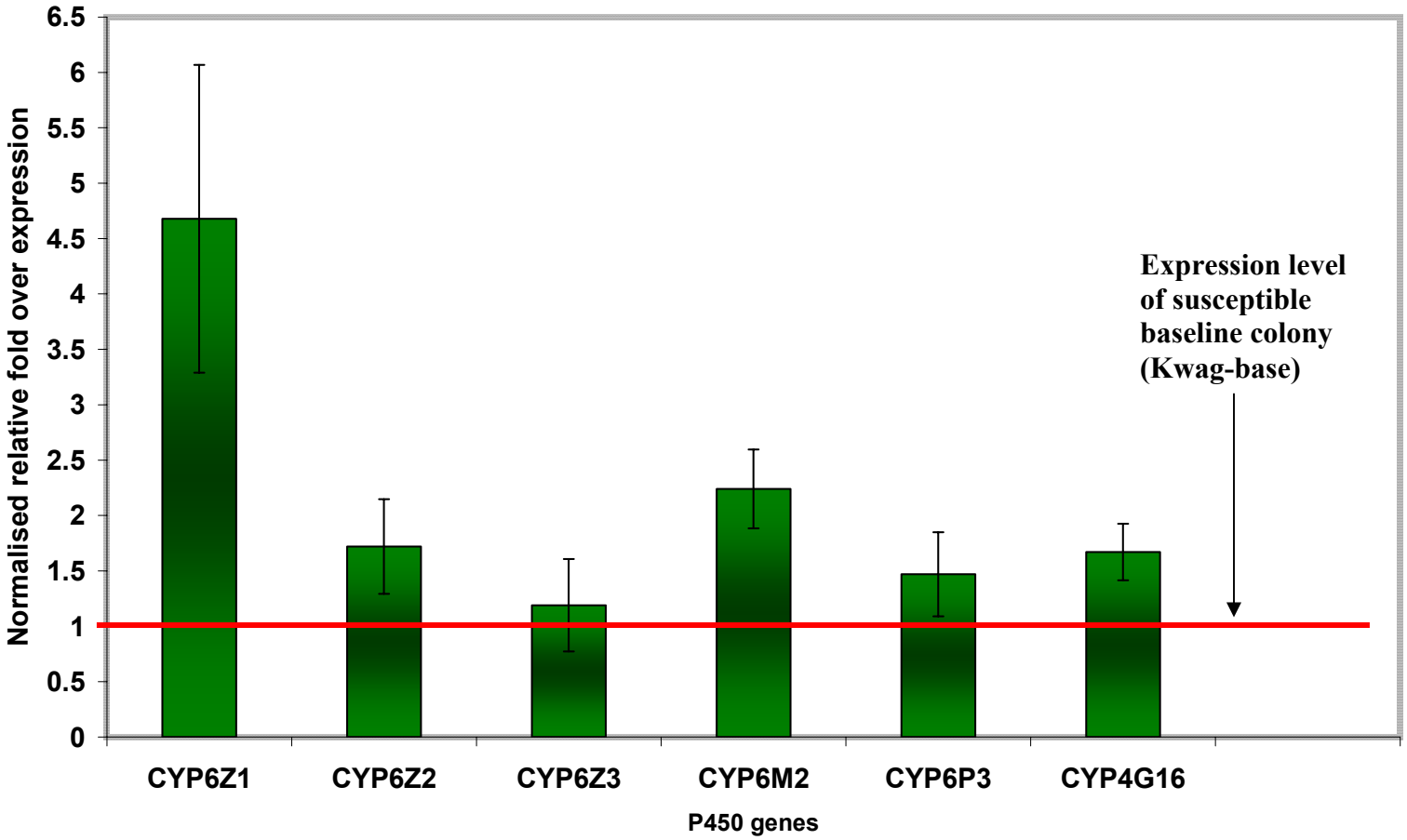



**Figure 5.4:** Constitutive expression of six P450 genes in permethrin selected *An. arabiensis* selected strain (KWAG-Perm $F_{12}$) normalised to 18S ribosomal RNA in susceptible (base) and resistant (selected) adult females. Data are presented as mean ± SE.

## 5.4 Discussion

Cytochrome P450 consists of a superfamily of enzymes that are capable of metabolizing a diverse range of exogenous and endogenous compounds inclusive of insecticides. In *An. gambiae* s.s, P450-mediated pyrethroid resistance is associated with overexpression of one or more P450 genes in the CYP families 4, 6, 12 and 325 (Nikou *et al.,* 2003; Muller *et al.,* 2007a; Djouaka *et al.,* 2008). Biochemical and synergistic studies of the permethrin selected colonies (KWAG-Perm F12) implicated elevated levels of monooxygenases activity (See Chapter 4).

In the present study permethrin susceptibility tests carried out simultaneously on parental and permethrin selected cohorts showed that there was a difference in susceptibility levels between the two cohorts. According to the WHO criteria KWAG-Perm $F_{12}$ is resistant to permethrin while the parental colony showed reduced susceptibility.

We investigated six reference genes that represent different functional classes and gene families for use as controls to normalise our qPCR data. These six genes, 18S rRNA (Morse *et al.,* 2005), rpl8 (Frost and Nilsen, 2003), gapdh (Nishimura *et al.,* 2006), tbp (Nishimura *et al.,* 2006), S7 (Muller *et al.,* 2008) and bactin (Morse *et al.,* 2005), have been validated previously for use as reference genes in many experimental systems. Gene quantification in mosquito work has been using S7 to normalise expression levels. However, none of the published studies provided information on the validation of this gene prior to its use. Our data clearly show that the expression level of S7 is affected when using colonies selected for permethrin resistance. Therefore, 18S rRNA was chosen as the reference gene in this study.

We identified four P450 genes, CYP6Z1, CYP6Z2, CYP6M2 and CYP4G16, whose expression levels were up regulated in a permethrin resistant line compared to the unselected baseline. The significant variation in expression levels of these four genes between the selected and parental cohorts suggest that one or several of these proteins may have roles in permethrin metabolism. Besides, three of these genes CYP6Z1, CYP6Z2 and CYP6M2 are located within a major quantitative trait locus (QTL) on chromosome arm 3R which has been associated with conferring pyrethroid resistance in *An. gambiae* (Ranson *et al.,* 2002; Nikou *et al.,* 2003; Ranson *et al.,* 2004). Two genes, CYP6Z3 and CYP6P3, did not show any significant difference in expression levels between the selected and parental cohorts. The exact role of the above mentioned genes in pyrethroid metabolism needs to be clarified.

CYP6Z1 recorded the highest fold-over increase in expression. The selected cohorts showed 4.7 fold over expression of CYP6Z1 compared to the parental colony. The strong overexpression of CYP6Z1 reported in this study is consistent with previous studies. CYP6Z1 has been recorded over-expressed with a quantitative ratio of 3.5 fold in adult females of a permethrin resistant laboratory (RSP) strain of *An. gambiae* collected in West Kenya (Nikou *et al.,* 2003). This was confirmed by David *et al.* (2005) using microarray analysis. To confirm that this gene is involved in insecticide resistance, it is essential to demonstrate that it is able to metabolize pyrethroids. Recent catalytic studies have shown that CYP6Z1 is capable of metabolising both DDT and carbamates (Chiu *et al.,* 2008), indicating its potential diverse role in insecticide detoxification. However, its role in pyrethroid resistance was not clarified in this study.

CYP6Z2 was significantly over-expressed in the selected cohorts by 1.7 fold. This gene has been previously found over expressed in permethrin-resistant adult males and females of *An. gambiae* from southern Ghana (Muller *et al.,* 2007a). A similar P450 in houseflies CYP6D3 with 44% amino acid sequence identity with CYP6Z2 is over-expressed in permethrin resistant strains (Kamiya *et al.,* 2001). Catalytic activity studies carried out by Mclaughlin *et al.* (2008) using CYP6Z2 isolated from a permethrin-resistant *An. gambiae* strain showed that this gene is capable of binding to both permethrin and cypermethrin but was not able to metabolise these pyrethroids. The same gene was however able to metabolise other xenobiotic compounds (Mclaughlin *et al.*, 2008). Mclaughlin *et al.* (2008) postulated that this could be due to the structural constraint within CYP6Z2 active site which prevented metabolism of these two pyrethroids. However, it could be possible that the absence of cytochrome $b_5$ in their functional enzyme system prevented metabolism of the two insecticides. Studies in other insect species have shown that some CYP genes confer pyrethroid resistance via alternative routes where cytochrome b5 is required to promote pyrethroid metabolism (Feyereisen, 2005). The exact role of CYP6Z2 in pyrethroid resistance in this permethrin selected cohorts needs further exploration.

Results of this study showed a 2.2 fold increase in CYP6M2 expression level in the selected colony compared to the parental colony. CYP6M2 has been previously associated with pyrethroids resistance in a colonised strain of *An. gambiae* from West Africa, Ghana (Muller *et al.,* 2007a) and field caught permethrin resistant *An. gambiae* (Muller *et al.,* 2008). Recently, Djouaka *et al.* (2008) demonstrated that the same gene is

over-expressed by a factor of 2.5 fold in a field resistant *An. gambiae* population sampled from southern Benin compared to susceptible populations sampled from the same geographical area. Involvement of this P450 gene in pyrethroid resistance in *An. gambiae* is now a subject of interest. Characterisation of *An. gambiae* CYP6M2 catalytic activity is currently underway (Muller *et al.,* 2008). The orthologous gene to CYP6M2 in other insect species, CYP6N3 in *Aedes albopictus,* CYP6BB1 in *Ochlerotatus sollicitans* and CYP6A5 in *M. domestica*, have not been implicated in pyrethroid resistance (Muller *et al*., 2007a; Zhu and Liu, 2008).

The fourth gene found over-expressed in this study was CYP4G16. Although the fold over expression was only 1.39 it was statistically significant. Recent studies in Cameroon showed that expression levels of this gene differed between *An. arabiensis* samples collected before the spraying season and after the spraying season. CYP4G16 expression level was higher in samples collected after the spraying season, indicating their potential role in pyrethroid resistance (Muller *et al.,* 2007b). The expression level in this study was only 1.39, compared to 4.5 fold recorded by Muller *et al*. (2007b). These finding differ with those of Strode *et al.* (2006). In their study, CYP4G16 was found consistently expressed in all life stages in *An. gambiae*. Strode *et al.* (2006) suggested that this might indicate a role in housekeeping or biosynthesis pathway rather than specialized detoxification pathway such as insecticide detoxification. Studies in other insect species show contrasting results. Orthologs of CYP4G16 in Drosophila, CYP4G15, has been shown to be localised to insect's nervous system suggesting they play a role in detoxification of endogenous compounds rather than xenobiotics metabolism (Maibeche-

Coisne *et al.,* 2000). In *Blatella germanica,* CYP4G19, an ortholog of *An. gambiae* CYP4G16 is five-fold over-expressed in pyrethroid resistant adults compared to susceptible strains (Pridgeon *et al.,* 2003). Independent records of overexpression of CYP4G16 in two different geographical locations might indicate possible association of this gene in pyrethroid resistance in *An. arabiensis.* This strengthens the theory that CYP genes can rapidly expand and have diverse functions in different insect species depending on ecological niches (Ranson *et al.,* 2002).

Two P450 genes, CYP6Z3 and CYP9P3 were expressed similarly in the permethrin selected and parental colonies. CYP6Z3 in *An. gambiae* is in the same cluster of CYP genes implicated in pyrethroid resistance; its expression is pronounced in earlier life stages and has not been associated with resistance in adults (Nikou *et al.,* 2003; Strode *et al.,* 2006). However, recently Muller *et al.* (2007a) found CYP6Z3 to be significantly elevated in permethrin resistant *An. gambiae* males from southern Benin. This highlights the complexity of functions of CYP genes in insect species. *Anopheles gambiae* CYP6P3 has been associated with pyrethroid resistance in a field population from Dodowa, Ghana (Muller *et al.,* 2008). Metabolic studies in the same strain showed that this gene was capable of metabolising both permethrin and deltamethrin in a NADPH regeneration system, highlighting its possible role in pyrethroid resistance (Muller *et al.,* 2008). Contrary, the present study showed that CYP6P3 is not associated with permethrin resistance. Similarly Muller *et al.* (2007b) showed the same gene to be down regulated in pyrethroid resistant *An. arabiensis* populations from Cameroon. This probably indicates

that this gene does not play a role in pyrethroid resistance in *An. arabiensis* further highlighting the complexity of functions of CYP genes in different insect species.

# CHAPTER 6

## GENERAL DISCUSSION AND CONCLUSIONS

Malaria in southern Africa remains the most important insect transmitted disease despite decades of concerted effort to control it. In addition to case management, use of insecticides is the major weapon in the fight against malaria in most southern African countries. Unfortunately, the emergence of insecticide resistance in countries of the region is threatening the efficacy and sustainability of insecticides for malaria control (Hargreaves *et al.,* 2000; Masendu *et al.,* 2005; Casimiro *et al.,* 2006a, b; Coleman *et al.,* 2008). Sustainability of insecticide based control strategies can only be achieved if proper resistance management programmes are in place. Resistance management programmes are only possible if vector dynamics and mechanisms underlying the phenotype are elucidated. Regular monitoring for the spread of insecticide resistance alleles forms part of a resistance management programme.

The present data showed that vector dynamics did not significantly change in the study area since the last anopheline survey carried out in 1999-2002 (Masendu *et al.,* 2005). *Anopheles arabiensis* remains the predominant species in Gokwe. This is in agreement with previous studies done in the same locality (Manokore *et al.,* 2003; Govere, 2003) and also tallies with reports from other southern African countries where *An. arabiensis* remains a vector of major concern (Ntomwa *et al.,* 2006; Coleman *et al.,* 2008; Mouatcho *et al.,* 2009). What was however, unique in this study was increase in proportion of *An.*

*merus* caught indoors. Although the sample size is too small too draw meaningful conclusions, most villagers have sold their domestic animals to try and sustain their daily livelihood. It is possible that due to the decimation of domestic animals *An. merus* is now predominantly feeding on humans. In future, studies to investigate the vectorial role played by this species should be carried out.

The sympatric occurrence of *An. arabiensis* with non-vectors such as *An. quadriannulatus* and *An. longipalpis* highlights the importance of species-specific identification in malaria vector monitoring programmes. In fact it has now been realized that if the current efforts to eliminate malaria is to be achieved emphasis should also go towards equipping National Malaria Control Programmes with reliable species identification tools to avoid channeling resources to non-vector species. In fact in this study *An. longipalpis* a species of little malaria epidemiological importance had been initially morphologically identified as *An. funestus* an efficient malaria vector. Recently a similar incident occurred when samples collected from Malawi were morphologically identified as *An. funestus* but turned out to be a completely new species after detailed molecular investigations (Spillings *et al.,* 2009). This further highlights the importance of species-specific identifications and remains the biggest challenge facing most malaria control programmes across Africa.

*Anopheles arabiensis* was the only species found infected with *P. falciparum* confirming its role in malaria transmission in Gokwe. There was conspicuous trend in the infectivity results. Only *An. arabiensis* caught indoors (n = 35) were infected despite the fact that

outdoor catches (n = 523) outnumbered indoor catches. This raises interesting questions on the population structure of *An. arabiensis* from Gokwe. It could be that *An. arabiensis* from this locality is sub-structured into populations that are either adapted to feeding and resting indoors or feeding and resting outdoors. We recommend detailed studies on the population genetic structure of indoor and outdoor catches to shed light on this peculiar observation. If population structuring is present, it will have a major impact on vector control programmes. Population structuring in relation to anthropophily is an important factor which determine approach to vector control.

There was an increase in mosquito *P. falciparum* infectivity over the two study periods which can be attributed to a number of reasons. Firstly, the NMCP is alternating DDT with pyrethroids and in this study there was evidence of resistance to both insecticides. It could be possible that resistance observed in *An. arabiensis* might be playing a role in increased infectivity rates. However, these results are not conclusive as we failed to obtain reliable malaria case data over the two year study period to compare parasitemia levels. Another contributing factor might be the collapse of the health system due to current economic challenges facing the country. IRS has been very erratic, resulting in some areas being sprayed late into the malaria transmission season. During the 2008 collection period most areas were yet to be covered by the NMCP spraying team. A vigilant epidemiological survey is recommended as an increase in infectivity rates in vector species is serving as a warning of a pending malaria epidemic.

This study provides the first record of pyrethroid resistance in a malaria vector species in Zimbabwe. Although we do not have evidence of epidemiological impact of this resistance it has potential implications for malaria control considering that pyrethroids, deltamethrin and lamdacyhalothrin are used interchangeably with DDT for malaria vector control in Zimbabwe. It is also a cause of concern for regional neighboring countries such as South Africa and Mozambique as the resistant populations might migrate to these countries.

The study showed the importance of continual insecticide resistance monitoring in vectors species. This is clearly illustrated by the difference in susceptibility to DDT between the two collection periods. Patterns of insecticide resistance may vary considerably in time and it is therefore important to have an active entomological surveillance system as part of a malaria vector control programme. From the surveys detailed here, resistance to permethrin was consistently recorded while the *An. arabiensis* population showed complete susceptibility to organophosphates and carbamates. We therefore recommend an operational change in insecticide application regime. DDT usage should be maintained but alternated with bendiocarb instead of pyrethroids to avoid continual selection of high levels of pyrethroid resistance which might compromise malaria control operations. A close cooperation between Ministry of Health and Ministry of Agriculture is strongly advised inorder to synchronise classes of insecticides being used in time and space.

Detailed study of the underpinning insecticide resistance mechanisms in both wild caught and laboratory colonies selected for permethrin and bendiocarb resistance confirmed that pyrethroid resistance in *An. arabiensis* from southern Africa is metabolically mediated with monooxygenases and esterases playing a central role. A low level of bendiocarb resistance in *An. arabiensis* has been previously recorded in Mozambique (Casimiro *et al.,* 2006b). However, data from this study suggests that there is complete absence of carbamate resistance in populations from Zimbabwe and it proved impossible to select for bendiocarb resistance in the South African population. Partial sequencing of the sodium channel gene and AChE gene showed absence of both the novel *kdr* mutations and insensitive acetylcholinesterase in *An. arabiensis* from Gwave, Zimbabwe and KZN, South Africa. This detailed knowledge about enzyme groups conferring insecticide resistance is highly important in implementing resistance management strategies. Synergist studies and enzyme assays showed that monooxygenases and esterases are the most important enzyme group associated with insecticide resistance in *An. arabiensis* from South Africa and Zimbabwe. Unfortunately almost all insecticides classes recommended for vector control are more or less detoxified by general esterases and monooxygenases. This highlights the importance of finding new alternative insecticides with a totally different mode of action. There are current efforts to come up with insecticides with novel modes of action. However, in the absence of such alternatives the best strategy is the rotation of insecticides and continual monitoring of susceptibility levels. This was successfully implemented in Mozambique where continual monitoring of resistance prompted a change over to DDT in 2006, since then bendiocarb resistance levels have gone down (Coleman *et al.,* 2008).

Gene profiling done in this study showed that over expression of four P450 genes appear to be associated with permethrin resistance in a laboratory colony of *An. arabiensis* from KZN, which has been artificially selected for permethrin resistance. Each of these P450 genes associated with pyrethroid resistance in this study has been previously associated with pyrethroids resistance in *An. gambiae* and *An. arabiensis* (Nikou *et al.,* 2003; Muller *et al.,* 2007b; Muller *et al.,* 2008). Each of these studies recorded different combinations of P450 with a maximum of two P450 genes being associated with pyrethroid resistance. This shows that any resistant population may contain a unique set of detoxification genes. Therefore, a P450 gene of pyrethroid resistant strains can not be extrapolated from one species to another or between different populations of the same species.

In conclusion, *An. arabiensis* remains the main malaria vector in Gwave and results provide a first instance of permethrin resistance in *An. arabiensis* from this locality. The study confirmed that mutation specific PCR assay developed to detect L1014F and L1014S *kdr* mutations is unreliable. The selections done in the laboratory gave an insight of the resistance mechanisms conferring permethrin resistance in *An. arabiensis* from KZN, which is a metabolic based resistance mechanism. Elevation of monooxygenases and general esterases are the main enzyme classes involved. These results provide information on P450 genes that may be useful in future studies on the possible role of this group of enzymes in permethrin resistance. Further studies to elucidate all possible genes which might be playing a role are highly recommended.

# APPENDIX I

## Preparation of chemicals and solutions

### A) PCR solutions

**TAE** (Tris Acetic EDTA) Buffer 50X (pH 8)

- 242g Tris
- 37.2 g $Na_2$ EDTA.$2H_2O$
- 57.1M glycial acetic acid
- Make up to 1 L

**2.5% Agarose gel**

- Dissolve 10g agarose in 400ml 1X TAE
- Mix and boil in microwave until solution is clear
- Cool for 5 mins
- Add 12µl Ethidium bromide
- Pour into trough

**Grinding buffer**

- 0.08M NaCl
- 0.16M Sucrose
- 0.06M EDTA
- 0.5% SDS
- 0.1M Tris-HCl

**TA (Tris EDTA) Buffer** (Sambrook *et al.,*1989**)**

- 100ml 1M Tris (pH 7.4)
- 20ml 0.5M EDTA
- Make up volume to 1L.

### B) Sporozoite ELISA solutions (Wirtz *et al.,* 1987)

**Phosphate Buffer Saline (PBS) 10X**

- Dissolve 1 tablet in 1 litre of distilled water and adjust pH to 7.4

Each 1 Litre contains

- 80g NaCl
- 2g KCl
- 2g $KH_2PO4$
- 19g $Na_2HPO4.7H2O$

**Blocking Buffer (BB)**

- 2.5% Casein
- 50ml 0.1N NaOH
- 450ml 0.01M PBS pH 7.4
- 0.002% Phenol red
- Suspend casein in NaOH and bring to boil.
- After casein is dissolved, slowly add the PBS and cool.
- Adjust pH to 7.4 using HCl
- Add phenol red.

**Phosphate Buffer Saline Tween 20 (PBS-Tween 20**)

- 500µl Tween 20 plus 1 litre 1 X PBS

**Grinding Buffer (BB-Nonident P-40)**

- 50ml BB plus 250 µl NP-40

## C) Biochemical Assays buffers

**1) 0.1M $Na_2HPO_4$ pH 7.4**

- Dissolve 14.2g $Na_2HPO_4$ (Sigma S-0876) into 1 litre $dH_2O$ (dibasic).
- Dissolve 12g $NaH_2PO_4$ (Sigma S-0751) into 1 litre $dH_2O$ (monobasic).
- Add monobasic to dibasic until a pH of 7.4 is reached.

**2) 0.05M $Na_2HPO_4$ pH 7.4**

- Add 350ml 0.1M $Na_2HPO_4$ to 350ml $dH_2O$.
- Adjust pH to 7.4 by adding monobasic.

**3) 0.02M $Na_2HPO_4$ pH 7.2**

- Add 100ml 0.1M $Na_2HPO_4$ to 400ml $dH_2O$.
- Adjust pH to 7.2 by adding monobasic.

**4) 0.1M $Na_2HPO_4$ pH 6.5**

- As in 1 but adjust pH to 6.5

**5) 0.1M $Na_2HPO_4$ 5% Sodium dodecyl sulphate (SDS- Sigma BDH 30175) pH 7.0**

- As in 1 and adjust pH to 7.0
- Add 25g SDS per 500ml buffer

**6) 0.1M $Na_2HPO_4$ pH 7.8 1% Triton**

- As in 1 and adjust pH to 7.8
- Add 1`ml Triton per 100ml buffer

**7) 0.25M sodium acetate ($NaC_2H_3O_2$ ) buffer pH 5.0**

- Add 10.2537g $NaC_2H_3O_2$ ( Sigma S-2889) and make up to 500ml using $dH_2O$
- Adjust pH to 5.0 using HCl

**8) 0.0625M $K_2HPO_4$ pH 7.2**

- Add 2.7219 $K_2HPO_4$ (Sigma P-8281), make up to 250 ml using $dH_2O$ (dibasic).
- Add 2.1265g $KH_2PO_4$ (Sigma P-5379), make up to 250 ml using $dH_2O$ (mono).
- Add monobasic to dibasic until a pH of 7.2 is reached.

**9) 30M α- Naphthyl acetate and β-Naphthyl acetate**

- 0.055g α or β NA in 10ml acetone

# APPENDIX II

## Standard Laboratory Methods

### A) *Anopheles gambiae* complex PCR protocol (Scott *et al.,* 1993)

One leg per specimen was dissected and placed in a micro centrifuge tube: 12.5µl of a master mix containing 10X PCR reaction buffer; 1.25mM 10X dNTPs; 25mM $MgCl_2$; 1.65pmol *An. quadriannulatus* species A and 3.3pmol each of *An. gambiae s.s*, *An. merus*, *An. arabiensis* and universal primers (Table 2.1); 4.9µl deionised water and 0.5 units of *Thermus aquaticus* (*Taq)* DNA polymerase enzyme were added. The micro centrifuge tubes were centrifuged for 10sec at 13,000 rpm to release template DNA. The reaction mixture was amplified in a thermal cycler (Primus 96, MWG Biotech) using the following thermal profile: 30 cycles consisting of 94ºC denaturation for 30 sec, 50ºC annealing for 30 sec, and 72ºC extension for 30 sec and a final autoextension step at 72ºC for 5 minutes. Ten microlitres of the amplified products were loaded onto a 2.5% agarose gel, stained with ethidium bromide (10mg/ml), submerged in 1X TAE buffer and subjected to electrophoresis at 100 V for 1 hour 30 minutes or until bromophenol blue was 3cm from origin to allow for proper separation of amplicons. Each gel contained four 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 documented in Syngene G-box sydr4/1152, gel documentation system. The expected fragment length for each species is as indicated on Table 2.1. The fragment lengths were determined by comparing amplicons to a 1 kilo base (kb) molecular marker (O'Gene ruler$^{TM}$, Fermentas Life Sciences. Cat no SM1153).

## B) *An. funestus* group PCR protocol (Koekemoer *et al.,* 2002)

Amplification was done in a 12.5µl reaction volume containing 8.25 pmol each of the following primers *An. vaneedeni*, *An. leesoni*, *An. parensis*, *An. funestus*, *An. rivulorum* and a universal primer (Table 2.1); 1.5mM $MgCl_2$, 200µM of each dNTP and 0.75 units of *Taq* DNA polymerase. The volume was made up to 12.5µl with double distilled water. Thermal cycling was carried out in Primus 96, MWG Biotech PCR machine with the following thermal profile: denaturation at 94ºC for 30 sec, annealing at 50ºC for 30 sec and extension at 72ºC for 30 sec. Each cycle was repeated 40 times followed by a final extension at 72ºC for 5 minutes. Ten microlitres of the amplified products were loaded onto a 2.5% agarose gel, containing ethidium bromide, submerged in 1X TAE buffer and subjected to electrophoresis at 100 V for 1 hour 30 minutes or until 1kb marker has separated properly. Positive controls consisted of DNA of *An. vaneedeni, An. leesoni, An. parensis, An. funestus s.s* and *An. rivulorum* while the reaction mixture without DNA was used as the negative control. The gel was photographed in a gel documentation box (Syngene G-box, sydr4/1152). The amplicon sizes were compared to a 1 kilo base DNA ladder (O'Gene ruler™, Fermentas Life Sciences. Cat no SM1153).

## C) *An. longipalpis* group PCR protocol (Koekemoer *et al.,* 2009)

The *An. longipalpis* multiplex PCR assay is used to distinguish two molecular types of *An. longipalpis*, type A and C was used to identify two specimens. Type A identifies *An. longipalpis* from South Africa and Type C correlates to specimens from Zambia (Kent *et al.,* 2006). Three primers, A3, C1 and a universal primer were used (Table 2.1). These primers are specific for Type A and C respectively. PCR conditions were as follows:

Initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 45°C for 30 seconds, 72°C for 30 seconds; and extension at 72°C for 10 minutes. 25 µl PCR mixture contained the following: 2.5µl of 10X reaction buffer, (100mM Tris-HCl pH 8.3, 500mM KCl), 200 µM of each dNTP, 1.5 mM $MgCl_2$, 6.6 pmol per primer, 0.5 units Taq DNA polymerase.

## D) *Plasmodium falciparum* infections (ELISA) (Wirtz *et al.,* 1987)

To determine infectivity, wells of a disposable polyvinyl chloride, flat bottomed microtiter plates (NUNC A/S, Denmark) were coated with a 0.200µg/50 µl volume of monoclonal antibody *P. falciparum* 2A10[Pf2A10] (Cat. No. 37-00-24-2, Kirkegaard and Perry Laboratories, Maryland, USA) and covered with aluminium foil paper and incubated overnight at 4ºC. The following day the plate contents were aspirated and washed three times with PBS-Tween (PBS pH 7.4 with 0.05% Tween 20) the plates were then blocked with blocking buffer and held for 1 hour in darkness at room temperature (23-26ºC). After aspiration of the blocking buffer, 50µl aliquots of mosquito triturate were added to the respective wells. The first well of the microtitre plate, was designated for the positive control and last seven wells had negative controls in all cases. Negative controls consisted of seven 50µl aliquots of triturate of laboratory-reared uninfected *An. arabiensis*. A positive control; (Pf 2+ (Cat. No. Pf-PC Washington DC, USA)) prepared at 100pg/50 µl of BB consisted of a synthetic peptide standardised against *P. falciparum*. The plate was covered with aluminium foil for two hours, after this incubation well contents were aspirated and the plates washed twice with PBS-Tween 20, (PBS-Tw) before the addition of 9 mg / µl 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 of freshly prepared ABTS peroxidase substrate (2,2'-azino-di-3 ethyl-benzthiazoline) (Cat. No.50-60-18, Gaithersburg, Maryland USA) was added to each well. After 30 minutes of incubation in the dark, plates were read at 405 nm using an ELISA plate reader (Labsystems Multiskan RC, Genesis software version 3.03).

## E) Biochemical assays (Penilla *et al.,* 1998)

### (i) Homogenate preparation

One to three days old individual mosquitoes from the $F_1$ generation of isofemale lines and or insecticide selected cohorts were transferred by means of forceps into a flat bottomed microtitre plate on ice. These were homogenised with plastic pestles in 50 µl of distilled water. The pestle was then rinsed with 200 µl of distilled water so that a total volume of 250 µl homogenate was obtained per well. A laboratory strain of *An. arabiensis* from Kanyemba (KGB) in Zimbabwe, susceptible to all insecticides, was used as a reference sample on all plates and was prepared as above. Negative controls consisted of wells containing only reagents without mosquito homogenate. The homogenate were left overnight for crude particles to settle at the bottom of the plate.

### (ii) Monooxygenase assay

The titration level of monooxygenases (cytochrome $P^{450}$) was determined as an end point assay by measuring the haem content of individual mosquitoes. The haem content of an

insects is used an indirect measure of $P^{450}$since the majority of haem is associated with cytochrome $P^{450}$ in non-blood fed insects.

Using homogenate of individual mosquitoes, two, 20µl replicates of homogenate was transferred into separate wells of a microtitre plate, following which 80µl of 0.0625M 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 0.25M sodium acetate buffer (pH 5.0) added to each of the replicates. Finally 25µl of 3% hydrogen peroxide was added to each well. Each plate was incubated at room temperature (23-26 ºC) for one hour before optical density was read at 650nm using a plate reader (Labsystems Multiskan RC, Genesis software version 3.03). Negative controls were run with 20µl of buffer instead of the insect homogenate.

**(iii) General esterase assay**

This assay is a direct end point measure of general esterases activity. In mosquitoes elevated esterases involved in resistance are generally active with two substrates alpha and beta naphthyl acetate. Therefore these two substrates were used to positively identify elevation of esterases in resistant mosquitoes.

Using the prepared homogenate, two 20µl replicates were pipetted into separate wells of a microtitre plate, 200µl of alpha-naphthyl acetate solution [130µl of 30mM alpha-naphthyl acetate (NA) dissolved in 13ml of 0.02M sodium phosphate buffer (pH 7.2)] was added to one duplicate and 200µl of beta naphthyl acetate solution [130µl of 30mM

beta-naphthyl acetate also dissolved in 13ml of 0.02M sodium phosphate buffer (pH 7.2)] was added to the other. After incubating on ice for 30 minutes, 50µl of: 0.023g fast blue salt dissolved in 2.25ml distilled water plus 5.25ml 5% sodium lauryl sulphate (SDS) dissolved in phosphate buffer (pH 7.0) were added to each well. A blank containing 20µl distilled water, 200µl of alpha-NA or beta-NA solution and 50µl of stain were also placed in separate wells. Optical density was read at 570nm as an end point.

### (iv) GST assay

The activity of GST an enzyme normally involved in detoxification of DDT was assayed kinetically. Using duplicates of each homogenate, 10µl was placed in each well of a 96 well microtitre plate. This was followed by addition of 200µl of: 10mM reduced glutathione prepared in 0.1M sodium phosphate buffer pH 6.5 and 65mM 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 10µl of distilled water with 200µl 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 five minutes at 340 nm.

### (v) Acetylcholinesterase assay

This was carried out as fixed point assay. The principle is to detect the presence of an altered acetylcholinesterase following inhibition by the carbamate propoxur.

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 0,1M 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 2ml of 0.1 phosphate buffer pH 7.0)] was added to each replicate. Finally, 25µl of acetylthiocholine iodide (ASCHI) was added to one replicate while 25µl of: ASCHI + 5µl propoxur was added to the other replicate. A control was set up with blanks without any insect homogenate. The optical density was read using the Multiskan plate reader at 570nm after 5 minutes of incubation.

### (vi) 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 inorder to establish a correction factor that could be used to adjust for the difference in sizes in the other assays.

Using a microtitre plate 2 x 10µl replicates per mosquito were placed in separate wells and 300µl 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 10µl of distilled water and 300µl of BIO- Rad solution. The reaction was read on a plate reader (Labsystems Multiskan RC, Genesis software version 3.03) at 570nm after incubation for five minutes.

## F) Molecular assays

### i) DNA Extraction (Collins *et al.,* 1987)

Individual mosquitoes were homogenized with sterile plastic pestles in 200µl of grinding buffer. (80mM sodium chloride; 160mM sucrose; 50.8mM EDTA pH 8; and 130mM Tris chloride pH 8), incubated at 70ºC for 30 minutes then 28 µl of 8M potassium acetate was added to each. The reaction mixture was then thoroughly mixed by inverting the epperndorf tubes before incubating on ice for 60 minutes. After incubation, samples were centrifuged at 13,000 rpm for 30 minutes at room temperature (23-26ºC). Aliquots of the supernatant containing suspended DNA was transferred into fresh tubes and incubated at -20ºC for 1 hour followed by centrifugation at 13,000 rpm for 60 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 13,000rpm for 15 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol and air dried for 5 minutes at room temperature (23-26ºC). The pellet was then resuspended in 200 µl of 1xTE.

# APPENDIX III

Ethical clearance certificate

# APPENDIX IV

## Published manuscript I

**Munhenga, G., Masendu, R.H., Brooke, B.D., Hunt, R.H., and Koekemoer, L.L.**

(2008) Pyrethroid resistance in the major malaria vector *Anopheles arabiensis* from Gwave, a malaria-endemic area in Zimbabwe. *Malaria Journal* **7**: 247.

## Contribution:

I carried out field work, species-specific identification, ELISAs, biochemical assays, interpretation of all results and wrote the first and subsequent drafts of the manuscript.

# APPENDIX V

## Published manuscript II

**Mouatcho, J. C., Munhenga, G., Coetzee, M., Brooke, B. D., Hargreaves, K., and Koekemoer, L. L.** (2009) Pyrethroid resistance in a major African malaria vector, *Anopheles arabiensis*, from Mamfene, northern KwaZulu/Natal, South Africa**.**

## Contribution:

Joel Mouatcho and I equally contributed to this manuscript. I carried out all the laboratory selections, biochemical assays of selected cohorts, interpretation of results and co-authored the first and subsequent drafts of the manuscript.

# APPENDIX VI

## Technical report

N. Lukwa, V. Gunguluzi, A. Makuwaza, L. Chibarinya, T. Dube, C. Kasambare and **G. Munhenga.** Supporting the National Malaria Control Programme through bioassays and insecticide resistance testing. *Zimbabwe National Institute for Health Research Technical Report 2008.*

## Contribution:

I conceived the project, carried out field work, helped in the interpretation of results and contributed to the subsequent writing of the report.

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