IMPLEMENTATION OF BIOCHEMICAL AND MOLECULAR TOOLS FOR RESISTANCE DETECTION, MONITORING AND MANAGEMENT OF MALARIA VECTORS IN SOUTHERN AFRICA

by

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DECLARATION

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DEDICATION

To my late parents Nkadi Welhelminah and Tsokolo Ernest Mohloai and Dr. Christopher Green. May their souls rest in peace.

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ABSTRACT

The study aimed to establish entomological baselines for operational insecticide resistance management programmes in African countries, with particular reference to southern Africa.

A small-scale malaria control programme at an expatriate area and local village at the Shell Rabi oil field in Gabon was used to develop the skill base in setting entomological baselines while also assessing intensive mosquito control operations conducted by a commercial company. Non-specific esterases conferring resistance to Organophosphates (OPs) and some pyrethroids and elevated Glutathione-S-Transferases (GSTs) conferring resistance to DDT and some pyrethroids were detected within local *An. gambiae* s.s. Results indicated that malaria control operations in Gabon could be improved and budgets reduced by discontinuing fogging, and by reverting to either indoor residual spraying (IRS) or providing pyrethroid impregnated bednets (ITNs). Combined with a robust monitoring system, this would allow operations to be targeted to those households or facilities where the highest levels of malaria transmission were occurring.

The programme was then expanded out to the national malaria programmes in a number of countries in the southern Africa region, where prior to this study, there was little or no expertise to assess possible insecticide resistance in malaria vector populations, despite the reliance on indoor residual house spraying and, increasingly, on insecticide treated bed nets for malaria vector control. In order to develop a vector insecticide resistance monitoring and management programme for the region, training of participants chosen from the three malaria control programmes in Botswana, Swaziland and KwaZulu/ Natal (KZN), South Africa and from two malaria research institutes in Mozambique and Zambia was undertaken in laboratory techniques, susceptibility testing, mosquito collections and field and insectary mosquito breeding techniques.

For all mosquito collections biochemical assays were performed on individual 1-3 day old adult F1 progeny for altered acetylcholinesterase (AChE), GSTs, esterase and monooxygenase-based resistance mechanisms. *Anopheles gambiae* and *An. funestus* complex mosquitoes were identified to species level using the PCR method. 'Resistant' and 'susceptible' kdr alleles were identified by two comparable methods (HOLA and PCR): both techniques gave reproducible results.

WHO discriminating dose assays showed pyrethroid resistance in Mozambique, cross-resistance between DDT/ permethrin in KZN and cross-resistance between DDT/deltamethrin at Fiwale, Ndola, in Zambia. Altered AChE conferring resistance to OPs and carbamates was found in all the three major malaria vectors in southern Africa. The presence of non-specific esterase-based pyrethroid and OP resistance mechanism was widespread in this region. An elevated monooxygenase-based mechanism was also present in all vector species. High levels of GST activity were detected in all study areas. Kdr-type based resistance mechanism conferring cross-resistance to DDT/pyrethroids were only detected in *An. gambiae* s.s at Fiwale, Ndola, Zambia.

Finally, an Ecosystem approach to health research was successfully implemented in KZN, South Africa. To facilitate this, a multidisciplinary forum was established to identify how communities perceive linkages between pesticide usage, vector resistance and malaria. Along with the vector resistance baselines established during this PhD, this has laid the groundwork for the next decade of insecticide based malaria control activities in southern Africa.

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LIST OF ABBREVIATIONS

ARC-PPRI	Agricultural Research Commission Plant Protection Research Institute
BHC	Benzene hexachloride
CGIAR	Consultative Group on International Agricultural Research
DBL	Danish Bilharziasis Laboratory
DDD	2, 4-dichlorophenoxy acetic acid
DDE	Dichloro-diphenyldichloroethylene
DDT	Dichloro-diphenyltrichloroethane
DFID	Department for International Development
DMC	Chlorfenethol
GIS	Geographic Information System
GPS	Global Positioning System
GABA	γ-amino butyric acid
GST	Glutathione S-transferase
HCH	Hexachlorohexane
ICIPE	International Centre of Insect Physiology and Ecology
ICRAF	International Council for Research in Agroforestry
IDRC	International Development Research Centre
IGR	Intergenic Region
INHEM	National Institute of Hygiene, Epidemiology and Microbiology
IPGRI	International Plant Genetic Resources Institute
IPT	Intermittent Presumptive Therapy
ITNS	Insecticide Treated Nets
IWMI	International Water Management Institute
IRS	Indoor Residual Spraying
KZN	KwaZulu/Natal Province
LC	Lethal Concentration
LD	Lethal Dosage
LT	Lethal Time
NGP	Non-Govermental Organisation
MIM	Multi-lateral Initiative on Malaria
MRP	Malaria Research Programme
MRU	Malaria Research Unit

MSS	Malaria Survey Section
OPs	Organophosphorus insecticides
PCR	Polymerase Chain Reaction
PB	Piperonyl Butoxide
PRA	Participatory Rural Appraisal
QTL	Quantitative Trait Loci
SAWRC	South African Water Research Commission
SIMA	Systemwide Initiative on Malaria and Agriculture
SNP	Single Nucleotide Polymorphism
TEPP	Tetraethyl pyrophosphate
UNEP	United Nations Environmental Programme
USAID	U.S. Agency for International Development
UV	Ultra Violet
WARDA	Africa Rice Centre
WRC	Water Research Commission
WW II	World War II
RF IHD	RockeFeller Foundation International Health Division

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

1.1.1 Malaria in Africa

Approximately 300 million people worldwide are affected by malaria with more than 90% of the disease occurring in Africa (WHO 1995). Malaria parasites are transmitted from one person to another by the female anopheline mosquito. The disease is still common throughout the warmer parts of the world, despite widespread and often intensive efforts at eradication. There are at least 380 species of anopheline mosquito, 60 of which are able to transmit malaria parasites. The larval and pupal stages of all mosquitoes are aquatic. Each species has its preferred breeding grounds, feeding patterns and resting places (Gilles and Warrell 1993). Their sensitivity to insecticides in different parts of the world is also highly variable and in many countries poorly documented. This study was undertaken to document potential insecticide resistance in Southern African mosquito vectors in countries, where little or no insecticide resistance testing had been done previously, and help establish an effective resistance management programme.

1.1.2 Transmission and Historical Distribution of Malaria

Malaria is caused by protozoan parasites of the genus *Plasmodium* comprising four species namely, *P. falciparum, P. malariae, P. ovale* and *P. vivax*. The most common of these species in Africa is *P. falciparum*. The parasite initially develops in the gut of the mosquito and is passed on to a new host after a 10 – 14 day incubation period, in the saliva of an infected insect when it takes a new blood meal. The parasites then invade the host's red blood cells where they multiply (Gillies et al., 1995).

Malaria is diagnosed relatively inaccurately by the clinical symptoms of fever, or more accurately by the presence of the parasite by microscopic examination or by Polymerase Chain Reaction (PCR) analysis of the host's blood. It can normally be cured by early use of appropriate anti-malarial drugs. The disease was known for centuries before the true causes were understood (Bruce-Chwatt 1985). Ronald Ross

discovered the parasite in mosquitoes more than 100 years ago (Rajakumar and Weisse 1999). Malaria was originally thought to be caused by "miasma" (bad air or gas) from swamps because of its occurrence in wet areas with high mosquito infestations (Schreiber et al., 1987 in Rajakumar and Weisse 1999). Ancient treatments included an infusion of ginghao (*Artemesia annua*), which has been used for the last 2000 years in China. The antifebrile properties of the bitter bark of *Cinchona ledgeriana* were known in Peru by the 15th century (Bruce-Chwatt 1980). Quinine, the active ingredient from this plant was first isolated in 1820 (Bruce-Chwatt 1980). Control of malaria started after the discoveries of the parasite by Laveran on November the 6th 1880 (Nobel prize in 1907) and the mosquito vector by Ross in 1897 (Nobel Price 1903) (Najera 1990).

The discovery of the insecticide DDT in 1942 and its first use in Italy in 1944 made the ideal of global eradication of malaria seem possible (Najera 2000). This was later developed into a global eradication campaign for malaria by WHO (1957). But the onset of DDT resistance and difficulties in funding and implementing these large scale campaigns through poor public health infrastructures in many countries made eradication impossible (Najera 1990). A malaria control strategy was subsequently adopted aimed at reducing mortality, prevention or control of epidemics, the protection of malaria-free areas, with the ultimate objective of eradicating the disease where it was deemed feasible (WHO 1978a).

The distribution of the disease varies greatly from country to country (Figure 1) and within the countries themselves. For example, in South Africa (Figure 2 and 3), malaria distribution is closely related to conditions that favour the survival of the *Anopheles* mosquito in the form of habitat and breeding sites. The life cycle of the parasite is also temperature dependent, hence climate and environment are very important (Martens 1995).

Distribution of endemic malaria

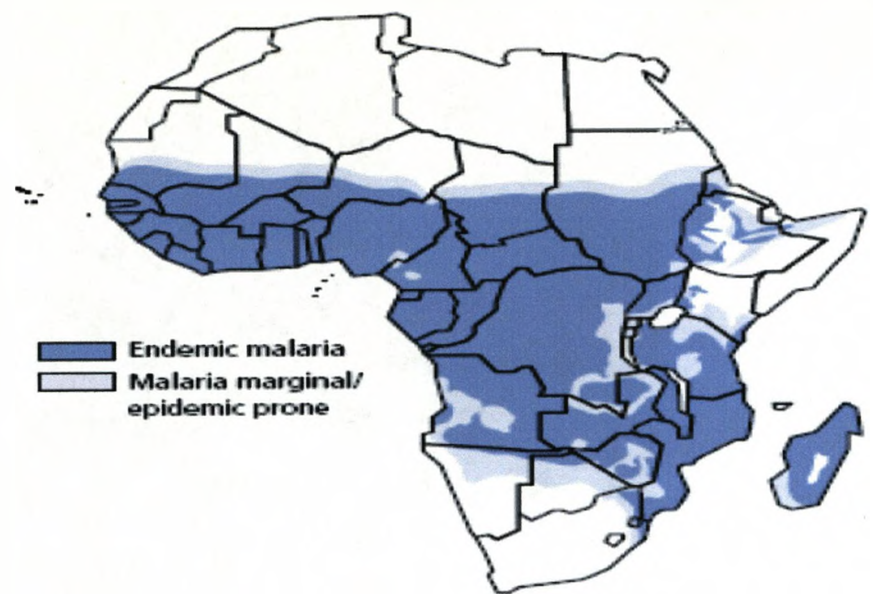


Figure 1. Distribution of malaria in Africa. A theoretical model of distribution of endemic malaria, based on continental long-term climate data. (Figure courtesy of the MARA network, http://www.mara.org.za).

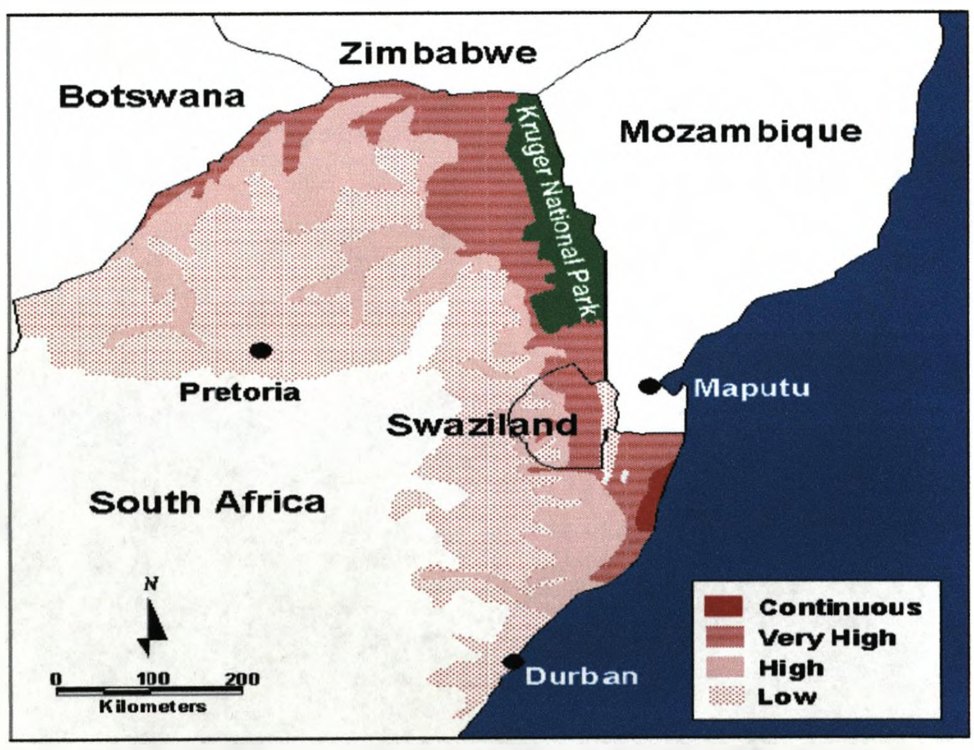


Figure 2. Distribution of malaria in South Africa prior to establishing malaria control. Disease was most severe along the coast of northern KZN (continuous-dark red) and in the hot and humid Lowveld flood plains (very high-light red, high-pink to low-dotted pink). This also shows severity dropping off at higher altitudes, with the Highveld being virtually malaria free (white parts of the country). (Source: http://www.mara.org.za).

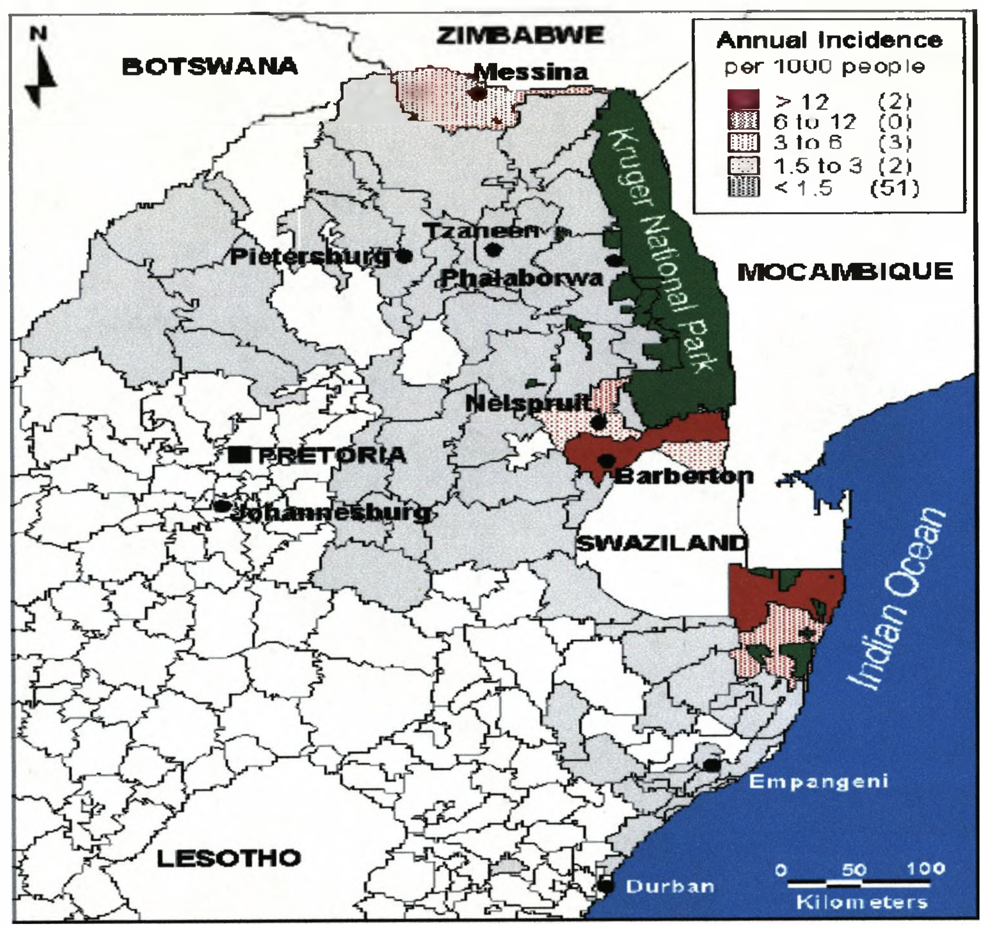


Figure 3. The distribution of malaria in South Africa at magisterial district level between 1987 and 1993. It is evident that high malaria risk areas are associated with corridors or proximity to Mozambique. The Kruger National Park still acts as a malaria buffer between Mozambique and South Africa. (Figure courtesy of the MARA network, http://www.mara.org.za.).

1.2 Malaria Vectors

A detailed understanding of the malaria disease cycle is expected to allow more effective control strategies to be developed (Hay et al., 2004). Studies on vector and parasite biology are required to be able to understand the factors affecting susceptibility of anopheline mosquitoes to human malaria parasite infection. More knowledge on vector and parasite biology is accumulating due to the *Anopheles gambiae* and *Plasmodium falciparum* genome sequences, together with the recent development of more sophisticated molecular tools. This knowledge will help to fine tune the current control strategies.

1.2.1 Molecular Methods for Mosquito Analysis

1.2.1.1 Population Analysis

Many methods have been used to identify and name the mosquitoes responsible for malaria and other disease transmission. Morphological identification of mosquitoes (Gillies and de Mellion 1968; Gillies and Coetzee 1987) has been complemented by molecular (Scott and Lee 1993) and cytogenetic (Coluzzi 1988) tools to better identify members of the *An. gambiae* complex to species level. In addition new molecular tools, for example, using single nucleotide polymorphisms (SNPs), are being developed using the *An. gambiae* genome sequence to facilitate population genetics approaches to study the distribution of genetic variability within and among populations of mosquitoes (Morlais et al., 2004). Molecular tools for genome-wide analysis will help to discover mutations underlying specific traits, which will help to develop new malaria control strategies. This could be in the reduction of the population density and/or longevity of the mosquito, alteration of their anthropophily level and disruption of the malaria parasite cycle in the vector (Morlais et al., 2004).

1.2.1.2 The Anopheles gambiae and An. funestus complexes

Members of the *Anopheles gambiae* Giles and *Anopheles funestus* Giles (Diptera: Culicidae) complexes, are the most important vectors of malaria in Africa (Gillies and De Meillon 1968; Gillies and Coetzee 1987; White 1974; Coetzee et al., 1993). The *An. gambiae* complex is composed of at least six species, namely *An. arabiensis, An. gambiae* s.s, *An. merus, An. melas, An. quadriannulatus* and *An. bwambae*. All these species differ widely in their biological attributes, such as larval habitats, host preference, anthropophagy and endophily (Figure 4). Studies have been conducted throughout much of Africa to understand these differences (Coetzee et al., 2000; le Sueur and Sharp 1988; Sharp and le Sueur 1991). Biting patterns, flight activities and host-seeking behaviour have also been studied in *An. gambiae* s.l (Adams 1940; Smith 1956; Haddow and Ssenkubuge 1962; Shelley 1973; Chandler et al., 1975).

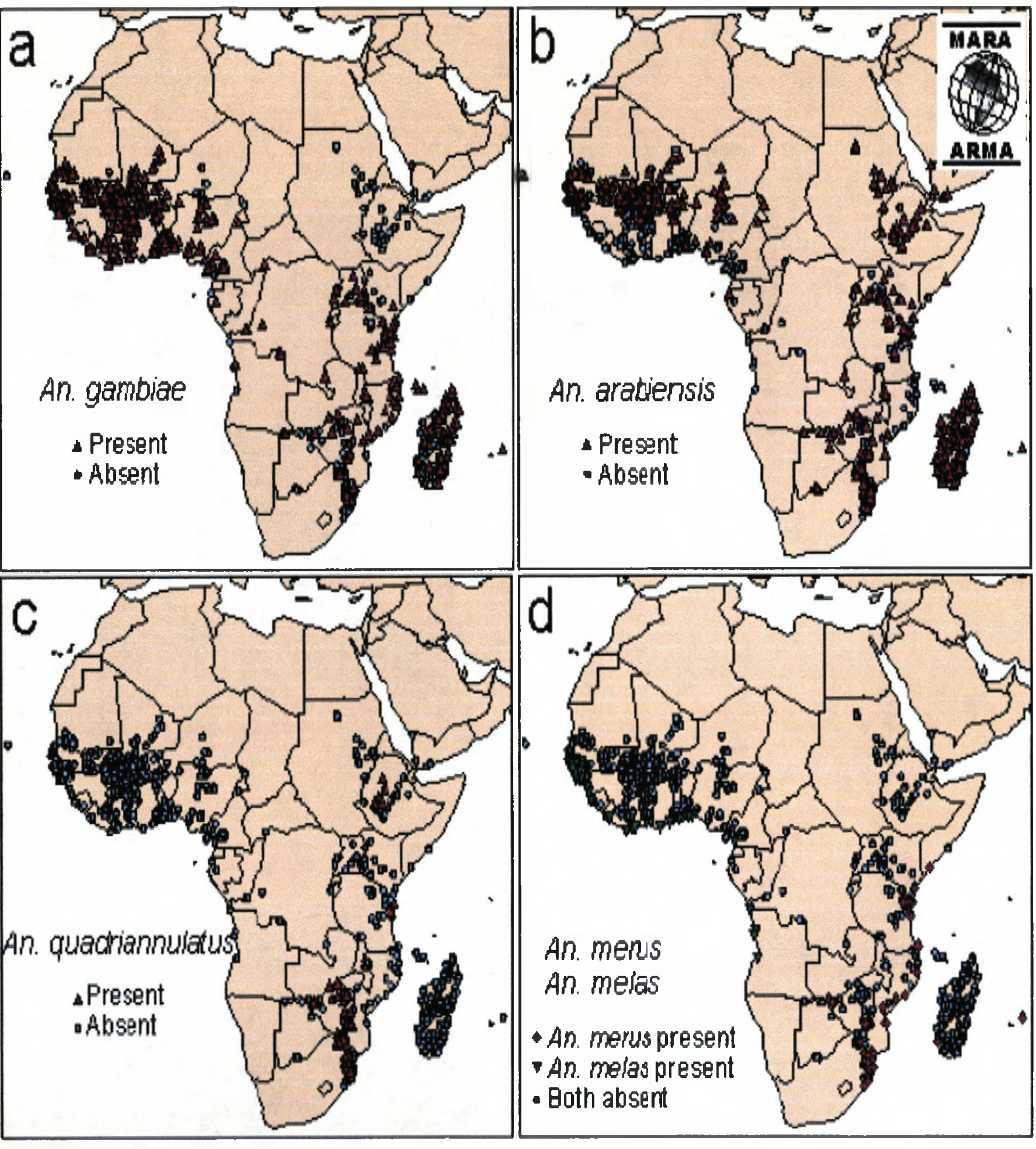


Figure 4. The distribution of the two major African malaria vectors. (4a and b *An. gambiae s.s* and *An. arabiensis*) and the three secondary vectors (Figure 4c and d *An. merus, An. melas and An.quadriannulatus*). (Figure courtesy of the MARA network, http://www.mara.org.za.)

The anopheline most adapted to human biting, with the highest malaria vectorial capacity, is *An. gambiae* s.s. This species can be further split into five taxonomic units, designated by non-Linnean nomenclature as chromosomal forms, namely Forest, Bissau, Bamako, Savannah and Mopti (Bryan et al., 1982; Toure et al., 1983; Coluzzi et al., 1985; Petraca et al., 1983; Appawu et al., 1994). The chromosomal forms, Bamako, Savannah and Mopti are more adapted to dry environments. These three forms are often sympatric and their distributions overlap with that of *An. arabiensis*, the second partially anthropophilic member of the *An. gambiae* complex. The Forest and Bissau forms are found in humid

and coastal areas and probably belong to a single panmictic unit widely distributed in East Africa (Favia et al., 1997). These forms, like all mosquitoes, share a mitotic karyotype with two pairs of autosomes and one pair of sex chromosomes. These can be visualized as polytene chromosomes from the adult nurse cells or larval salivary glands. Figure 5 shows the five polytene chromosome arms and their banding patterns among the different species.

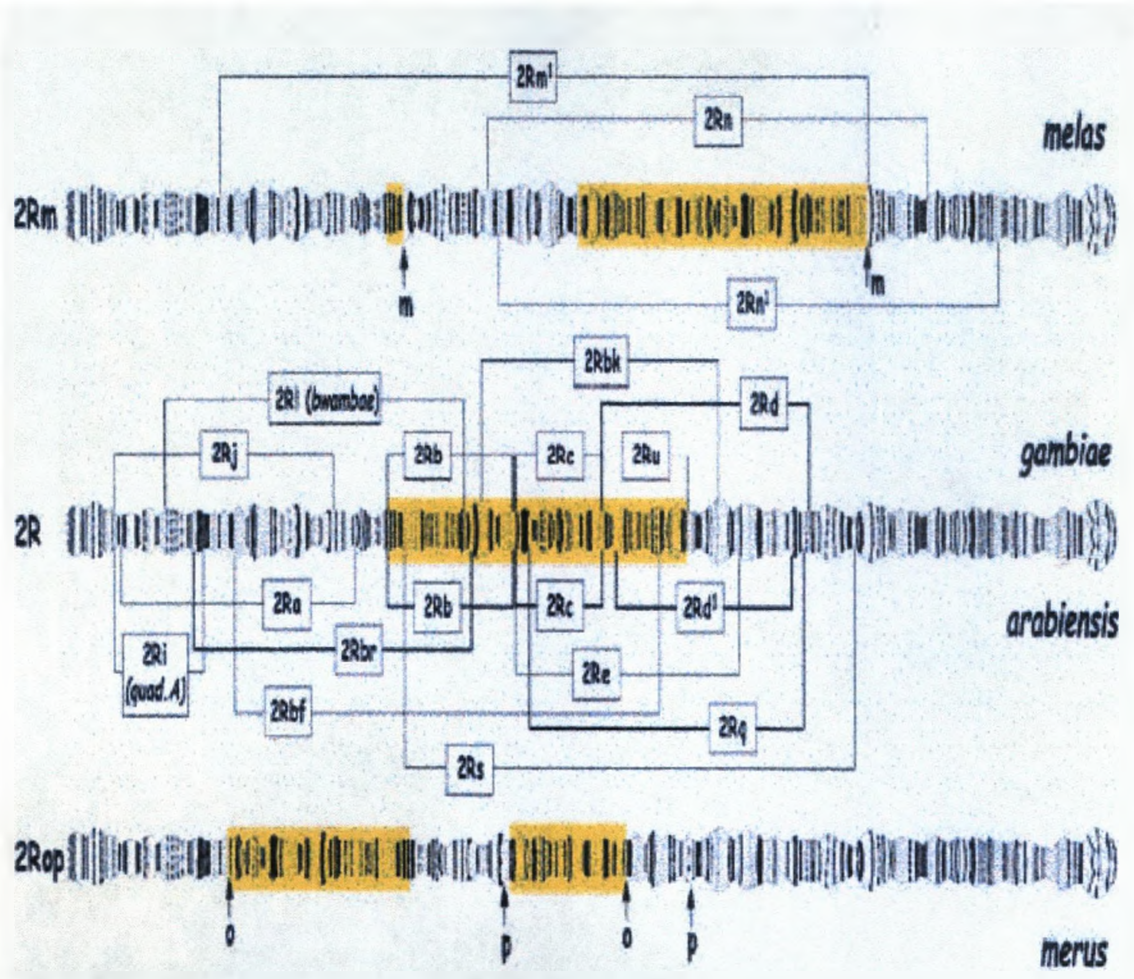


Figure 5. A diagrammatic representation of the different chromosomal inversion polymorphisms found in four members of the *An. gambiae* complex. ↑m indicate the boundaries of the inversion in 2R of *An. Melas* (yellow colour). ↑o and ↑p indicate the boundaries of the overlapping o and p inversions of *An. Merus* (yellow colour). (Source: Coluzzi et al., 2002).

An abundance of paracentric chromosomal inversions is to be found in this complex, ten of which are fixed. These fixed inversions, found as inverted homozygotes in natural populations, are used to differentiate mosquito species within the *An. gambiae* complex (Coluzzi et al., 2002). The two most widely distributed species (*An. gambiae* and *An. arabiensis*) have the highest number of inversion polymorphisms (Coluzzi et al., 1979).

These are followed by *An. melas*, with few occurring in *An. bwambae* and *An. quadriannulatus*. No inversions have been observed in *An. merus*. Figure 6 shows diagrammatically the polytene chromosome relationships among taxa (Coluzzi et al., 2002). An independent speciation processes for *An. melas* and *An. merus* may be supported by this chromosomal differentiation, with *An. gambiae* and *An. arabiensis* being regarded as the most similar species ecologically in view of their closely related adaptation to human environments (Coluzzi et al., 2002).

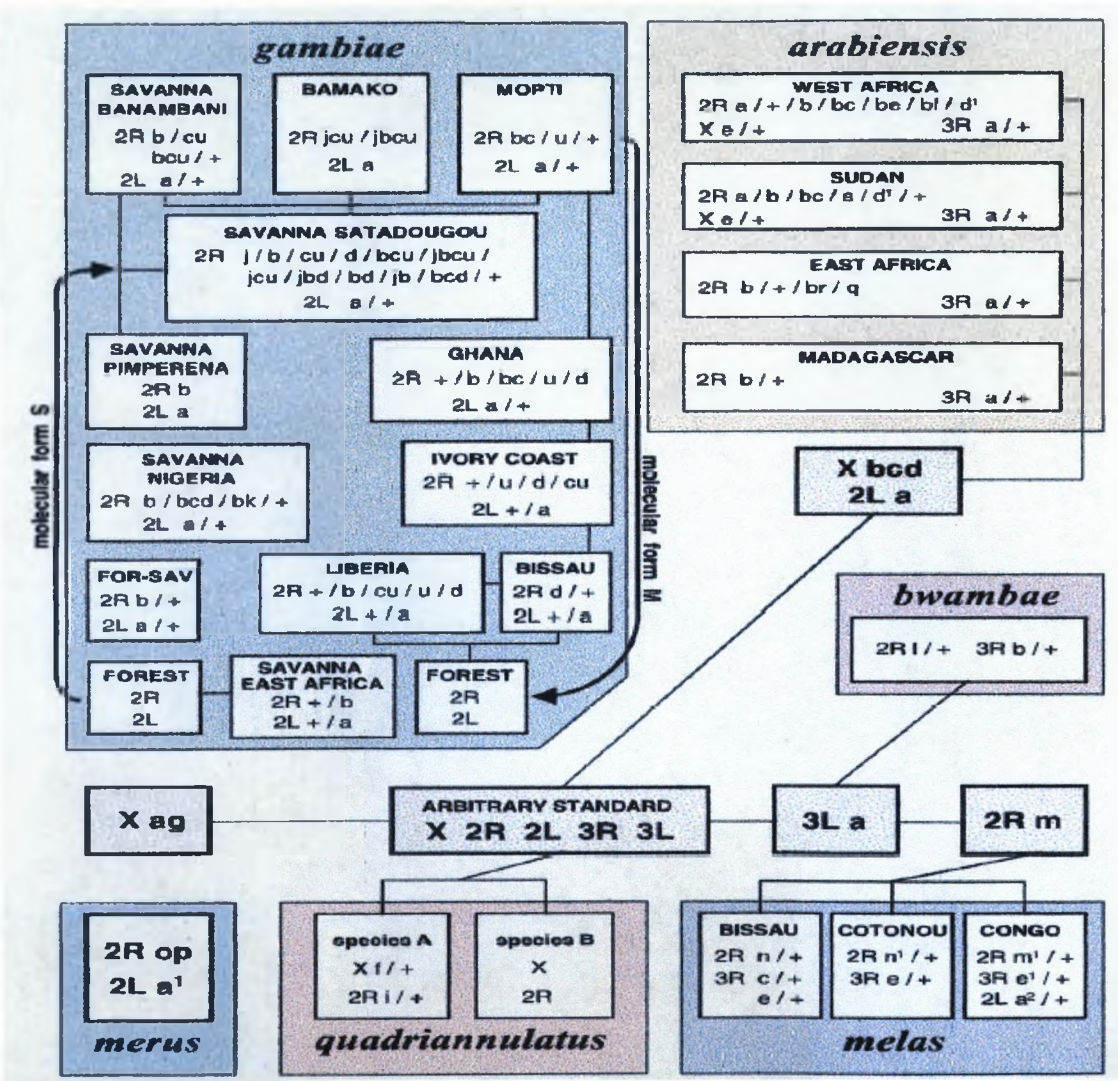


Figure 6. *An. gambiae* chromosomal inversions. The relationships of chromosomal inversions among the sibling species and chromosomal forms of the *An. gambiae* complex. (Source: Coluzzi et al., 2002).

1.3 Vector Biology and Ecology

While most studies have concentrated on the adult mosquito and the parasite, little attention has been given to the aquatic stages of the malaria mosquitoes. Survival analysis of mosquitoes has been based on adults, not aquatic stages (Clements and Paterson 1981). Vector abundance has been correlated with disease risk and this has often been used to model disease transmission. Adult vector density is to an extent dependent on its larval aquatic stages.

The longevity of the aquatic stages dictates the rate of production of adults and hence the intensity of disease transmission (Bayoh and Lindsay 2004). The risk of infection with a mosquito-borne disease, such as malaria, depends critically on the number of vectors per host (Garett-Jones 1964). Larval survival is dependent on a number of factors, including temperature, moisture, nutrient competition, predation and disease. Recent studies by Bayoh and Lindsay (2004), looked at the effect of temperature on the length of the aquatic stages of *An. gambiae* s.s. Temperature was a critical regulator of growth and development for each instar and each life stage, affecting the end of one stage and the beginning of the next and influencing the regulation of the length of the gonotrophic cycle. Bayoh and Lindsay (2003, 2004) had reported studies that suggested an extreme sensitivity to temperature exposure above 40°C during development of immature stages of most species. They showed that in general, life expectancy declined in a linear manner with age, although at temperatures between 14°C and 20°C, survival of older individuals was greatly extended. They found that the maximum life expectancy within this temperature range was approximately 25 days at 16°C. The shortest life expectancies of adults were approximately 2 days at 10°C and 40°C. Neven (2000) reported that as the body temperature of an insect rises, the rates of both metabolism and respiration increase up to a critical thermal limit and that death occurs soon after respiration begins to drop, even if the insect is returned to normal temperatures. This is indicative of systematic cell death at high temperature.

Shelton (1973) reported how larvae that survive high temperatures produce smaller, less successful adults. At high temperatures a large proportion of larvae died at pupation or pupae failed to emerge into adults, a finding attributed to disruption of the highly complex process of metamorphosis. One of the effects of high temperatures on larvae is disruption

of critical mass development for pupation to occur (Clements 1992; Chambers and Klowden 1990). An understanding of the ecology of the vector informs the design of effective malaria control strategies. Larval abundance and distribution are important factors affecting successful control of adults or larvae. Muirhead-Thomson (1951) found that *An. gambiae* s.l larvae develop in freshwater habitats that are small, temporary, clean and exposed to sunlight. These are not the only type of habitats encountered by these vectors. Holstein (1954) claimed that it is difficult to attribute a definite type of breeding place to *An. gambiae* s.l and that this vector species complex can potentially breed in almost any fresh or brackish water body that happens to be available. The larval collection design for use in this study, described in the material and methods section, was based on these observations and the fact that even though *An. gambiae* s.l prefers small sunlit pools, it does not mean that they are restricted to these characteristic breeding places.

An. gambiae s.l was found breeding in urban areas at the Kongola Mines in Zambia and Mamfene village in KwaZulu/Natal (KZN). The village was more urban-like than the surrounding rural areas. The adaptation to more urban situations by *An. gambiae* s.l. was observed by Chinery (1984). Fillinger et al. (2004) point out that the flexibility of this species complex should never be underestimated in operational larval control programmes where high effective coverage is necessitated by high levels of endemicity.

Depinay et al. (2003) produced a simulation model of African *Anopheles* ecology and population dynamics for the analysis of malaria transmission. Their model combines biological and environmental variables and incorporates the egg, larvae, pupae and adult life stages. This model considers five factors, namely temperature, moisture, nutrient competition, predation and disease and lastly dispersal. From their model, it is clear that temperature is the most critical variable in malaria epidemiology, e.g. between 18°C – 26°C, a 1°C change alters the mosquitoes' life span by more than a week.

Fillinger et al. (2004) state that although the range of variation of water temperature is very wide, it is rarely taken into account in the literature. Forty degrees celsius is considered as the upper thermal death point for *An. gambiae* s.l and as Fillinger et al. (2004) report this temperature has been recorded by some authors in small pools. Larval collections were obtained from cattle hoof prints in this study, but temperature recordings were not made. Breeding in temporary pools such as hoof-prints leaves the eggs

susceptible to desiccation as the water evaporates. *Anopheles* eggs cannot survive more than 15 days in dry soil (Depinay et al., 2004).

Swaziland experiences highland malaria transmission, that is, higher than expected from general experience of seasonal malaria due to unusual weather conditions. This also occurs in some parts of KZN, South Africa. Mean temperatures that allow larval survival were recorded at high altitude villages (>1400m) during the cooler months of the year in the Usambara Mountains in Tanzania (Bodker et al., 2003). The low entomological inoculation rate (EIR) in the Usambara Mountains was attributed to low anopheline densities during the cold season.

Highland malaria has serious implications for malaria control in regions where malaria is seasonal like Swaziland and South Africa. Larval populations are maintained at low densities at these cool temperatures and any rise in temperature results in a rapid increase in mosquito abundance. Bayoh and Lindsay (2004) demonstrate how temperatures recorded at meterological stations are usually lower than those experienced by the immature stages in *An. gambiae* s.l in small sunlit pools. If larval habitat water temperatures are used to predict conditions favouring larval development as well as adult mosquito production in highland regions, it is necessary to monitor these temperatures in order to establish the relationship between standard meteorological data and larval breeding sites.

From this literature survey, it is clear that more research is needed to gain insights into vector biology and ecology in Africa. Remote determination and extrapolation of the role of temperature, moisture (which interacts with temperature), along with detailed regional understanding of nutrient competition (minimum weight requirement for the transition from larva to pupa and through its influence on adult weight, that is, the relation of larval weight to fecundity), predation and diseases and dispersal should allow accurate fine tuning of control operations.

Dispersal is defined by Depinay et al. (2003) as the adult female mosquito's movement in space, a critical factor in the insect's life cycle for seeking blood meals and oviposition sites. Little is known in detail about what drives this behaviour in *An. gambiae* s.l., although groups such as Dr. McCall's in Liverpool are looking at the behaviour of

individual mosquitoes. He states that, 'While they have predetermined mechanisms – genetic memory or instinct- there is a degree of flexibility or plasticity within this. Like many animals, the mosquito can be moulded by its environment. If we can find out how, this would lead to a greater understanding of how they find resources and to the design of new ways to prevent or divert their biting behaviour (McCall and Kelly 2002).'

1.4 Malaria Control

The discovery of the role of the *Anopheles* mosquito in the transmission of malaria prompted the goal of mosquito control as a public health measure (de Zulueta 1990). These were the discoveries that led to the eventual implementation of control strategies (Najera 2000). Below is a summary of historical-epidemiological evaluations of several anti-malaria campaigns and the relative contribution of various direct measures employed and indirect factors operating during these campaigns. This is followed by an outline of approaches and factors that are essential or at least useful in successful malaria control programmes.

Organization and co-ordination of anti-malaria efforts, research and understanding of vector biology, accompanied by socio-economic and agricultural development are key factors in malaria control (Kitron 1987; Najera 2000). Human factors include demographic and migration patterns (Kitron 1987). Agricultural practices can affect malaria, by increasing breeding sites, modifying the habitat through deforestation and putting selection pressure on the vectors through insecticide use (Kitron 1987; Najera 2000; this PhD).

1.4.1 History of Malaria Control

The earliest reference to malaria was described by Hippocrates during the 5th century BC (Bruce-Chwatt 1985). Malaria outbreaks were also noted by Plato (427 to 347 BC) and Aristotle (384 to 322 BC) (Schreiber et al., 1987). When reviewing the past malaria interventions based on environmental management and manipulation for malaria control in Asia, the anti-malarial operations at Mian Mir in Punjab, which was later called the Lahore cantonment (Watson 1924), stand out as the malaria intervention strategy that was

analysed in greatest detail. This experimental site for larviciding operations in Mian Mir in 1901 is well documented in the Systemwide Initiative in Malaria and Agriculture (www.iwmi.cgiar.org/pubs/working/WOR47.pdf). It was also referenced in Watson (1924), in his presentation on the observations on malaria control, with special reference to the Assam Tea Gardens, and some remarks on Mian Mir, Lahore Cantonment. The Mian Mir interventions were based on initial observations and preliminary anti-mosquito efforts undertaken at Freetown, Accra and Lagos in West Africa, inspired by the initial vector-control activities in Ismailia, Egypt, by the Suez Canal Company under the guidance of Ross (Boyce 1904 and Stephens 1904 in www.iwmi.cgiar.org/pubs/working/ WOR47.pdf).

The Mian Mir area included an army garrison and scattered traditional, residential and farming areas. Assessment of malaria transmission and identification of potential breeding sites for malaria vectors were included in the baseline data collected in Mian Mir (www.iwmi.cgiar.org/pubs/working/WOR47.pdf). These investigations were designed to determine the burden of malaria among the army personnel. Findings from the baseline entomological investigations showed that *An. culicifacius* was the main malaria vector in Mian Mir (James 1903a) (www.iwmi.cgiar.org/pubs/working/ WOR47.pdf).

The Mian Mir area was characterized by very flat terrain, resulting in very limited natural drainage due to the impervious nature of the soil to water. The nature of the soil made the region susceptible to surface puddles despite its arid nature. *An. culicifacies* was found breeding extensively in the smaller irrigation channels associated with agricultural activities. More breeding sites were formed from the overflow from the watercourses along the channels. The breeding sites were further expanded to surrounding homesteads due to the provision of excessive irrigation water to small gardens. While *An. culicifacies* at that time was not known to be a species complex, these remain the traditional breeding sites of *An. culicifacies* species B, the main malaria vector in Sri Lanka. Rainfall, temperature and irrigation water releases were correlated with seasonal pattern of malaria and the army quarters near the irrigation canals were shown to be at the highest risk of malaria (James 1903a; 1903b in www.iwmi.cgiar.org/pubs/working/ WOR47.pdf). Measures were subsequently implemented to reduce vector breeding sites, which are briefly mentioned in Watson (1924). These included repairing the banks of irrigation channels, clearing these of silt and vegetation to reduce overflow and reducing

depressions next to channels by levelling to remove surface water. Activities included the daily inspection of the ten watercourses near the army barracks during the high transmission season, removal of debris and attempts to reduce pooling. Suspected *anopheline* breeding pools were filled with earth, large pools were emptied with buckets or larvae were killed by the application of kerosene oil during the rainy season of June and July. The identification of potential breeding sites was a continuous process, which was accompanied with superficial small drainage lines created to remove water from large open areas. The environmental modifications and manipulations undertaken in Mian Mir were also complimented with mass distribution of quinine (Christophers 1903; James 1903b in www.iwmi.cgiar.org/pubs/working/ WOR47.pdf).

The results showed that anti-larval measures could affect larval breeding as monitored in the specific habitats. However, there was little effect on adult mosquito abundance within the settlements (Watson 1924). This led to many heated debates around the Mian Mir experiment, as there was no clear consensus of opinion about the beneficial effects of larviciding and environmental management on malaria transmission (Watson 1924). The committee also stated how the problem of malaria control in India was one of great complexity, and that large and costly schemes, as carried out in other countries, were beyond the resources available in India. Malaria today remains a disease of poverty with many effective interventions beyond the means of the poorest communities where transmission is highest.

Ross (1910) in (www.iwmi.cgiar.org/pubs/working/WOR47.pdf) made several contributions to debates on the Mian Mir data. He claimed that the committee did not have a sufficient evidence base for their conclusions, and therefore felt strongly that the experiences in Mian Mir should not determine the future of vector control for all of India. Ross clearly believed that different species of mosquitos must be dealt with in different ways. The experimental design followed at Mian Mir for malaria interventions was seriously criticised by both Ross and Watson. They believed that not enough had been done to prevent vector breeding over a sufficiently large area to actually produce a measurable reduction in vectors within human habitations. This was attributed to a lack of knowledge about the vector behaviour, for example the flight range of the *anopheline* species. Funding was also a limiting factor as the cost of larviciding and environmental management in this format was completely out of proportion with the cost of malaria to

the government. Watson (1924) concluded that the area was never sufficiently drained to achieve mosquito control and that proper outlets should have been constructed from the drainage canals, possibly with pumping devices, to assist the drying of canals.

The Mian Mir experiment is a classic example of an early malaria intervention from which many lessons were learned. It was the first case resulting in a heated debate over the role of irrigation in the spread of malaria and how to combine the necessity of food production and livelihoods with public health (Giles 1904 in www.iwmi.cgiar.org/pubs/working/WOR47.pdf). It is also a good example of moving away from a vertical approach to a horizontal one in addressing malaria interventions. This experiment gave rise to fundamental questions about effective malaria vector control, such as how large an area should be covered to provide protection to human settlements. Information on vector biology and ecology, which helps to design effective malaria vector control strategies, is now available and research is still on going on the optimum role that larviciding and environmental management could play in malaria control.

Another question raised from the Mian Mir experiment was how much an adult vector population had to be reduced to achieve a measurable impact on malaria in human beings. The cost-effectiveness of proposed interventions was central to discussions of the Mian Mir experiences. Similar discussions are currently underway with regard to the cost-effectiveness of bed nets and indoor insecticide residual spraying (Curtis and Mnzava 2001) that now forms the basis for implementation of malaria control strategies in most endemic countries.

The Mian Mir experiment in India shows that malaria epidemiology and therefore its control is dependent on many factors such as ecology and biology of the vectors and parasites involved. The other factors include political economy of a country or region, socioeconomic, demographic and migration patterns, agricultural practices and health services (Kitron 1987). Agricultural practices play an important role in malaria transmission, i.e. by increasing breeding sites and inducing insecticide resistance in the vector (described in detail in Chapter 4). Housing, nutrition, migrations and urbanization are some of the factors influenced by agriculture (Kitron 1987). Kitron also states how education is important to health as it determines the effectiveness of dissemination of information on malaria and its control, and the degree of community participation in

conducting control work (see Chapter 4). In Israel/Palestine, between 1919 and 1925 anti-malarial work was based on extensive surveys of mosquito vectors, mapping of breeding sites, and efficient gathering of data on malaria prevalence and incidence (Kitron 1987).

Three different populations were involved, the indigenous Moslem and Christian populations, who were largely rural and the Bedouins (also Moslems), who were nomads in continuous migration (Kitron 1987). The immigrating Jews settled mostly in towns, but a significant proportion established agricultural settlements in highly malarious areas. Kitron states that in this region, migration played an extremely important role in the epidemiology of malaria. Due to Bedouins' migration patterns, they transported malaria throughout the country. The Jewish immigrants provided a continuous influx of susceptibles into this malaria endemic region. Matters were further complicated by the mass pilgrimages to religious shrines, especially by Moslems, among whom malaria was endemic (Kitron 1987).

In Israel/Palestine, anti-malaria work was undertaken by the Department of Health of the Government of Palestine and two private institutions which were incorporated into the department: the Malaria Research Unit (MRU) and the Malaria Survey Section (MSS) supported by the Rockerfeller Foundation (Kitron 1987). Kitron states that by 1925, the towns of Palestine were comparatively free of malaria through the control efforts of the Department of Health, the malaria research unit, and the malaria survey services in co-operation with landowners.

In 1921, Kligler and Weitzman received external funds to conduct control projects in three highly affected Jewish settlements, to determine whether malaria control was possible with limited means and to tabulate exact data on all factors associated with malaria epidemiology and control (Kitron 1987). Comparisons were undertaken among four control strategies, namely the detection and treatment of infected people, larviciding, quinine prophylaxis, education and propaganda. An essential part of the Jewish anti-malaria campaign was community participation and this was used in all the four above mentioned strategies through popular lectures, distribution of pamphlets, personal talks by physicians during examinations, repeated visits of inspectors to families and health days in schools (Kitron 1987).

The outcome of these four strategies illustrates the fact that health problems, including malaria, that have an impact on human development can not be solved by a single scientific discipline working alone as emphasized in Chapter 4. The four malaria control strategies promoted interactions between different community sectors and led to greater awareness, exchange of information and a joint action in anti-malaria activities. Kitron states that however, anti-malaria and agricultural requirements can conflict if not well managed, e.g., the British decided not to grow rice in the high malarious Huleh area between 1925 and 1948. The anti-malaria work there was based on weekly drainage of the irrigation canals. The Department of Health ordered an end to this malaria control effort in May 1942, because it ran counter to the needs of the rice-farmers (Kitron 1987).

In 1938 a good measure of malaria control was achieved in the Mediterranean countries and in the United States by the judicious use of the anti-malarial drugs Atabrin and Plasmoquine, and the use of quinine (de Zulueta 2000). Drainage of mosquito breeding sites was successful in Italy and the United States, e.g., by the Tennessee Valley Authority (de Zulueta 2000). In the tropics, subsoil drainage was used with success in Puerto Rico and Malaysia (de Zulueta 2000). The Second World War saw the introduction of DDT for malaria control. Malaria was controlled initially with Paris green and atabrin and later with DDT and chloroquine (de Zulueta 2000). DDT was used in Castel-Volturno and the Tiber Delta in 1944-45 resulting in almost complete interruption of transmission (Soper et al., 1947). Alberto Coluzzi achieved complete eradication of malaria in Cassino, Italy from 1946-48 with only two cycles of DDT house spraying (Raffaele and Coluzzi 1949).

Zulueta's fieldwork in the Llanos or plains of Eastern Colombia from 1947 to 1951, at the Rockerfeller Foundation Laboratory of Villavicencio, was based on investigations on the behaviour and habits of the larvae and the adult mosquitoes dwelling in the savannah county of Eastern Colombia (Bates and de Zulueta 1949; de Zulueta 1950 in de Zulueta 2000). They showed how the density and prevalence of anophelines were primarily determined by the adult rather than the larval habitat (de Zulueta 2000).

After leaving Colombia in 1952, de Zulueta had his first assignment with the World Health Organisation in Sarawak. He was a leader of a Malaria Pilot Project to survey Sarawak and neighbouring Brunei for malaria (de Zulueta 2000). They undertook a trial

of indoor residual insecticides spraying, primarily with mainly using DDT, carried out along one of the largest rivers in Borneo, the Baram (de Zulueta and Lachance 1956). The Baram River's tributary, the Tinjar, was used as an unsprayed control area. These two areas were well defined, with population movements restricted to their own river valleys (de Zulueta 2000). With the choice of such a well-defined control area, de Zulueta's group experienced transport problems as road communications within the chosen areas was nonexistent and therefore transport of insecticides and drugs as well as personnel was undertaken by canoe or walking along forest paths. Excellent co-operation between the malaria personnel and the local population was established despite the difficulties of transport, and the group managed to carry out four DDT spray cycles in two years (de Zulueta 2000).

De Zulueta's group used infant malaria parasite-rates as a measure of the success or failure of the trial. All infants in the control area examined during the two-year treatment period were parasite negative, with the exception of two individuals, who were thought to have visited the untreated control area (de Zulueta 2000). The WHO global malaria eradication campaign was launched in 1955 as a result of such experiments.

De Zulueta working with the WHO moved to Uganda in 1959 to launch a malaria eradication pilot project in Northern Kigezi (Western Uganda) where hyperendemic and mesoendemic malaria prevailed (de Zulueta 2000). In this area, three cycles of DDT spraying were carried out in one year in the hyperendemic area of the project and two cycles in the mesoendemic area, with both areas supplemented by single-dose treatment of chloroquine-pyrimethamine (de Zulueta 2000). Infant parasite rates were used as a measure of the efficacy of the two implemented strategies, namely, insecticide residual spraying and prophalaxis. The results showed a complete interruption of transmission, as judged by the infant parasite rates. De Zuleuta (2000) also reported a substantial reduction, but not complete eradication of *An. gambiae* and *An. funestus* in the area.

De Zulueta's group also carried out another malaria eradication experiment, in the Highlands of Kigezi, using the same methods employed in the Northern Kigezi pilot project. The experiment resulted in the eradication of *An. funestus* the only vector species and the rapid disappearance of malaria. De Zulueta demonstrated with these experiments how it was possible to control or even eradicate malaria with DDT treatment in medium

and high altitude regions with hyperendemic and mesoendemic malaria. However, the problem of how to interrupt transmission of holoendemic malaria in Africa remained. The difficulty of malaria intervention in areas of high transmission has been suggested as a tenet of malariology (Hay et al., 2004) with the debate often centred on the holoendemic areas of sub-Saharan Africa.

The first reports of insecticide resistance were by de Zulueta during his work with WHO, where he documented the initial selection of insecticide resistance in malaria vectors of the Eastern Mediterranean and the Middle East (de Zulueta 2000). In Iran, incipient DDT-resistance was detected in *An. maculipennis* in 1956 during a six months mission (de Zulueta et al., 1957 in de Zulueta 2000). Two years later in 1958, a high level of DDT resistance in *An. sacharovi* was detected in the Tarsus area in Turkey (de Zulueta 2000). Later on, dieldrin-resistance as well as a moderate degree of DDT-resistance was found in *An. sacharovi* in southern Greece (de Zulueta 1959). Malaria control problems created by the selection of insecticide resistance in Turkey are said to still persist today, unlike Greece where malaria was eradicated (de Zulueta 2000). The last Greek indigenous malaria cases occurred in 1960 (Bruce-Chwatt 1980). The contrast between the results obtained by these two countries facing the same problem show the influence of cultural and economic conditions on the course of malaria campaign, as pointed out by Coluzzi (1960).

De Zulueta (2000) suggested a need for an interdisciplinary approach to malaria control during the study of the persistence of malaria transmission in Mexico, which showed the importance of social and economic conditions in the course of malaria campaigns. Oaxaca State was sprayed with DDT for five years, but had a low persistent level of malaria transmission in remote parts of the study area. Low levels of malaria transmission were found during Zulueta's study, even though the vectors *An. pseudopunctipennis* and *An. albimanus* were still susceptible to DDT. This was because vectors were still able to enter houses with incomplete walls and little or no contact with the DDT deposits. De Zulueta (2000) further points out that the same scenario seen in Oaxaca State was also evident in eastern Columbia.

Based on the success of Gorgas in Panama from 1904-8, Hackett (1937) believed that malaria was a simple function of anopheline density and being quickly responsive to changes in density (Najera 2000).

Three approaches to malaria control were reported as successful (Najera 2000). These were:

1. Koch's use of systematic quinine at Stephansot.
2. Ross's campaigns at Freetown, Sierra Leone, aimed at breaking the breeding cycle of anophelines.
3. The segregation of Europeans from Indian dwellings in the Gold Coast.

The third method was introduced because of the problems encountered with continuous clearing of breeding sites, and in 1902 a decision was made, based on these problems, to move all Europeans to a segregated settlement above Freetown that had fewer mosquitoes and therefore a reduced risk of malaria (de Zulueta 2000). Koch and Ross's analyses clearly showed that successful anti-malaria campaigns were not the result of mosquito control alone. They concluded that where mass labour movement occurred in developing tropical countries malaria would continue to be a serious disease. The human factor was as important as simple anopheline density in determining whether malaria was prevalent in a given locality, since the incidence of malaria could not always be correlated directly with the number of mosquito vectors. Scientific control of malaria started with the discovery of the malaria parasite, by Laveran in 1880 and its mosquito vector by Ross in 1897. Great successes were achieved in major economic development projects, such as the Panama Canal, the rubber plantations of Malaysia, the Tennessee Valley and the Pontine Marshes in the USA.

Tropical Africa and some areas of South East Asia had extreme disease control problems because of their very high malaria endemicity, primitive state of development and lack of human and economic resources (Najera 1990). Evidence started to accumulate that, although it was possible to reduce and even interrupt malaria transmission by insecticide spraying in large areas, it was very difficult if not impossible, to establish effective surveillance in the absence of a solid public health infrastructure as stated in Najera (1990).

Species eradication was a strategy championed by Soper after his successes against *Anopheles gambiae* in Egypt and Brazil (Brown 1998). This led to implementation of an eradication project in Sardina sponsored by the RockerFeller Foundation International Health Division (RF IHD) against *Anopheles labranchiae* (Brown 1998). The problems encountered during the project were firstly that no entomological research was conducted about the vector and secondly, that having been planned based on Sopers successes, the Sardina project covered a huge area which affected amongst others, the time limit and both human and financial resources allocated for the project (Brown 1998). The great battles between eradication and sustainable control that resulted in the global eradication campaign being replaced in favour of the present 'sustainable' malaria control strategies, were principally over developing country health services (Bradley 1998). Several particular features of malaria affected the broader discussion. A major feature was that malaria is a vector-borne disease, and it therefore introduces a large specialized non-medical aspect to transmission control (Hackett 1937).

The great controversies about how best to prevent malaria in expatriates and in the local population, tended to lead to the association of particular approaches with specific colonizing countries in the earlier part of the 20th century (Bradley 1998). The United Kingdom and the Netherlands emphasised environmental control of vectors. Germany advocated quinine and Italy emphasised mosquito proofing of dwellings. Within the United Kingdom tropical establishment alone, two approaches were identified namely Ross tending towards epidemiology as well as public health action and Manson representing clinical and research interests (Bradley 1991).

1.4.2 Current methods of Vector Control in Africa

Strategic planning and implementation have been cited as major difficulties in control strategies. Implementation of insecticide treated nets (ITNs), now the most commonlly used vector control tool, has met many obstacles. Numerous trials of pyrethroid treated nets have consistently shown 50 – 60% reduction in mild malaria fever and in anaemia (Curtis and Mnzava 2001). Four large trials showed a significant reduction in all-cause child mortality (Lengeler 2003), but scale-up of ITN use has proved difficult. The most successful national or provincial operations with treated nets are in Vietnam, China and Vanuatu, where major reductions in malaria incidence have occurred in the last 10 – 15

years (Cheng et al., 1995; Verle et al., 1998). In Vietnam, China and Vanuatu, insecticides for net re-treatment have been supplied free of charge by government agencies organizing the programmes in the same manner as residual spraying (Curtis and Mnzava 2001). Net coverage has been very low in Africa, with less than 2% of people having ITNs in most countries, excluding the Gambia and Sao Tomé & Principe (Figure 7).

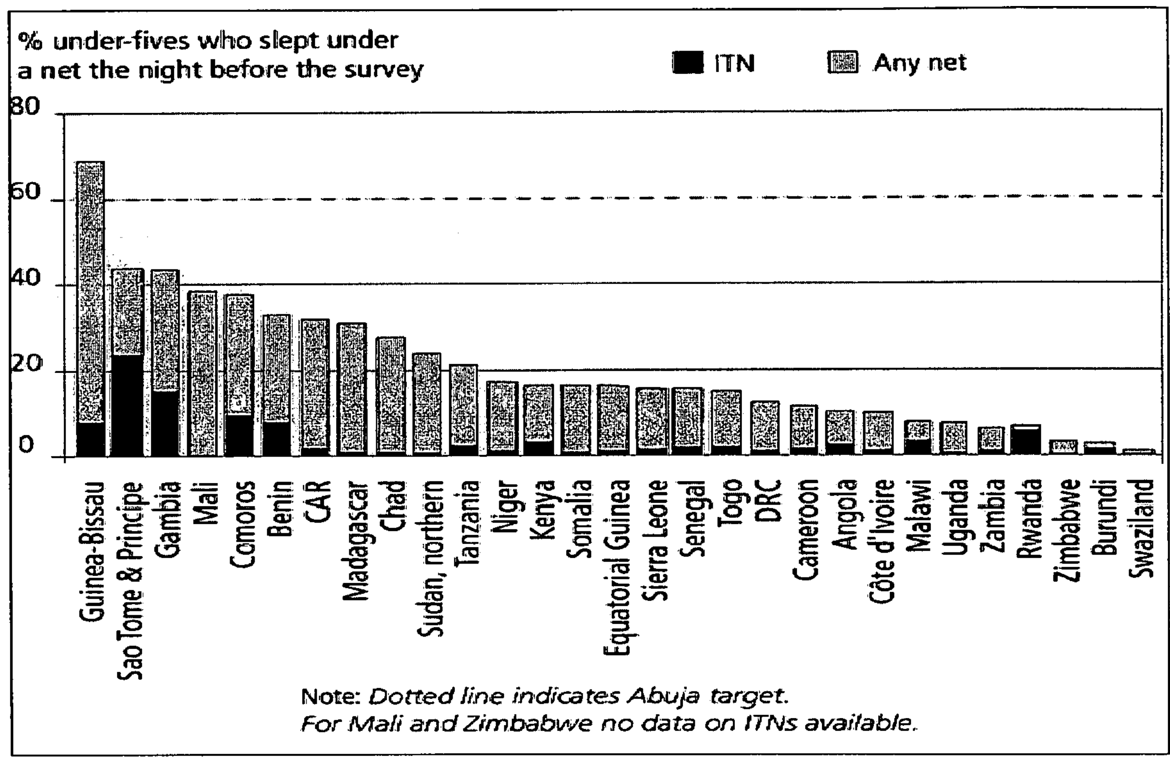


Figure 7. ITNs coverage in Africa 1998-2001. The percentage of children under five sleeping under ITNs in Africa 1998-2001.
(Source: Africa Malaria Report 2003, http://www.rbm.who.int/amd2003/amr2003/amr_toc.htm).

In tropical Africa, where 90% of the world's malaria occurs, there is very little national vector control apart from an increasing number of small ITN projects (Chavasse 1999). ITNs reduce the number of under five deaths by around 20% in randomized controlled trials in African settings of different transmission intensities (Africa malaria report 2003) (Figure 7). Problems with ITN coverage have been associated with cost, which prompted the Abudja Declaration for African governments to commit to reduce or waive taxes and tariffs for nets and materials, insecticides, anti-malarial drugs and other recommended goods and services that are needed for malaria control strategies. As a result of this, 60% of the population at risk (children & pregnant women) should have ITNs by 2005.

The main mandate of Roll Back Malaria is to reduce the global malaria burden of risk, morbidity and mortality by half by 2010. Three other main targets stipulated in Hay et al. (2004) are 60% of malaria cases receiving effective treatment within 24 hours of onset of symptoms, 60% of pregnant women receiving intermittent presumptive therapy (IPT) and lastly 60% of epidemics to be detected within two weeks of onset and responded to approximately within a further two weeks. One of the most important reasons cited in Hay et al. (2004) for failing to reach this target is funding. The Commission for Macroeconomics and Health estimated that $1 billion per annum is needed to enable the RBM to start to work towards its goals, with additional required boosts of $1.5 – 2.5 billion annually by 2007 (Hay et al., 2004). Another suggestion by Hay et al. (2004) is that the global maps of malaria endemicity, such as those in Figure 1, needed updating in order to be able to provide regional consistent measures of population at risk that could contribute to continuing efforts to refining global burden of disease estimates for malaria.

In Africa, during the 1950-70s, indoor insecticide residual spraying (IRS) had a major impact on malaria (Curtis and Mnzava 2001). For example, the Pare-Taveta dieldrin house spraying halved all-cause childhood mortality. There was a reduction of holoendemic malaria and parasite prevalence fell to below 5% in Zanzibar after DDT residual spraying. A 47% reduction in under five-year olds all-cause mortality near Kisumu was achieved by fenitrothion insecticide residual spraying. In South Africa, 50 years of DDT use successfully eradicated one of the vectors, *An. funestus*, and drove malaria back to the northern frontier. Deltamethrin was then used for house spraying in 1996. This co-incided with an increase in malaria cases when pyrethroid resistant but DDT susceptible *An. funestus* reappeared in South Africa (Hargreaves et al., 2000). The southern African region relies heavily on residual house spraying for vector control and increasingly on insecticide treated bed nets (ITNs) (Tables 1 and 2).

Country	Indoor Residual House spraying	ITNs
Botswana	Primary vector control and in high risk areas only.	Trials undertaken since the early 1990s but not yet implemented operationally.
Mozambique	In place in certain high risk areas in major cities.	Government policy is to introduce ITNs on a cost recovery basis and a large project is in place in Zambesia.
Namibia	Primary vector control in high risk areas.	Implemented on a minimal scale.
South Africa	Primary vector control method and in place in all 3 malaria endemic provinces.	A secondary control measure to house spraying. 3000 people under a structured bed net project in KwaZulu-Natal Province and ITNs sold on a cost recovery basis.
Swaziland	Primary vector control method in the low lying eastern part of the country.	Not currently used.
Zambia	Not undertaken.	Policy is to introduce ITNs on a cost recovery basis.
Zimbabwe	Primary vector control method in high risk areas.	Only used on an experimental basis to date.

Table 1. A summary of the present Malaria Control Strategies employed by countries in the southern African region.

Country	Year	Start of IRS and changes of insecticides over time
South Africa	1931	Pyrethrum (experimental IRS)
	1946	DDT and BHC introduced
	1958	Coverage of all malarial areas achieved
	1960–96	DDT
	1997–99	Deltamethrin (policy change)
	2000	DDT (resistance to pyrethroids)
Swaziland	1945	IRS introduced and programme launched
	1947–50	DDT (coverage of all malarial areas in 1950)
	1951–60	BHC (shortage of DDT) dieldrin tried but was costly
	1960–67	BHC and DDT (focal spraying)
	1968–2000	DDT (cyfluthrin in houses with painted walls)
Botswana	1946	IRS introduced (limited scale)
	1950–71	DDT (improved coverage)
	1972	Fenitrothion tried and abandoned (low efficacy)
	1974	Programme launched
	1973–97	DDT
	1998–2000	Deltamethrin and lambda-cyhalothrin (policy change)
Namibia	1965	IRS introduced (limited scale)
	1970	Coverage of all malarial areas achieved
	1965–2000	DDT (bendiocarb in western type residential areas)
Zimbabwe	1945	IRS introduced (pilot projects)
	1949	Programme launched
	1957–62	DDT and BHC
	1972–73	BHC (equally effective as DDT but cheaper)
	1974–87	DDT (resistance to BHC)
	1988–2000	Deltamethrin and lambda-cyhalothrin (policy change)
Southern Mozambique	1946	IRS introduced (selected southern areas)
	1946–56	DDT and BHC (coverage of all targeted areas in 1950)
	1960–69	DDT (only in Maputo region)
	1993	Deltamethrin and lambda-cyhalothrin (major towns)
	2000	Bendiocarb (selected southern areas)

Table 2. Indoor residual house spraying (IRS). The changes in indoor residual house spraying (IRS) and insecticide use in the southern Africa region from 1931-2000. (Reproduced from Mabaso et al., 2004).

A regional collaborative Multi-lateral Initiative on Malaria (MIM) project was initiated in 1999 to develop and implement a molecular and biochemical capability for insecticide resistance monitoring and management in southern Africa (during this PhD). Prior to this project there was little or no expertise in southern Africa to assess possible insecticide resistance in natural mosquito populations. In the same year the Lubombo Spatial Development Initiative (LSDI) was established in southern Africa. The LSDI programme encourages new investment in the geographical area Swaziland, southern Mozambique, and northeastern KZN in South Africa. Malaria control forms a component of the programme (http://www.malaria.org.za/lsdi/home.html). The LSDI malaria control aims at protecting communities in the area in order to enhance development potential and therefore to protect economic investments. The general Protocol which put in place a platform for regional cooperation and delivery was signed in July 1999 by President Mbeki (South Africa), President Chissano (Mozambique) and His Majesty, King Mswati III (Swaziland) for implementation of the initiative (http://www.malaria.org.za/lsdi/home.html).

The aim of the current WHO global malaria control strategy is to halve the number of annual deaths from malaria by 2010 (WHO 1998). An essential part of this strategy is the implementation of efficient vector control, the idea being to reduce the levels of mortality and morbidity by reducing transmission of the disease. The four strategies put forward to achieve this goal are:

1. Provision of drugs and treatment.
2. Implementation of sustainable and effective preventive measures, including vector control.
3. Prevention or early detection and containment of epidemics in high-risk areas.
4. Strengthening local capacities through research and development (WHO 1998).

Problems in achieving these goals were stated earlier with funding being the most important as also noted in Narasimhan (2003).

1.5 History and Development of Pesticides

Man has always had to cope with disease, discomfort and economic loss due to the presence of insect pests. In order to improve health and socio-economic well being,

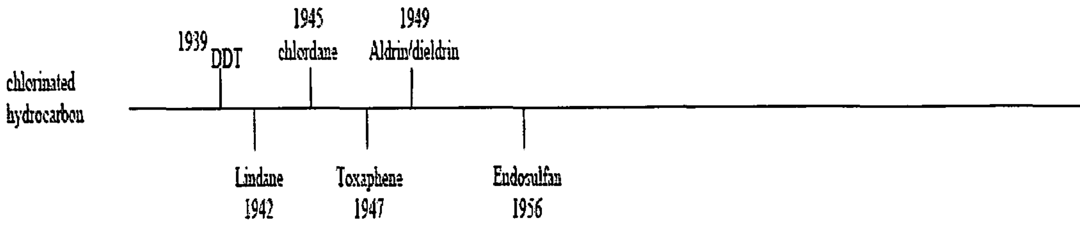
methods to cope with human environmental demands were developed, amongst which was the emergence of pesticides for the control of insect pests responsible for both the transmission of disease and for the destruction of crops (Table 3). Before the Second World War (WWII), most chemical pesticides were inorganic followed by few organic chemicals of plant origin. During and post WWII in the 1940s, there was a shift in pesticide technology, with the introduction of DDT. Zeidler first synthesized DDT in 1874 and Paul Muller discovered its insecticidal properties in 1939. It was first commercially manufactured in 1943. Other groups of synthetic insecticides followed after the discovery and application of DDT (Figure 8).

The success in malaria reduction due to pesticide usage in many parts of the world prompted the WHO assembly in 1955 to vote for a global campaign to totally eradicate the disease (Brown 1998). This idea was abandoned in 1976 by the WHO, which officially changed its policy of eradication to that of control. This policy change was also influenced by the sudden emergence of DDT resistance in vectors and difficulties in control implementation through inadequate public health infrastructures (Brown 1998).

Name	Period	Use
Sulfur	100BC	fumigant
	1800s	fungicide to control powdery mildew on fruit
Mixed poor quality whale oil with vinegar	16th century	on rice paddies to prevent development of insect larvae
Water extracts of tobacco leaves	17th century	sprayed on plants
Rotenone from *Derris eliptica* & pyrethrum from flowers of chrysanthemums	19th century	
Arsenic Trioxide Copper arsenite (Paris Green) Bordeaux mixture (copper sulfate, lime and water)	19th century	weed killer colorado beetle vine downy mildew
Sulfuric acid (10%)	20th century	Destroy weeds
	1920s saw an increase in public concern over pesticide contamination of fruits and vegetables	
Dramatic increase of pesticide development and usage	Post World War II	In agriculture & public health

Table 3. The dates on introduction of the first pesticides and their uses.

(Source: www.pestmanagement.info/pesticide_history/two.pdf)



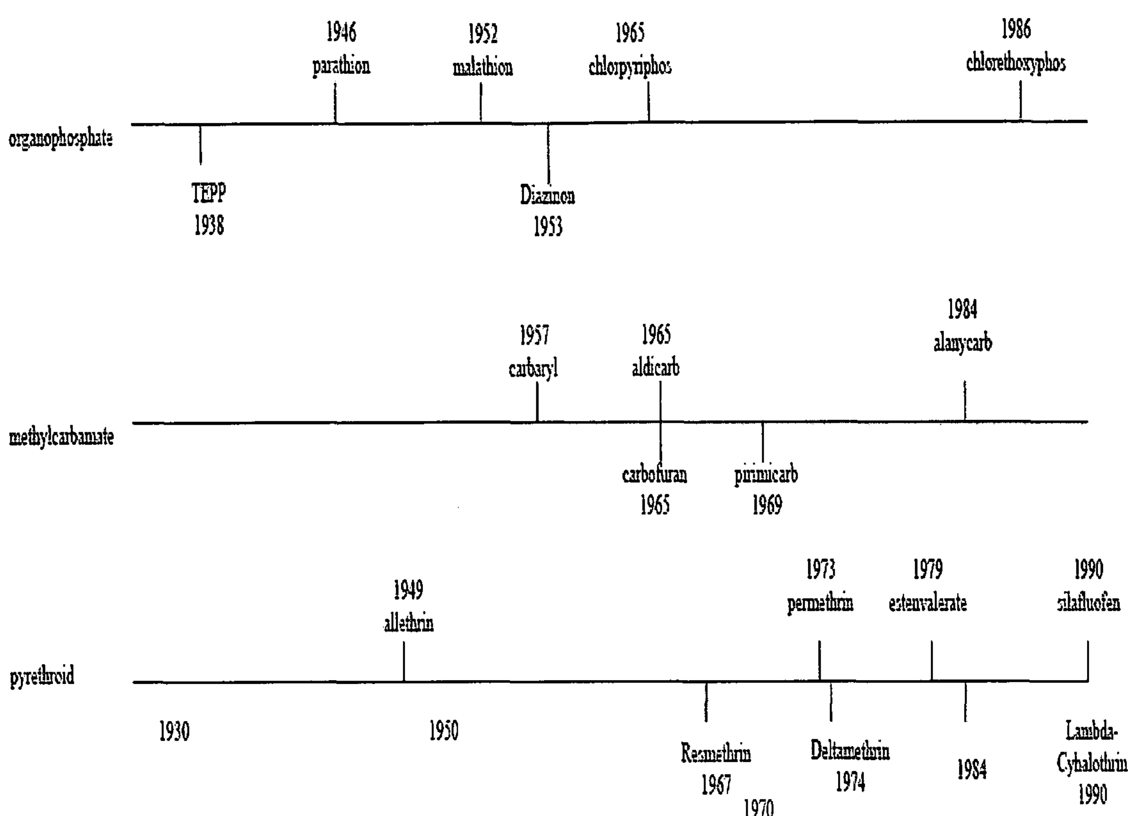


Figure 8. Chronology of insecticide discovery or introduction by chemical class. (Reproduced from Casida and Quistad 1998).

1.5.1 Classification of Pesticides

Pesticides are classified according to their chemical nature and origin. Pesticides in use for public health can be divided into three broad groups: the organochlorines, the acetycholinesterase inhibitors (carbamates and organophosphates) and the pyrethroids (Table 4).

Insecticides class and indicative chemical structure	Other examples	Natural analogues
Chlorinated hydrocarbons: DDT	Endrin Aldrin Dieldrin Endosulfan γ-Hexachlorocyclohexane (lindane)	No natural analogues
Organophosphates: Malathion	Fenitrothion Dichlorvos Diazinon	No natural analogues
Pyrethroids: Permethrin	Permethrin Deltamethrin Cypermethrin Flumethrin	Pyrethrin I from *Chrysanthemum cinerarifolium*:
Carbamates: Carbaryl	Carbaryl Aldicarb Propoxur Bendiocarb	Eserin (Physostigmin) from Calabar bean (*Physostima venenosum*):

Table 4. Chemical structures of the four main groups of insecticides.
(Source: www.agrocourier.com/medien/pages/997/efficacy_e.pdf and Ray 1991).

1.5.1.1 Dichlorodiphenyltrichloroethane (DDT) and DDT Analogues

DDT is stable under most environmental conditions and is resistant to complete breakdown by enzymes present in soil microorganisms and higher organisms. DDE a primary metabolite of DDT, has stability equal to or greater than that of the parent compound. DDT and DDE are both persistent in the environment. Both are soluble in fat and insoluble in water. DDT was first synthesised in 1874 and its effectiveness as an insecticide discovered in 1939 (Carter 2004), DDT was deployed to protect military

personnel, mainly against malaria, typhus and other vector borne diseases in WWII. It was first released for commercial sale in August 1945 in the USA and slightly later in most other countries. Its use peaked in the USA in 1959, but production of DDT continued to increase, driven by the export market, until 1963. The DDT ban, first introduced by Sweden was mainly due to ecological considerations, first highlighted by Rachel Carson (SIMA working paper 95, 1962) (www.iwmi.org/sima).

DDT can be absorbed by mammals after inhalation and ingestion, the latter being the more important route of absorption. Absorption of large doses is facilitated by solubilisation in animal or vegetable fat. Absorption of small doses, such as those found in the residues of food, is virtually complete and is facilitated by the presence of fat in food. Even in solution, DDT is poorly absorbed through the skin. Recently there has been a considerable debate about its potential harmfulness and the possible discontinuation of its application as an insecticide of choice for malaria control (Curtis 1994). WHO (1994) have requested more evidence on harmful effects before any decision is taken to recommend that DDT should no longer be promoted as an insecticide of choice for malaria control. However, the increasing trend of vector resistance to organochlorines and the shift from IRS to ITNS is likely to lead to its natural discontinuation.

Many analogues of DDT were synthesised following its success, the most important ones being methoxychlor, dicofol (kelthane), DDD (2, 4-dichlorophenoxy acetic acid), chlorobenzilate and chlorfenethol (DMC) (Matsumura 1975). The latter has been used as a dehydrochlorinase inhibitor to protect DDT from metabolic attack (Matsumura 1975). Hexachlorohexane (HCH), also known as benzene hexacloride (BHC), was first synthesized in 1825 by Michael Faraday, and its insecticidal properties were discovered in 1942. It was used infrequently to replace DDT in areas where DDT resistance emerged (Matsumura 1975). Several cyclodiene compounds, which are the collective group of synthetic cyclic hydrocarbons, were introduced in 1945 (Matsumura 1975). These included aldrin, chlordane, dieldrin, heptachlor, endrin, and endosulphan. After DDT, dieldrin was the most extensively used organochlorine for malaria control. Dieldrin is more toxic than DDT and HCH to insects, human and animals, while less excito-repellent than DDT (Matsumura 1975). Resistance to dieldrin is usually associated with cross-resistance to other cyclodienes (ffrench-Constant et al., 2000). Dieldrin resistance, along with concerns about toxicity and its long half life led to a decline in its use. Dieldrin is

now only recommended for long-term use for inaccessible pest control treatments, such as termite control in house foundations.

1.5.1.2 Organophosphorus Insecticides (OPs)

All insecticides inherently have acute toxicity since they were developed to kill organisms. Organophosphorus insecticides were first recognized in 1854 with their insecticidal properties being established in the 1930s (Matsumura 1975). They represent another extremely important class of organic insecticides. The first OP insecticide, tetraethyl pyrophosphate (TEPP) was developed in Germany during WWII as a by-product of nerve gas development (Matsumura 1975). OPs are generally among the most acutely toxic of all pesticides to vertebrate animals. They are unstable and break down relatively quickly in the environment. The first example of a broad-spectrum organophosphorus insecticide with the added advantage of low mammalian toxicity was malathion, produced in the 1950s (Matsumura 1975). Most OPs are esters or amides of organically bound phosphoric or pyrophosphoric acid. They can be divided into five classes according to their phosphorous moiety. The most important OPs used for insects of both economic and health importance belong to the two classes, phosphorothionates (e.g. fenthion, temephos, chloropyrifos, fenitrothion etc.) and phosphorothiolothionate (e.g. malathion) esters.

1.5.1.3 Carbamates

Carbamates, the derivatives of carbamic acid were originally extracted from the calabar bean grown in West Africa. Their use began in the 1950s and they are among the most popular pesticides for home use, indoors as well as on gardens and lawns. Carbaryl is the best-known and most applied carbamate pesticide. Other examples of this group are propoxur and bendiocarb, which have been used in malaria control programmes, especially in areas where vector resistance to DDT, pyrethroids and OP's has emerged. Bendiocarb and propoxur are the only two carbamates registered for IRS use and the latter is likely to be withdrawn in the near future as production ceases.

1.5.1.4 Pyrethroids

Pyrethroids are the newest generation of highly toxic insecticides used to control insect pests in agriculture, households, stored products and disease vectors. Pyrethroids are based on the chemical structure and biological activity of pyrethrum that was extracted from plants of the genus *Chrysanthemum* (Matsumura 1975; Ray 1991). Based on the low toxicity and rapid degradation of pyrethrins, the development of pyrethroids involved extensive chemical modifications in order to to make compounds that were more toxic and less rapidly degraded by UV light than the natural products. Their toxicity is highly dependent on stereochemistry, meaning the three dimensional configuration of the molecule. Each *cis* or *trans* isomer has its own toxicity, with the *cis* isomer being generally more toxic than the *trans* isomer (Mueller-Beilchmidt 1990; Ray 1991).

The ratio of the amounts of the isomers within a mixture determines the acute toxicity of that mixture. Permethrin, for example, is sold in 60:40 or 75:25 *cis-trans* ratios. The emergence of resistance to organophosphates, carbamates and organochlorine insecticides, together with the pyrethroid's low volatility and polarity (which result in less movement in the air or soil from the point of application), increased attention and focus on these insecticides for public health use. They now represent the most important insecticides class for the control of insects of medical importance. Currently the imidacloprids outsell pyrethroids in the agricultural market by a large margin, but they are not registered for public health use.

The main classes of insecticide used in public health operate through poisoning the nervous system of target organisms. The mechanism is an alteration in the transfer of a signal along a nerve fibre and across the synapse from one nerve to another or from a nerve to a muscle fibre (Applied Toxicology NURS 735). The different classes of insecticides act in separate ways but all effectively block normal nerve signals (Figure 9).

1.6 Factors Influencing Development of Insecticide Resistance

Resistance is defined as "the inherited ability of a strain of an organism to survive a dose of a toxicant that would kill the majority of individuals in a normal population of the same species" (WHO 1957). It is multidimensional and depends for its evolution on the

interaction of multiple influences (Table 5). The multiple factors that influence the development of resistance to insecticides can be classified into the following categories.

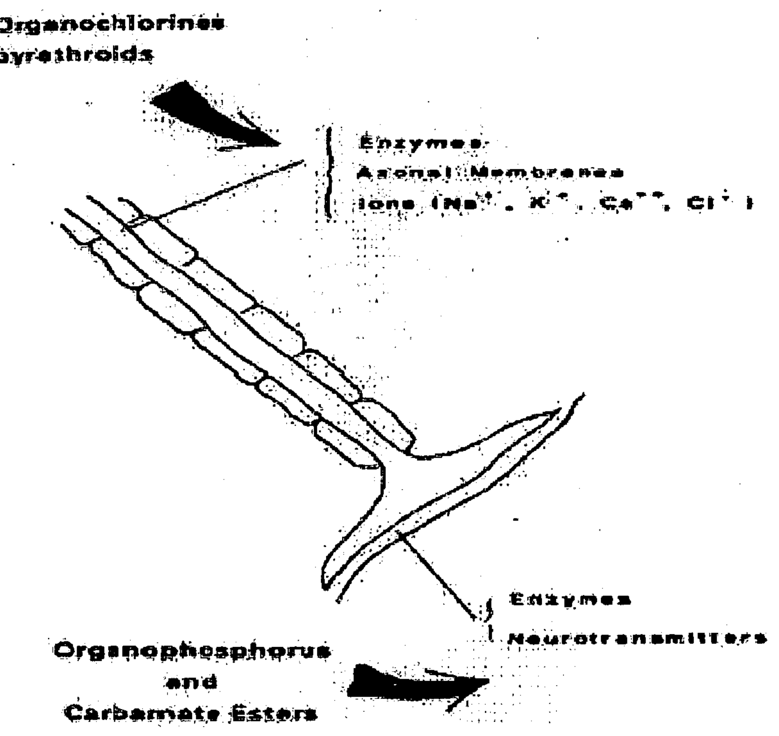


Figure 9. Target sites of insecticides.A schematic presentation of sites of action of the four main classes of insecticides used for control of insects of public health importance.
(Source: Applied Toxicology NURS 735 (http://aquaticpath.umd.edu/appliedtox/).

Genetic	Mutation rate and frequency of R genes Penetrance, expressivities and dominance of R genes Relative fitness of genotypes
Reproductive	Generations per year Rate of increase, and fluctuations in population size
Behavioural	Migration in and out of exposed population Response to repellent effects of insecticide
Operational	Relationship of chemical to insecticides used earlier Level of insecticide exposure

Table 5. A summary of influences on the development of insecticide resistance.

1.6.1 Insecticide Resistance and Dominance levels

The level of dominance is a measure of the relative position of the heterozygous phenotype relative to the phenotype of the two corresponding homozygotes (Bourguet et al., 1996). If a wild type gene (A) mutates to a deleterious allele (a), the Aa heterozygote often displays a wild-type phenotype: the deleterious effects of mutations are fully recessive. This depends on where the mutation is and the normal function of the mutated gene. Insecticide resistance provides a good model to study dominance relationships (Bourguet et al., 2000) because many of the genes and mutations responsible for resistance have been identified, and the physiological processes in which the resistance genes are involved are known. Additionally there is a large variation of the level of dominance of resistance, e.g., the insecticide resistance phenotype conferred by mutations decreasing the affinity of insecticide target sites varies from complete recessivity to complete dominance. A resistance allele (Table 6) may be dominant (over a susceptible allele) for one species and recessive for another.

Resistance mechanisms	**Insecticide resistance**	**Dominance levels**	**Species**	**References**
Insensitive sodium channel	DDT and pyrethroids	Recessive to codominant	*Musca domestica* *Culex pipiens complex* *Leptinotarsa decemlineata*	Farnham et al. (1984) Halliday and Georghiou (1985a,b) Argentine et al. (1989)
Insensitive GABA receptor	Cyclodienes	Codominant to dominant	*Boophilus microplus* *Musca domestica* *Anopheles sp.* *Culex sp.* *Tribolium castaneum*	Georghiou (1969) Georghiou (1969) Georghiou (1969) Beeman and Stuart (1990)
Insensitive AChE	Carbamates and OPs	Codominant to dominant	*Boophilus microplus* *Musca domestica* *Nephotettix cincticeps* *Leptinotarsa decemlineata* *Culex tritaeniorhynchus*	Stone et al. (1976) Plapp and Tripathi (1978) Hama and Iwata (1978) Ioannidis et al. (1992) Takahashi & Yasutomi (1987) Georghiou (1996)
Monooxygenases	Carbamates	Codominant to dominant	*Culex pipiens complex*	Tate et al. (1974) Cochran (1994)
Esterases	Organophosphates	Codominant to dominant	*Musca domestica* *Blattella germanica* *Culex pipiens* complex *Culex pipiens* complex	Pasteur and Sinegre (1978) Pasteur et al.(1984)

Table 6. Resistance mechanisms and their dominance levels. (Source: Bourguet et al., 1996).

Martinez-Ramirez et al., (1995) suggested that it is inappropriate to talk about the dominance of a resistance allele in the way used in many papers. For instance one should always specify the environmental parameters, since dominance describes the relationship between the phenotypes of the three genotypes, which may vary between traits and environments.

1.6.2 Defining and Measuring Dominance

There are several ways to measure dominance (Figure 10). Dominance may be based on:

a. The position of the mortality curve for heterozygous individuals relative to those for both homozygotes, at a given mortality level (Bourguet et al., 1996).
b. The mortality of heterozygous individuals relative to that of both homozygotes, at a given insecticide concentration (Curtis et al., 1978; Roush and Mckenzie 1987).
c. A comparison of the fitness of the heterozygotes relative to that of the two homozygotes at a given insecticide dose (Bourguet et al., 1996).

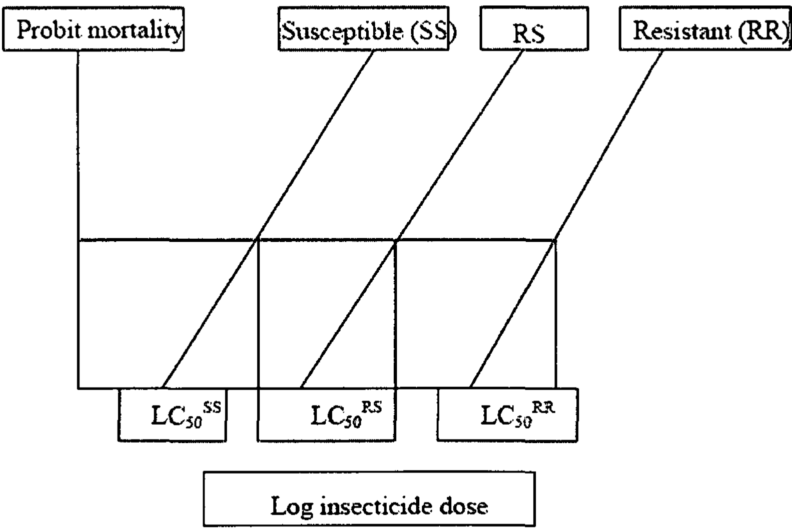


Figure 10. Probit mortality. The use of probit mortality curves to measure the level of dominance of insecticide resistance phenotypes. (Reproduced from Milani 1963).

1.6.3 The Dominance of Insecticide Resistance

In toxicology, dominance level was initially determined by comparing the mortality curves of homozygous susceptible, resistant and heterozygous individuals (Figure 10) (Milani 1963). Resistance was qualitatively classed as recessive or dominant according to whether the heterozygote mortality curve was closer to the homozygote susceptible or resistant mortality curve, respectively and codominant if the heterozygote mortality curve was equidistant from those of the homozygotes. A quantitative measure was introduced by (Stone 1968), using Falconer's formula (1964):

$$D = (2\log LC_{RS} - \log LC_R - \log LC_S)/(\log LC_R - \log LC_S)$$

LC_R, LC_{RS} and LC_S = lethal concentrations for the resistant, hybrid and susceptible individuals.
D varies from –1 to 1 (-1 = complete recessivity and 1 = complete dominance)

It is an established principle of population genetics that a rare recessive gene will increase in frequency under selection less rapidly than a rare dominant gene. This fact formed the basis of a strategy proposed to slow down the evolution of insecticide resistance by making the responsible gene 'effectively recessive'. It depended on raising the quantity of insecticide picked up by an exposed insect to a level that killed all RS individuals. This strategy, which could be applied only to monogenically inherited resistance, was proposed by Wood and Cook (1978) in a WHO Working Document and discussed further by Curtis et al (1978). Independently it was proposed by Taylor and Georgiou (1979) under the name 'functional dominance'. Computer simulations showed that the technique could only be effective if a small proportion of the population under control had the chance to escape exposure, thereby preserving a 'reservoir' of susceptible insects (Wood and Mani 1981).

1.6.4 Determination of Insecticide Resistance Status of Mosquitoes

The first stage in the investigation of any insecticide resistant mosquito population is the determination of the general resistance spectra (Hemingway 1981). Methods of measuring the resistance spectra are shown in Figure 11.

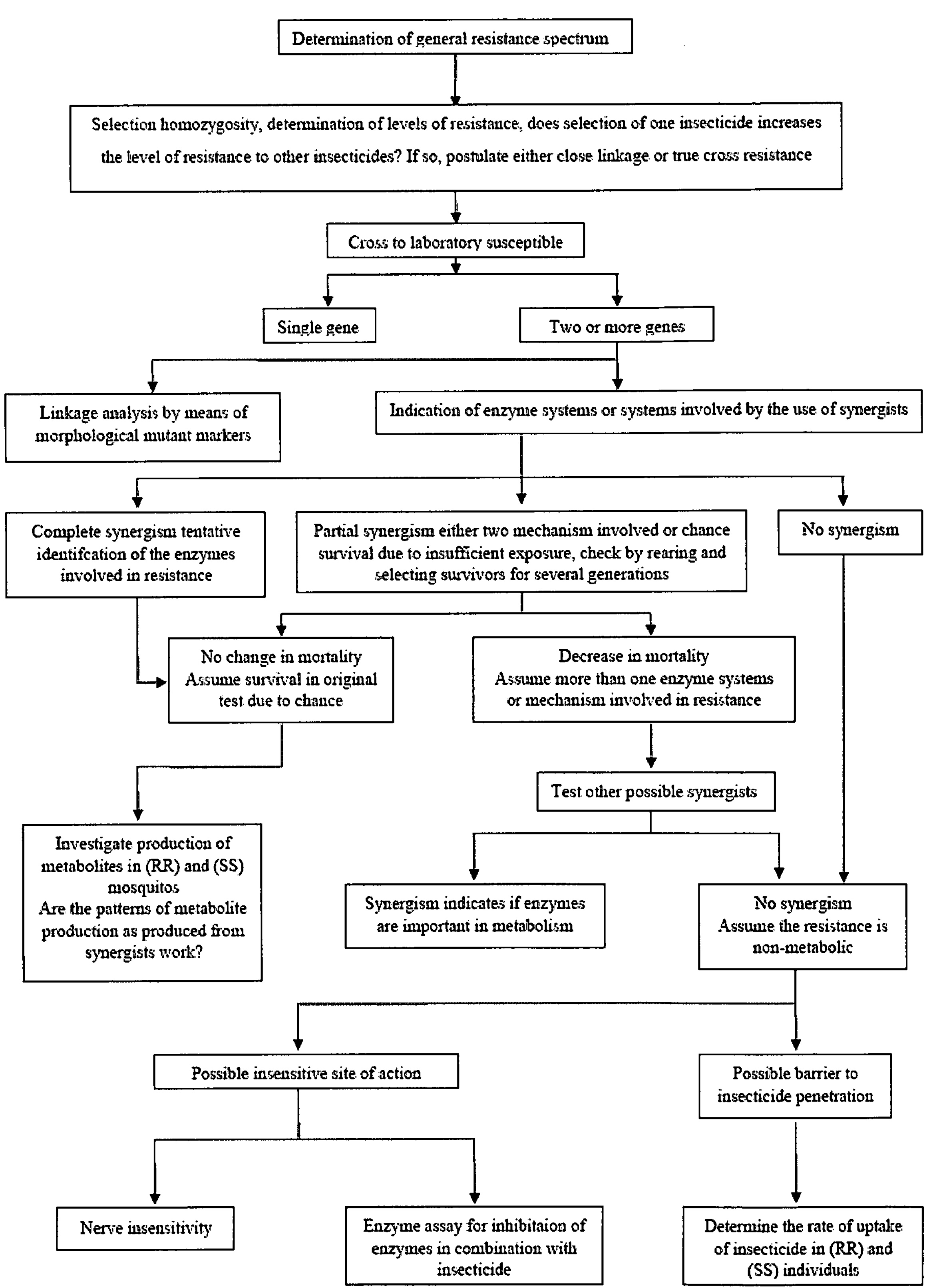


Figure 11. Stages in the investigation of resistance mechanisms in mosquitoes. (Reproduced from Hemingway 1981).

1.6.5 Methods of Detecting Resistance

Detection and monitoring of insecticide resistance in malaria vectors is an important activity, which has to be carried out along with other entomological surveys (Brent 1986). There are different approaches for the detection of resistance status in insects that are described below. These form part of the pest control and management strategies implemented in various countries, e.g. South Africa. The lack of some of these tools, namely biochemical and molecular assays, for the detection and monitoring of insecticide resistance in malaria vectors in southern African region and the need to implement them for successful resistance management prompted this programme. Establishing the resistance baseline in Southern Africa formed the basis of this PhD.

1.6.5.1 WHO Susceptibility Tests

Bioassay refers to methods used to determine the relation between a physiologically active agent and the effect that it produces in a living organism (Hoskins and Craig 1962). Bioassays with the dosage or the exposure time as the variable are carried out to test the resistance status of insect populations.

The lethal dosage (LD), lethal concentration (LC) or lethal time (LT), which kills 50% ($LD_{50}/LC_{50}/LT_{50}$) or 90% ($LD_{90}/LC_{90}/LT_{90}$) of the population can be calculated from such bioassay data (Matsumura 1985) and compared with a known susceptible population of the same species. The resistance in this case is expressed in relative terms. Discriminating dosages (twice the LD_{99} that kills homozygote susceptible *Anopheles*) have been determined by the World Health Organization (WHO 1963; WHO 1980) for many insecticides for anopheles of medical importance. The resistance status detected using bioassays, can then be further studied by looking at the mechanisms responsible for resistance using synergists or biochemical and molecular assays.

1.6.5.2 Synergists

Synergists are used to suggest the type of metabolic resistance mechanisms present in insect field populations (Scott 1990). They are used in bioassays to counteract or inhibit the enzymes responsible for resistance to the insecticide. Some of these chemicals have also found uses in control, where they are used to reduce the dose or rate of pesticide

application (Devine and Denholm 1998). For example piperonyl butoxide is commonly added to pyrethorid – based aerosol formulations to decrease the time to knockdown and increase the time to recovery from the insecticide.

Insecticide synergists inhibit specific detoxification enzymes and thus can reduce or eliminate the selective advantage of individuals possessing such enzymes (Matsumura 1985). Examples of these synergists are piperonyl butoxide (PBO) and S,S,S-tributylphosphorotrithiote (DEF) inhibitors of oxidases and esterases respectively (Devine and Denholm 1998; Soderlund et al., 1990).

1.6.5.3 Biochemical Assays

There are two major ways that metabolic enzymes can produce resistance (Hemingway 1981), that is, overproduction of the enzyme, which leads to either increased metabolism or sequestration of the insecticide and an alteration in the catalytic centre activity of the enzyme, which increases the rate of insecticide metabolism by the enzyme. Sequestration occurs when the overproduced enzyme rapidly binds and slowly metabolises the insecticide, therefore preventing it from reaching the target site within the insect (Aldridge 1993). With sequestration the resistance level is proportional to the increase in the quantity of the enzyme produced because of the slow insecticide turn-over rate (Aldrige 1993). Biochemical assays are used to give a first indication of the enzyme system (e.g. esterases, glutathione S-transferases or monooxygenases) involved in resistance (Hemingway 1981).

1.6.5.4 Molecular Assays

Molecular techniques can also be used to detect some well characterized resistance mechanisms. Most techniques employ the method of Polymerase Chain Reaction (PCR). A PCR method can be used to detect cyclodiene resistance due to an alteration in the GABA-gated chloride channels (ffrench-Constant 1995). DDT and pyrethroid cross-resistance due to the kdr mutations in the para voltage gated sodium channel in *An. gambiae* s.s and other insects can also be detected by specific PCR reactions (Martinez-Torres et al., 1998; Ranson et al., 2000).

1.7 Mechanisms of Insecticide Resistance

The four most important types of mechanism for the four main insecticide groups used in public health are (Hemingway 1981):

- increased metabolism to non-toxic products,
- decreased target site sensitivity,
- decreased rates of insecticide penetration and
- increased rates of insecticide excretion.

This PhD deals with the first two, which are by far the most important. Penetration rate changes in isolation generally produce insignificant (<5-fold) levels of resistance, and are important only when found in combination with other resistance mechanisms. Increased rates of insecticide excretion are very uncommon and also produce low levels of resistance.

1.7.1 Metabolic Insecticide Resistance Mechanisms

Increased metabolism to non-toxic products and decreased target site sensitivity produce the highest levels of resistance and these can further be sub-divided into enzymes groups involved in insecticide metabolism, which are:

a. esterases
b. monooxygenases
c. glutathione-S-transferases

The pesticide target sites are the sodium (Na+) channels for the pyrethroids, DDT and acetycholinesterase for the organophosphates and carbamates, and GABA receptors for the cyclodienes and fipronils.

Insecticide metabolism is the type of resistance conferred by changes in enzyme activity, which increases the rate of detoxification or sequestration of the insecticide (or in the case of OPs their toxic metabolite), thereby reducing the amount that reaches the insecticides target site. The three enzyme classes that are involved in insecticide resistance in insect of

both medical and agricultural importance are: esterases, glutathione S-transferases and cytochrome P^{450}s (monooxygenases).

1.7.1.1 Esterases

Esterases have diverse functions in insects, which include proteolysis, nervous system function, hormone metabolism, and xenobiotic metabolism or sequestration (Aldridge 1993). They hydrolyse insecticides with ester bonds, such as OPs as well as some carbamates and pyrethroids. Fifty one and thirty six putative esterase genes were identified in *An. gambiae* and *D. melanogaster* respectively after data from both insect genomes became available (Ranson et al., 2002). Hydrolysis of p-nitrophenyl or naphthyl esters in simple biochemical assays is frequently used to detect elevated esterase activity in individual insects (Siegfried and Scott 1992; Hemingway et al., 1993). Polyacrylamide gel electrophoresis (Scharf et al., 1996), starch gel electrophoresis (Pasteur et al., 1988) and thin-agarose gel electrophoresis (Lee et al., 1994) have been used to visualise the elevated esterase electromorphs with novel properties in resistance (Scharf et al., 1996). Insecticide resistance can be due to qualitative or quantitative changes in the esterases.

Carboxylester hydrolases are classified as acetyl, carboxyl or cholinesterases depending on their inhibition with sulfhydryl reagents, OPs and eserine sulfate (Holmes and Masters 1967). Over thirty have been identified in *Drosophila melanogaster.* They belong to one superfamily of B hydrolases. Fifteen esterase genes have been mapped and 12 are clustered at 2 chromosomal sites. The constitution of each cluster varies between Drosophila species but two carboxylesterases in one cluster are sufficiently conserved that their homologous can be identified among enzymes conferring insecticide resistance in other Diptera (Oakeshott et al., 1993).

The interaction of elevated esterases with insecticides has been shown for enzymes purified from at least three different insect species: the peach potato aphid *Myzus persicae* (Devonshire 1977; Devonshire and Moores 1982), the mosquito *Culex quinquefasciatus* (Ketterman 1992; Karunaratne et al., 1993a) and the brown planthopper *Nilaparvata lugens* (Vontas et al., 2000). Two esterases, Estα2[1] and Estβ2[1], are the enzymes commonly most elevated in approximately 90% of all the OP-resistant *Cx. quinquefasciatus* strains so far analysed (Mouches 1986; Vaughan 1995; Hemingway and

Karunaratne 1998). These are encoded by genes on a single amplicon in resistant insects. The esterase genes are in a head to head orientation, separated by a non-coding sequence of 2.7Kb in resistant insects (Vaughan 1997). The elevation of esterases in many OP-resistant *Culex* species and populations was found to be due to gene amplification (Karunaratne et al., 1993a; Jayawardena et al., 1994; Vaughan 1997; Karunaratne and Hemingway 1998, Small and Hemingway 2000). Their elevation is caused by up to 80-fold gene amplification with the amplicons stability integrated sequentially into the chromosome (Paton et al., 2000). In contrast Raymond et al. (1998) found no evidence of gene amplification in esterase Estα[1] involved in OP-resistance in *Culex pipiens* from France.

Pyrethroid insecticide resistance due to elevated esterase was first suggested in the late 1980s in *Anopheles albimanus* in Guatemala (Beach et al., 1989). Brogdon and Barber 1990 and (Georghiou 1972) described resistance to both fenitrothion and deltamethrin in *An. albimanus* populations that were heavily selected by years of organophosphorus insecticide use on cotton crops. Some permethrin hydrolysing esterases have been characterized in the agricultural pests *H. armigera*, *H. punctigera* and *B. tabaci* (Gunning 1996; 1999). The elevated E4 and FE4 esterases in the aphid *M. persicae* may contribute to permethrin metabolism as well as producing high levels of OP resistance (Devonshire and Mores 1982; Devonshire et al., 1998).

Pyrethroid resistance due to elevated esterase activity has been detected in the tobacco budworm, *H. virescens* and the apple bud moth *Platynota idaeusalis* which also includes *Musca domestica* and *Culex* mosquitoes (Soderlung et al., 1990).

1.7.1.2 Glutathione S-transferases (GSTs)

GSTs are a diverse family of dimeric proteins found in almost all living organisms (Ranson et al., 2001). These proteins were originally studied for their role in detoxification of endogenous and xenobiotic compounds. They have additional roles as transport proteins and in protection against oxidative stress (Hayes et al., 1995). Each GST subunit consists of two domains, each containing two binding sites, the G site and the H site. The highly conserved G site binds the tripeptide glutathione and is largely composed of amino acid residues found in the N-terminal domain. The H-site or substrate

binding site is more variable in structure and is largely formed from residues at the C-terminal.

Purification of independent homogenous GST preparations with differing substrate specificities indicated the presence of multiple forms of GSTs. The recognition of five classes of cytosolic GSTs in mammals, the alpha, mu, pi, theta and sigma classes was due to the availability of N-terminal sequence data (Mannervik 1985; Meyer et al., 1991; Meyer and Thomas 1995). More GST classes have recently been recognized, the omega and zeta classes represented from a range of species extending from plants to animals (Board et al., 1997).

The mammalian kappa class, the insect epsilon class, the plant tau and phi classes a more restricted distribution (Ding et al., 2003). Most of these classes are encoded by multigene families. Alternative splicing and the formation of heterodimers also add a further level of heterogeneity to some GST classes, which makes assigning physiological functions to individual GSTs, a complex task (Ranson et al., 2001).

GSTs in insects are involved in the detoxification of a number of insecticides largely organophosphates, organochlorines and cyclodienes (Reidy et al., 1990; Clark et al., 1986; Grant and Matsumura 1989; Fournier et al., 1992). They are involved in the O-dealkylation or O-dearylation of organophosphorus insecticides (Hayes and Wolf 1988; Huang et al., 1998), as a secondary mechanism in the detoxification of the active oxon-analogues of OP insecticides (Hemingway et al., 1991) as well as in the dehydroclorination of organochlorines (Clarke and Shamaan 1984). GSTs confer resistance to pyrethroids by detoxifying lipid peroxidation products induced by the pyrethroids, thereby protecting tissues from oxidative damage and/or by binding pyrethroid molecules in a sequestration mechanism, thus offering passive protection (Vontas et al., 2001).

The epsilon class of insect GSTs has previously been implicated in conferring insecticide resistance in several insect species. (Ding et al., 2003) compared the expression level of all members of this GST class in two strains of *An. gambiae* s.s to determine whether epsilon GST expression was correlated with insecticide resistance and found that GSTe2 is associated with DDT resistance in several mosquito species. There are at least six

different classes of GSTs in insects, but 20 of the *An. gambiae* s.s GSTs belong to the two insect specific classes, delta and epsilon. Members of these two GST classes are clustered on chromosome arms 2L and 3R respectively. The *An. gambiae* s.s GSTs genes are located on all three of the mosquito's chromosomes but two large, gene-specific clusters occur (Ding et al., 2003) (Figure12).

Advances in the field of genomics, being the comparative study of the structure and function of entire genomes (Heckel et al., 1998), are now being used to make inroads into the study of mechanisms of insecticide resistance (Ranson et al., 2000). Many genomic approaches have the advantage that they do not make any prior assumptions about the resistance mechanisms involved.

Two loci affecting DDT resistance have been genetically mapped using a QTL mapping approach in *An. gambiae* s.s (Ranson et al., 2000), utilizing recently compiled genetic and physical maps in this species (Zheng et al., 1996). This approach was successful in identifying threshold traits (discrete traits, which are either present or absent in any one individual e.g, in mosquitoes, either resistant or susceptible to insecticide) by identifying two quantitative trait loci (QTL) that explain the majority of the variance between DDT resistant and susceptible *An. gambiae* s.s individuals (Ranson et al., 2000).

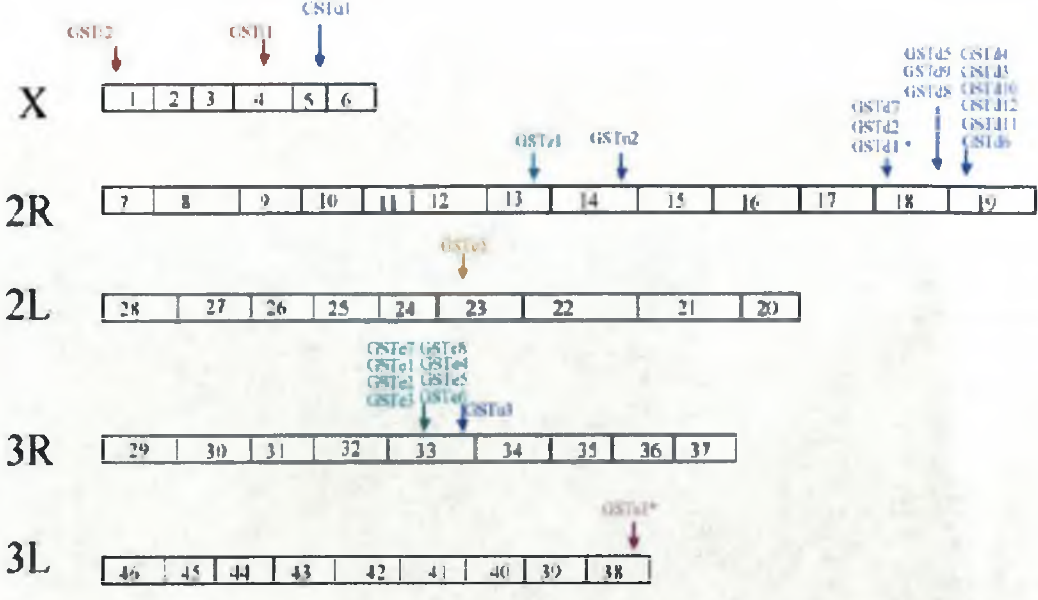


Figure 12. A schematic diagram indicating the organisation of GST genes in the *An. gambiae* genome. The numbers represent polytene chromosome divisions. Those genes marked with * are alternatively spliced to produce multiple transcripts. (Source: Ding et al., 2003).

DDT in malaria control programmes is mostly used as an adulticide. The original class 1 insect GSTs now classified as Delta GSTs and class 2 as members of the Sigma genes in *An. gambiae* s.s, have been studied to ascertain their role in conferring DDT resistance (Ranson et al., 2001). The single class II GST, aggst2-1, was found highly expressed in *An. gambiae* s.s larvae (Reiss and James 1993) but barely detectable in adult insects. The class I GSTs are expressed at high levels in both larvae and adults (Ranson et al., 2001). Several recombinant *An. gambiae* GST enzymes are able to metabolise DDT, demonstrated by using antibodies raised against these class I GSTs (Ranson et al., 2001). Ranson et al. (2001) demonstrated that these enzymes are not the most important family in DDT resistance.

1.7.1.3 Cytochrome P^{450s} Monooxygenases

Williams in 1955 was the first to discover cytochrome P^{450s}, when he noticed a pigment from rat liver microsomes, which had a peculiar carbon monoxide binding spectrum. He observed an intense but broad absorption maximum at 450nm using a double-beam recoding spectrometer. Klingenberg and later Garfinkel independently examined the data in 1958. Their research, even though the chemical nature of the pigment was unknown, indicated that a heavy metal ion was involved in carbon monoxide binding. Omura and Sato (1964) showed cytochrome P^{450} was a haemoprotein of the b-type class. It was named P^{450} because of its strong absorbance at 450nm when combined with carbon monoxide.

Cytochrome P^{450} monooxygenases are important in the metabolism of a wide range of xenobiotic and endogenous compounds through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidaiton, aromatic hydroxylation, ester oxidation, nitrogen and thioether oxidation. A large number of substrates can be metabolized by this enzymatic system due to the multiple isoforms of P^{450} that exist in each organism and the broad substrate specificity of some of these isoforms (Scott et al., 2000). Their roles include the synthesis and degradation of ecdysteroids and juvenile hormones to the metabolism of foreign chemicals of natural or synthetic origin in insects. Insect genomes carry about 100 P^{450} genes, all of which have evolved from a common ancestral gene. They are sometimes arranged in clusters within the chromosomes (Feyereisen 1999).

Both microsomal and mitochondrial P^{450}s are present in insects and are best studied by heterologous expression of their cDNA and reconstitution of purified enzymes. They are under complex regulation, with induction playing a central role in the adaptation to plant chemicals and regulatory mutations playing a central role in insecticide resistance. Polymorphisms, induction or higher constitutive expression allow insects to scan their P^{450} gene repertoire for the appropriate response to chemical insults, and these evolutionary pressures in turn maintain P^{450} diversity.

The nomenclature for P^{450}s, based on amino acid sequence identity, was introduced by Nelson et al. (1993). All genes encoding the P^{450} superfamily are designated with a CYP prefix followed by a numeral for the family, a letter for the subfamily, and a numeral for the individual gene. Members of a family all share >40% identity at the amino acid sequence level, while members of a subfamily share >55% identity. The genes are described in italics, the gene product and enzyme in capitals. Most P^{450} proteins are approximately 500 amino acids long, and their characteristics cysteine axial ligand to the heme iron is located toward the C terminus of the protein and is a highly conserved region.

Insect cytochrome P^{450} knowledge is largely based on assumptions of homology to well studied mammalian P^{450} systems. This indicates the structure and mode of action of P^{450} enzymes is well conserved, for example from bacteria to *D. melanogaster* (Feyereisen 1999).

The catalytic cycle involves the heme protein in the iodized form, by P^{450}(Fe^{III}) binding the substrate to form a P^{450}-substrate complex, which in turn receives a single electron from a redox partner and P^{450} (Fe^{III}) then binds oxygen (Feyereisen 1999). The second one-electron reduction step involves a molecular oxygen being split and the reactive oxygen complex which is sometimes described as P^{450} (Fe^{V}=O) inserts an atom of oxygen into the substrate in a radical reaction. Although the formal reaction is the insertion of an atom of oxygen into a substrate, the other atom being reduced to water (hence the term mixed-function oxidase), the nature of the oxidizing species (iron oxene or iron peroxy) and the stability of the initial formal product can vary. The overall chemistry can be diverse from hydroxylation to epoxidation, O- N- and S-dealkylation, N and S-oxidations.

Insect cytochrome P^{450}s can be detected in a wide range of tissues. The developmental regulation of P^{450} gene expression is well documented. Some P^{450}s are larval specific, for example, CYP6B2, whereas others, such as CYP6D1, are adult specific (Feyereisen 1999). High levels of P^{450} activity are associated with the midgut, fat bodies and Malpighian tubules (Hodgson 1983), but their relative expression levels vary between tissues (Scott et al., 1998). In the housefly, CYP6D1 is found throughout the body (Korytko and Scott 1998; Scott and Lee 1993), while Drosophila CYP6A2 is expressed in the midgut, pericuticular fat bodies and Malpighian tubules (Brun et al., 1996). Insect P^{450}s are studied because of their role in resistance through the oxidative metabolism of insecticides. P^{450} genes, CYP6A1 (Feyereisen et al., 1989), CYP6A2 (Waters et al., 1992) and CYP6D1 (Tomita and Scott 1995) are found overexpressed in resistant strains of the housefly, *M. domestica* and in *D. melanogaster*. CYP6A2, CYP6A1 and CYP6D1 were all overexpressed in malathion, diazinon (10-fold) and pyrethroid (9-fold) resistant strains of *M. domestica* compared to susceptible strains. CYP6A1 and CYP6D1 overexpression was due to enhanced gene transcription (Liu and Scott 1998) but not gene amplification (Feyereisen 1995); Tomita and Scott 1995). A semi-dominant factor on autosome 1 and an incompletely recessive factor on autosome 2 are factors involved in the high level of expression of CYP6D1. The location of the several mutations that result in amino acid substitutions in CYP6A2 protein suggests by 3D modelling that some are important for enzyme activity (Berge et al., 1998). Table 7 gives examples of insect P^{450}s involved in insecticide metabolism (Scott 1999).

P^{450}s	Insect species	Strain	Insecticide metabolism	Reference
CYP6A1	*M. domestica*	Rutgers	Pyrethroids	Kasai and Scott, 2000
CYP6D1	*M. domestica*	Learn-PyrR	Organophosphates	Andersen et al., 1994
CYP6G1	*D. melanogaster*	Hikone-R, WC2 etc.	DDT	Daborn et al., 2002
CYP12A1	*M. domestica*	Rutgers	Various insecticides	Guzov et al., 1998

Table 7. A summary of insects P^{450}s involved in insecticide metabolism.

Nikou et al. (2003) investigated the CYP6 P^{450}s in pyrethroid resistant *An. gambiae* s.s to establish if there was a causative link between increased P^{450} activity and insecticide resistance. An adult CYP6 P^{450} was overexpressed in the *An. gambiae* s.s pyrethroid

resistance RSP strain and CYP6Z1 was indirectly implicated in conferring pyrethroid resistance in this malaria vector. More work is still being undertaken to elucidate whether CYP6Z1 is responsible for metabolic resistance to pyrethroids in the RSP strain of *An. gambiae* s.s. Figure 13 is a polytene chromosome map showing the location of two QTLs for pyrethroid resistance in *An. gambiae* s.s investigated in the above mentioned study.

Another important reaction catalysed by oxidases is the activation of phosphorothionates to their oxon analogues within the insect. OPs are usually applied as thionates because of their low toxicity to humans and their relatively high solubility in lipids that enables them to penetrate the insect integument rapidly. Once inside the body, the formation of oxon analogues, which are highly toxic, is catalysed by MFOs. Malaoxon has approximately 2000 times greater anticholinesterases activity than malathion in *Cx tarsalis* (Matsumura and Brown 1961). Antiserum raised against purified P^{450s} of housefly inhibited the activation of chloropyrifos to its oxon analogue in housefly microsomes (Hatano and Scott 1993). The use of oxidase synergists such as PB in bioassays increased the level of resistance to organophosphates due to the inhibition of the formation of the toxic oxon analogues.

An increase in resistance to OPs was observed in *An. stephensi* (Hemingway 1982) and in *Cx quinquefasiatus* (Magnin et al., 1998; Khayrandish and Wood 1993a) after pre-treatment of the mosquito larvae with PB. Isoform specific inhibitors that have been identified for mammalian P^{450s} are considered to be useful for *in vivo* and *in vitro* studies as well as holding potential for therapeutic modulation of human drug metabolism (Scott et al., 2000).

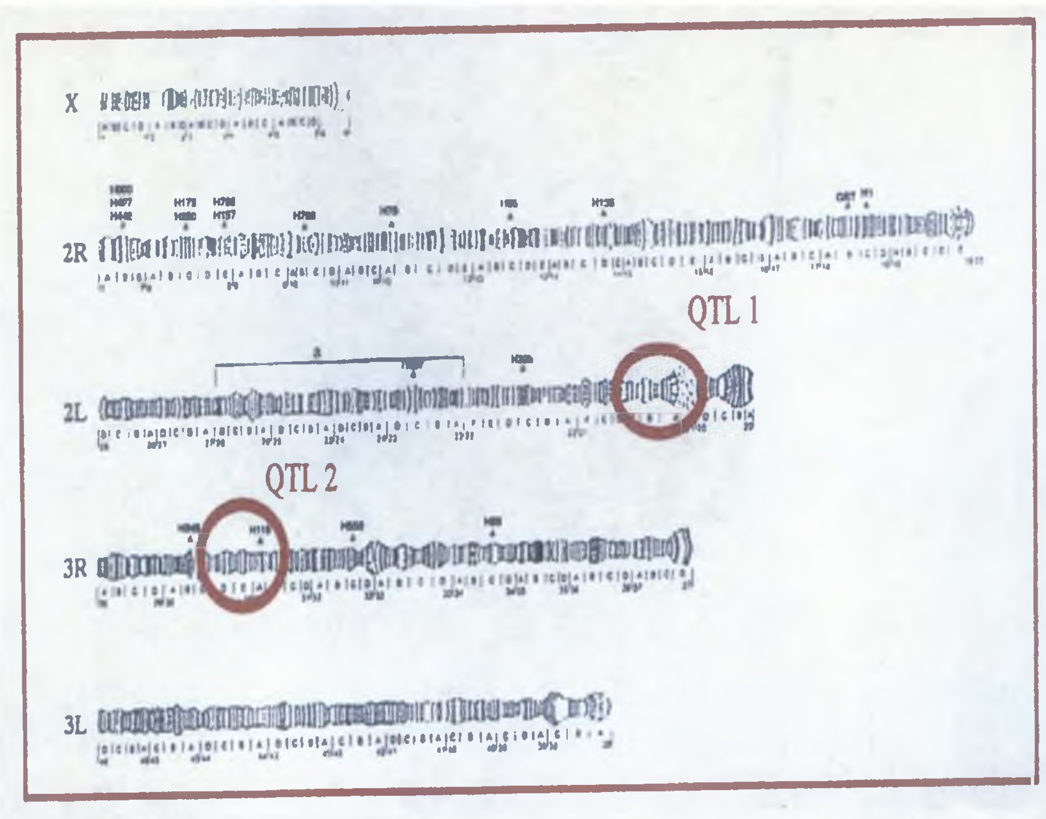


Figure 13. An *An. gambiae* s.s polytene chromosome map.It is showing the position of two quantitative trait loci for pyrethroid resistance. (Reproduced from Nikou et al., 2003).

Identification of specific inhibitors of insect P^{450s} could lead to the identification of selective insecticide synergists. Evaluation of inhibitors of individual insect P^{450s} have received little attention, even though several insecticide synergists have been identified together with elucidation of mechanism of inhibition for certain classes of synergists.

Alkynylarenes and methylenedioxyarenes were evaluated for their role in inhibition of housefly CYP6D1 (Scott et al., 2000). Their results showed that both ethynyl and propynyl substituted pyrenes are potent inhibitors of CYP6D1. The level of potency was dependent on the position of the substituent, with the greatest inhibition seen for the 4 position and the least for the 2 position.

Methylenedioxyphenyl compounds (Table 8) have been extensively evaluated as insecticide synergists in houseflies and other species (Scott et al., 2000), but the potency of these compounds against individual P^{450}s has received little attention. Piperonyl butoxide ($C_{19}H_{30}O_5$) and myristicin ($C_{11}H_{12}O_3$) were the most potent of the

methyleenedioxyphenyl CYP6D1 inhibitors. The length of the alkyl substitution caused considerable change in the inhibitory potency with $C_3H_7>C_6H_{13}>H>C_8H_{17}>C_{12}H_{25}$.

X	Y	Z	Compound	I_{50} [M][a]
$-CH_3CH_2CH_3$	$-CHCHCH_2CH_3$	H	Piperonyl	$4.3(2.1)x10^{-7}$
$-CH_3CHCH_2$	H	-OCH3	butoxide[b]	$6.5(0.8)x10^{-7}$
$-CH_3CH_2CH_3$	H	H	Myristicin	$1.4(0.5)x10^{-6}$
$-CHCHCH_2CH_3$	H	H	C_3MDP	$3.4(1.0)x10^{-6}$
n-hexyl	H	H	Isosafrole[b]	$7.3(3.7)x10^{-6}$
$-CHCHCH_2CH_3$	H	H	C_6MDP	$1.6(0.6)x10^{-5}$
H	H	H	Verbutin	$1.7(0.5)x10^{-5}$
H	$-CHCHCH_2CH_3$	H	C0MDP	$6.6(1.6)x10^{-5}$
n-octyl	H	H	M1[b]	$\sim10^{-5}$
n-dodecyl	H	H	C_8MDP	$>10^{-5}$
			C_{12}MDP	

Table 8. Substituted methylenedioxyphenyls evaluated as inhibitors of cytochrom CYP6D1. (Source: Scott et al., 2000).
[a]Values = the mean (SE) for a minimum of three replications
[b]Data from Scott 1996

Two OPs, malathion and parathion, were both substantially less potent CYP6D1 inhibitors than chlorpyrifos. Parathion is an inhibitor for CYP6D1 while another organophosphate insecticide (chlorpyrifos) is not. A possible explanation for the difference is that the putative intermediate formed during P^{450} mediated metabolism of OPs could preferentially lead to inhibition, that is, generation of thionophosphoric acid without inactivation of the P^{450}, as observed for chlorypyrifos. The size and shape of the aromatic moiety of the alkynylarenes, the position of substitution by the alkylnyl group and the presence of a terminal acetylene (ethyne) or a methyl acetylene (propyne) all affected the selectivity of the observed P^{450} inhibition.

The result of Scott et al. (2000) showed that it should be possible to develop new insecticide synergists, which inhibit insect P^{450}s. These inhibitors could be useful tools for enhancing the toxicity of insecticides to insects without increasing the toxicity to mammals.

Monooxygenase-mediated resistance is due to increased expression of one or more P^{450}s through increased transcription. Resistance may also be due to a change in the structural P^{450} gene itself (Scott and Kasai 2004).

An important question regarding cytochrome P^{450}s is how much plasticity exists within this family, in terms of the development of insecticide resistance to populations selected with the same insecticide. Will all populations utilize the same P^{450} or not? To answer this question, (Scott and Kasai 2004) investigated the genetic basis of resistance in a strain of housefly (NG98) from Georgia, USA that had evolved 3700-fold resistance to permethrin, and compared that to other permethrin resistant strains of houseflies from the USA and Japan. Their results indicated that the genes, which evolve to produce moonoxygenase-mediated resistance to permethrin, are different between different populations and that P^{450}s have some degree of plasticity in response to selection. They further concluded that this may present a problem in diagnostic assay development for monitoring monooxygenase-mediated pyrethroid resistance as the genetic basis of P^{450} mediated resistance is variable in different populations. This also shows that pyrethroid resistance can evolve using different P^{450} and possibly different regulatory signals controlling P^{450} expression even in strains selected with the same insecticide.

1.7.2 Altered Target Sites

There are three major target sites for current public health insecticides:

1. Acetylcholinesterase (AChE), which breaks down the neurotransmitter acetylcholine;
2. Ligand-gated ion channels (Rdl) that bind chemical signals, such as y-aminobutyric acid (GABA), which is then converted into electrical signals via the opening of their integral ion channels;
3. Voltage-gated channels, such as the sodium channel that are triggered by changes in membrane voltage rather than changes in the concentration of a neurotransmitter.

1.7.2.1 Acetylcholinesterase (AChE)

Acetylcholinesterase (AChE) is a glycosylated dimer attached to a membrane via a glycolipid anchor (Fournier et al., 1988; Chaabihi et al., 1994). The AChE enzyme catalyses the hydrolysis of the neurotransmitter acetylcholine. It is a major target for organophosphate and carbamate insecticides, which inhibit enzyme activity by convalently phosphorylating or carbamylating the serine residue within the active site gorge (Corbett 1974). Over expression of AChE in Drosophila (Fournier et al., 1992) was correlated with insecticide resistance. Point mutations accompanied by a modification of the kinetic parameters of acetylcholine hydrolysis, have been identified as responsible for insecticide resistance in several insects (Mutero et al., 1994; Zhu et al., 1996; Devonshire et al., 1998; Walsh et al., 2001; Kozaki et al., 2001). These mutations associated with resistance, involve substitutions at key sites located within the active site gorge of the enzyme. The altered amino acids have a steric effect or alter the orientation of the active sites, so that insecticides bind less effectively while the natural substrate acetylcholine can still bind (Mutero et al., 1994; Walsh et al., 2001). An exception to this is in *Leptinotarsa decemlineata*, the Colorado potato beetle, where substitution of a smaller amino acid, remote from the catalytic region of the enzyme, confers insensitivity through altering the secondary structure of the AChE protein (Zhu et al., 1996). In general the levels of organophosphate insensitivity conferred by each individual amino acid replacement are low, but in combination they produce increasingly insensitive enzymes (Mutero et al., 1994; Walsh et al., 2001).

Site-directed mutagenesis studies (Vaughan et al., 1997) confirmed that many potential resistance-associated point mutations may result in AChE insensitivity, without significantly reducing AChE catalytic efficiency. Mutations producing insensitive AChE have been reported in *Drosophila melanogaster* (Mutero et al., 1994), *Musca domestica* (Devonshire et al., 1998; Walsh et al., 2001; Kozki et al., 2001) and *L. decemlineata* (Zhu et al., 1996) to date.

Sequencing of the AChE genes in *Nepholitillisc* and *Boophilus microplus* indicated that the insensitive AChE phenotype in these insects does not result from amino acid substitutions in the AChE protein itself, suggesting the involvement of another mechanism (Baxter and Barker 1998). Vontas et al. (2001) reported a novel resistance

mutation in *B. olaeae,* providing strong evidence that many resistance-associated AChE mutations may occur in different insect species. Recently, Weill et al. (2004) detected a unique mutation in the ace-1 gene giving high OP resistance in *An. gambiae* s.s. The high AChE insensitivity in *An. albimanus* is the result of the same mutation G119S found in both *Cx. pipiens* and *An. gambiae* s.s. The mutation was detected in both the M and S forms of *An. gambiae* s.s from the Ivory Coast. This mutation is located in the oxyanion hole (Figure 14) and alters the active site by placing a serine at position 119, which reduces the access to the catalytic triad. It has been shown that the ability of organophosphates to inhibit acetylcholinesterase is in part due to the electronegativity of the central phosphate ion of the insecticide.

1.7.2.2 Altered GABA Receptor

The insect γ-amino butyric acid (GABA) receptor is the site of action of cyclodiene insecticides and second generation insecticides called phenylpyrazoles, such as fipronil, which block the chloride channel. It controls Cl^- flux across the nerve membrane. The GABA-receptor-subunit gene was cloned from a field-isolated *Drosophila* mutant that was resistant to dieldrin and was termed Resistance to dieldrin or Rdl (ffrench-Constant 1993). The resistance, conferred by a single gene, mapped to chromosome III at position 66F (ffrench-Constant and Roush 1991). It involves replacement of an alanine or a lycine at position 302 that play an important role in insecticide binding (Zhang et al., 1994). The same mutation has been found in *Ae. aegypti* (Thompson et al., 1993), *M. domestica* (Anthony et al., 1991) and *An. gambiae* (Brooke et al., 2000). The ligand-gated ion channels are also the site of action of insecticide classes, such as neonicotinoids and ivermectins (Clark et al., 1995).

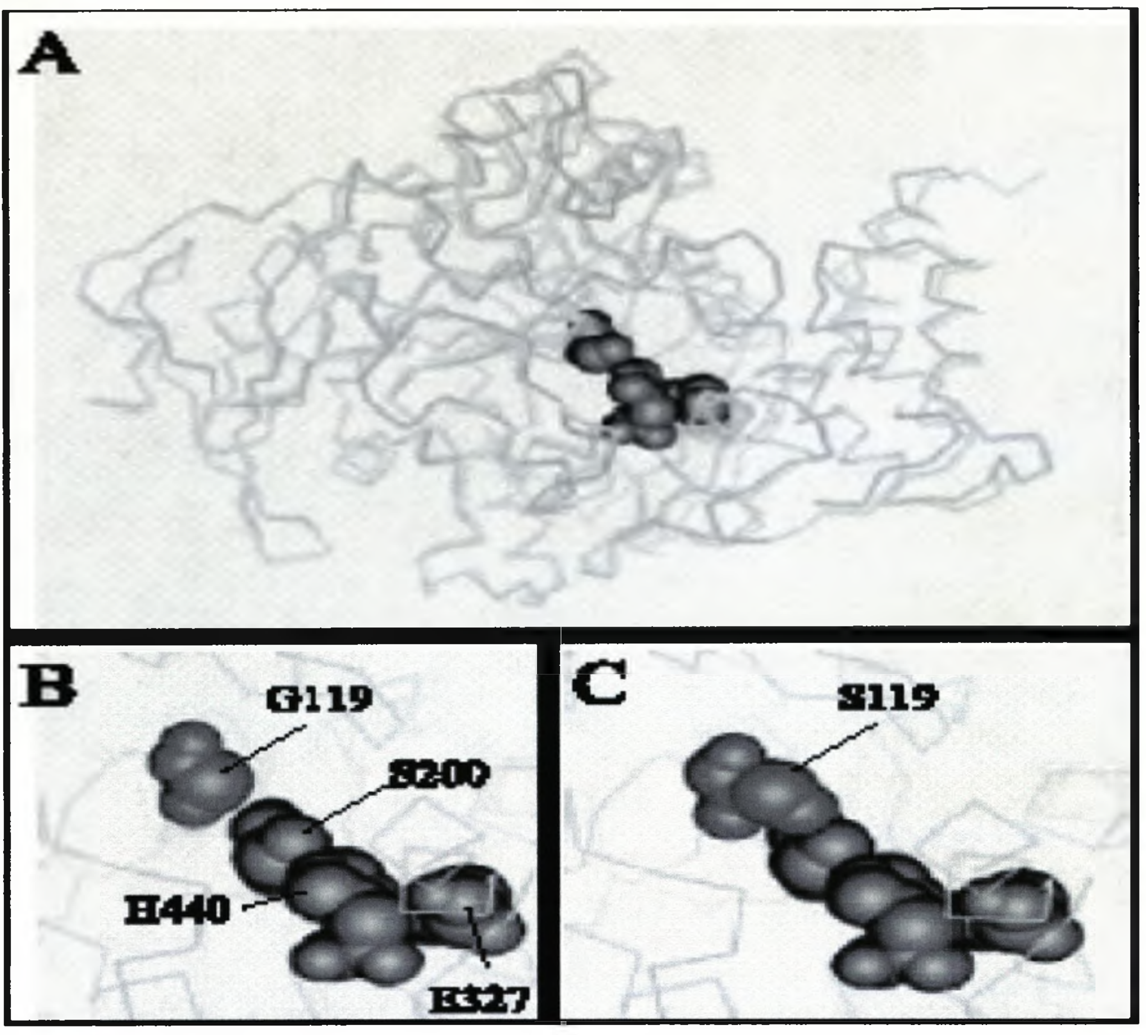


Figure 14. The position of the G119S mutation in ace-1 of *An. gambiae* s.s (A) is the alpha-carbon skeleton of the modelled 3D structure of ace-1 overlain on that of the AChE of *T. californicus*. The view is down the catalytic gorge. (B) is a close-up of the catalytic triad of ace-1 of the susceptible strain of *An. gambiae* s.s. showing G119 at the edge of the oxyanion hole. (C) is a close-up of the catalytic triad of ace-1 of the resistant strain of *An. gambiae* s.s showing the S119. (Source: (Weill 2004).

1.7.2.3 Sodium Channel- Voltage-Gated ion Channels

This volgate-gated channel, unlike the ligand-gated channels, is triggered by changes in membrane voltage rather than changes in the concentration of a neurotransmitter. The insect sodium channel is the site of action of DDT and pyrethroid insecticides. Resistance to pyrethroids due to changes in this target-site was first characterized as knockdown resistance (kdr) in houseflies (Farnham 1977). It can be produced by a single Leu – Phe substitution in the S6 segment of domain II of the sodium channel gene in *D. melanogaster* (Jackson et al., 1984), *M. domestica* (Williamson et al., 1996) and *Blattella germanica* (Hemingway et al., 1993; Dong and Scott 1994). Resistance to pyrethroids due

to the voltage-gated sodium channel type of mechanism (kdr) in the main malaria vector *An. gambiae s.s* was first reported in West Africa (Martinez-Torres et al., 1998). This resistance mechanism also confers cross-resistance to DDT. It results from a single point mutation (leucine TTA to phenylalanine TTT) in the sodium channel gene (Martinez-Torres et al., 1998). It gives the characteristic “knockdown resistance” or kdr phenotype. This type of resistance mechanism has been reported in the Ivory Coast and Burkina Faso (Martinez-Torres et al., 1998; Chandre et al., 1999) and Kisumu, Kenya (Ranson et al., 2000). The Kenyan mutation (RSP-ST) differs from the West African one, and involves the mutation of the same leucine to serine.

Cross resistance between DDT and pyrethroids in West Africa and the initial selection of this mechanism was associated with DDT and pyrethroid usage in cotton farming (Martenez-Torres et al., 1998). In Kenya the mutation was present before the introduction of insecticide impregnated bednets and therefore the initial selection was from agricultural use of pyrethroids or DDT based anti-malarial activities, rather than public health use of pyrethroids (Ranson et al., 2000).

1.8 Insecticide Resistance Management

Development and deployment of sound insecticide resistance detection and monitoring tools form an essential base for implementation of resistance management strategies for efficient and sustainable malaria control programmes. In this study, development of monitoring strategies initially involved looking at the available tools, both biochemical and molecular, that could easily be implemented by existing malaria control programmes in southern African countries based on their current infrastructure.

As part of the study, insecticide resistance was documented at a mechanistic level in field populations of the main malaria vectors, *An. arabiensis*, *An. gambiae* s.s and *An. funestus*. Target populations must be monitored for the magnitude of resistance, frequency of resistance genes and the effect of resistance on practical field performance of pesticide for control.

1.8.1 Resistance Detection and Monitoring

In this study, the initial evidence "baseline" for the presence of resistance in the three malaria vectors, *An. arabiensis*, *An. gambiae* s.s and *An. funestus* was undertaken in southern Africa. This included determining the relative importance of anti-malarial and agricultural pesticide usage in the selection of insecticide resistance and bringing together the communities and private and public organizations that are directly or indirectly involved with pesticide usage. This was facilitated to document the different factors that have a direct or indirect influence in selecting for insecticide resistance, especially from the agricultural sector. The initial step in determining the potential impact of resistance on control strategies is to detect changes in the susceptibility of a natural population. Initially in southern Africa, this was done through bioassays and now, biochemical and molecular tools have been implemented operationally through this study.

Monitoring of insecticide resistance in malaria vectors is an important activity that has to be performed along with other entomological studies. It involves a routine continuous programme of surveillance aimed at detecting and following the spread of resistance. This can include more specific, shorter-term investigations that are done either to gain initial or baseline susceptibility data before the use of a new insecticide, or to examine individual cases of suspected resistance indicated by obvious loss of field insecticide efficacy.

Resistance studies are useful for formulating suitable situation specific insecticide-based vector control strategies and most importantly for management of insecticide resistance in malaria vectors. In this study, spatial and temporal detection was performed to measure the development and the spread of insecticide resistance.

1.8.2 The Aims of Insecticide Resistance Detection and Monitoring

Resistance to insecticides (Figure 15) in insect pests of both medical and agricultural importance has resulted in increased insecticide application rates and this may in turn have resulted in environmental damage without necessarily producing the required reduction in diseases associated with both humans and animals (Liu and Scott 1998).

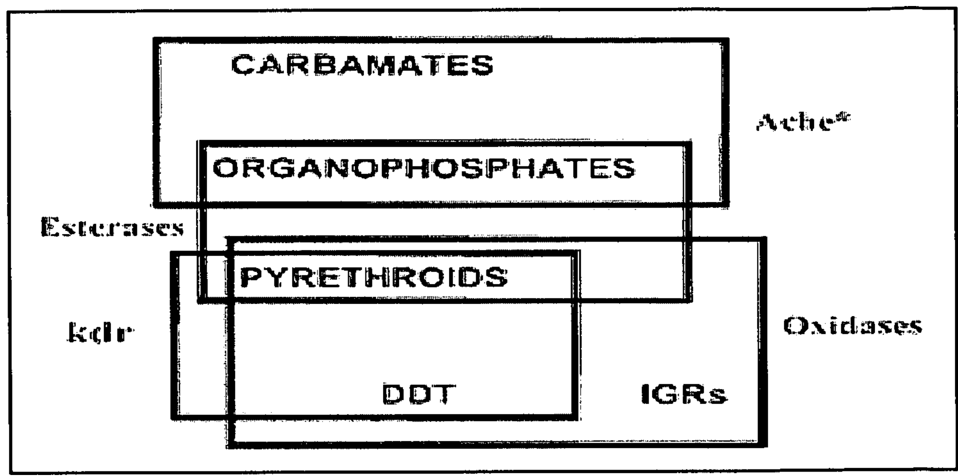


Figure 15. Resistance mechanisms to the four main insecticide groups. AChE and kdr represent target site mechanisms and esterase and oxidase represent metabolic mechanisms. (Source: Brogdon 1998).

An overview of insecticide resistance, detection and monitoring activities were summarised by (Brent 1986) as follows:

- Check for the presence and frequency of occurrence of the basic genetic potential for resistance (expressed resistance genes) in target organism populations.
- Gain early warning that the frequency of resistance is rising and/or that practical resistance problems are starting to develop.
- Determine the effectiveness of management strategies introduced to avoid or delay resistance problems.
- Diagnose whether rumoured or observed fluctuations or losses in the field efficacy of an agrochemical are associated with resistance.
- If resistance has been confirmed, determine subsequent changes in its incidence, distribution, and severity.
- Give practical guidance on pesticide selection in local areas.
- Gain scientific knowledge of the behaviour of resistant organisms in the field in relation to genetic, epidemiological and management factors.

A major aim of this study was establishing the resistance spectra present in southern African vectors. Where resistance had been reported before the inception of this study, for example in some South African vectors, further work was undertaken to determine the mechanism and current frequency of resistance. Biochemical and molecular tools were

used in this study to assist the current vector control programmes in determining the efficacy of their control programmes and to introduce effective management strategies to avoid or delay resistance problems. A problem mapping exercise was used and a stakeholders' forum established involving different, transdisciplinary community sectors in a participatory approach. This forum was used to help diagnose whether observed increases in malaria transmission were associated with the loss of insecticide field efficacy due to insecticide, resistance or other operational factors. Spatial and temporal mapping of the resistance was undertaken at a mechanistic level to determine the changes in resistance gene frequency and distribution. Resistance management was then implemented in southern Mozambique and South Africa based in part on the results of this study. The integration of bioassays, biochemical and molecular assays into monitoring systems have assisted the southern African malaria control programmes in avoiding the potential impact of resistance which may otherwise only have been detected after a rise in clinical malaria cases.

1.8.3 Resistance Management Strategies

Insecticide resistance is an ideal phenomenon for studying natural selection in field populations (Scott and Kasai 2004). The selection pressure is strong, the selective agent (the insecticide) is known, the evolution of resistance is rapid and the experimental populations can be easily manipulated.

Resistance studies also have practical relevance to operational control programmes. For example, chemical control programmes are likely to fail unless strategies for insecticide use that do not rely entirely on continuous use of a single insecticide are followed (Hemingway et al., 1993). The goals of resistance management with the tools available to date, is to delay or retard resistance selection in pests that are important in health and agriculture. Computer simulations and mathematical models have been employed for devising strategies to retard pesticide resistance in pests (Table 9). Most of these models assume that resistance to each insecticide is monogenic and independent of resistance to other insecticide classes (Tabashnik 1989).

Studies	Factors Emphasized		
	Biological	Operational	Economic
Anyalytical			
MacDonald, 1959	X		
Commins, 1997a	X		
Curtis et al., 1978	X	X	
Taylor and Georghiou, 1979	X	X	
Cook, 1981	X		
Skylakakis, 1981	X	X	
Wood and Mani, 1981	X	X	
Muggleton, 1982	X		
Simulation			
Georghiou and Taylor, 1997a,b	X	X	
Greever and Georghiou, 1979	X	X	
Plapp et al., 1979	X		
Kable and Jeffrey, 1980		X	
Curtis, 1981	X	X	
Taylor and Georghiou, 1982	X	X	
Tabashnik and Croft, 1982, 1985	X	X	
Levy et al. 1983		X	
Taylor et al., 1983	X	X	
Knipling and Klassen, 1984		X	
Dowd et al., 1984	X	X	
Optimization			
Hueth and Regev, 1974	X	X	X
Taylor and Headley, 1975	X	X	X
Guttierrez et al., 1976, 1979	X	X	X
Commins, 1997b, 1979	X	X	X
Shoemaker, 1982	X	X	X
Statistical/Empirical			
Georghiou, 1980	X		
Tabashnik and Croft, 1985	X		

Table 9. A summary of the models of insecticide resistance management strategies that have been published. (Source: Tabashnik 1986 where all the studies are referenced).

Tabashnik (1986) listed the key assumptions of resistance management models as:

- Resistance is controlled primarily by a single-gene locus with two alleles, R (resistant) and S (susceptible), with a fixed dose-mortality line for each genotype.
- The dose-mortality line for RS heterozygotes is intermediate between the SS (susceptible) and RR (resistant) lines. At low pesticide doses RS heterozygotes are not killed, and the R gene is effectively dominant; at high doses RS heterozygotes are killed, and the R gene is effectively recessive.
- The insect life cycle is divided into sub stages, with transition probabilities between sub stages determined by natural and pesticide mortalities.

- Immigrants are primarily susceptible and have at least one day to mate and reproduce before being killed by a pesticide.

Recent advances in research tools are now leading to more information on vector biology, resistance genes and vector behaviour (e.g. gene flow) being documented. This will assist in future manipulation of biological factors in resistance management programmes. This present study looked at how well operational and economic factors affecting resistance management can be captured. Information was gathered through a stakeholders' forum. This is expected to assist in developing an integrated pest and pesticide management and usage programme with the objective of sustainable and efficient insecticide-based vector control. To achieve this objective, vector resistance monitoring, agricultural pesticide usage and pesticide residue monitoring programmes have been developed.

These activities will promote interactions between communities and the environmental, health and agricultural sectors, which will lead to increased awareness, exchange of information and joint action in anti-malaria and agriculture activities.

Recommendations for policy decision making in both the agricultural and health sector will be made for appropriate interventions, which will result in effective malaria control. Technological improvement (pesticide choice) should also be adopted that will benefit the health and well-being of the local communities.

The response of resistant and susceptible populations to pesticide application is predicted (Figure 16), which models the effects of dose on the rate of resistance evolution when there are 0% to 100% immigrants daily, with two weekly insecticide treatments of adults. With no immigration resistance develops faster as the insecticide dose increases, but with immigration, of susceptible pests from untreated areas, two scenarios emerge (Tabashnik and Croft 1982):

- At low insecticide doses resistance develops faster as the dose is increased, being parallel to the case without immigration. This is determined by the rate at which the S genes are removed from the population.
- At high doses, resistance development is slower as the dose is increased.

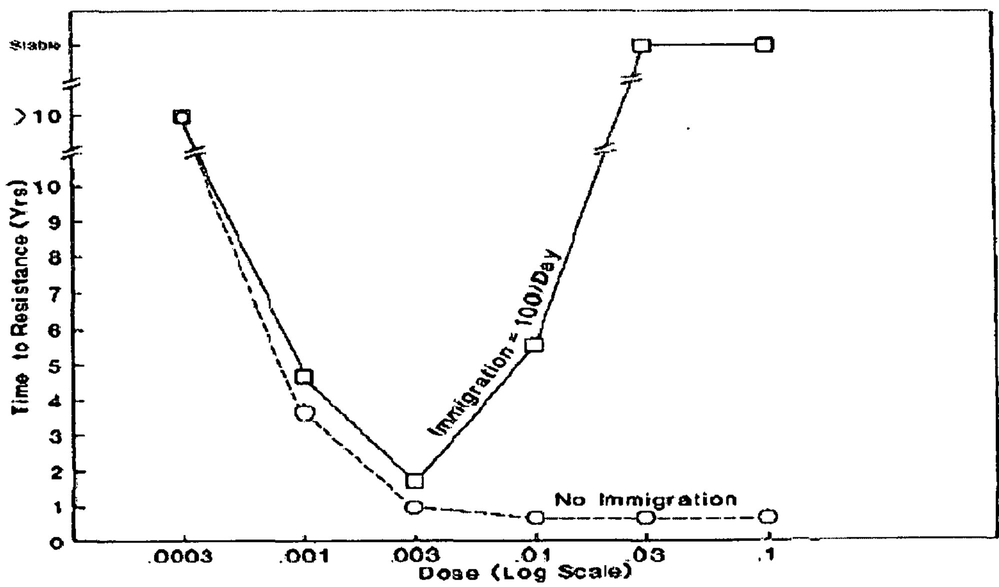


Figure 16. Effects of dose on the rate of evolution of resistance. Conditions: 0 to 100 immigrants daily, biweekly treatments of adults. (Source: Tabashnik and Croft 1982).

However this model may not mimic reality as in many areas a complex interaction of factors exists from area to area. Different populations will also have different factors as is observed in areas of high agricultural activity compared to those with low or no agriculture activity. A holistic approach is needed to study the influence of pesticide dose on the resistance selection operationally.

In the models, as the insecticide dose increases, S genes are removed rapidly and this leads to resistance being selected faster, as in the situation with low insecticide doses and immigration. In a situation where there is immigration combined with high doses and selection is high enough to kill RS heterozygotes, mortality from pesticide exposure also removes R genes from the population. More RS individuals are killed as the dose increases, leaving a small number of homozygous resistant (RR) individuals, which are then diluted by the incoming SS immigrants. This scenario retards resistance selection. Studies on field observations of resistance selection in soil and apple arthropod pests by Georghiou et al. (1980) and Tabashnik and Croft (1985) were consistent with the hypothesis that the number of generations per year is an important factor influencing the rate of resistance evolution, as suggested by model simulations.

The influence of various factors (Table 9) on resistance development highlights the interactions among factors. More information is now available on some factors. With

regard to biological factors, many resistance mechanisms can now be accurately monitored and therefore their frequencies can be well documented. Present studies on the molecular basis of insecticide resistance will provide more knowledge on the population dynamics of resistance and therefore new insights into the factors that determine the spread of resistance. The earlier that resistance can be detected, the greater the chance that management strategies can be effective in suppressing it.

Several authors have proposed possible resistance management strategies (Immaraju et al., 1990; Wood 1981). For example, rotation was first proposed by Coyne (1951), and computer simulations and models have been used to investigate these strategies. Most experimental work to test these models was laboratory-based. The predictive value of investigating resistance through laboratory selection is limited, as shown in the 1950s, when laboratory strains of houseflies were selected in Riverside, California for 19 to 149 generations with various OP insecticides. Only increases in low level tolerance were obtained, which bore no resemblance to what later developed in the field (Pal and Brown 1971 in Johannes Keiding 1986). A mathematical model to analyse the relationship between pesticide dose and the rate of development of resistance was developed by Birch and Shaw (1997). They found that mixtures only reduced the build-up of pesticide resistance if the dosages of pesticides in the mixtures were low. However, with mixtures, both components have to be used at their toxic dose, which reduces the practicality of this model.

Lenormand and Raymond (1998) proposed a stable zone strategy for resistance management, which could be used even if resistance genes were already present. The stable zone strategy consists of applying an insecticide in a treatment area that is smaller than the critical size of the adaptive pocket. This strategy considers different factors that can be manipulated, i.e. the size of the treatment area, the dose of insecticide, the proportion of treated sites within the treated area and the quality of the environment in the treated area relative to the untreated area.

The size of the treated area relative to gene flow in the insect population is a critical factor in the rate of increase in frequency of resistance genes (Lenormand and Raymond 1998). The stable zone strategy could be extended to mosaics, by using different insecticides

associated with different resistance genes (Lenormand and Raymond 1998). This type of mosaic strategy would need to be optimized as follows:

- By avoiding the selection of closely linked resistance genes in adjacent areas;
- By avoiding treating adjacent areas with insecticides that may select for resistance genes that show cross-resistance;
- By changing the distribution of insecticides through time, as directed by monitoring of resistance gene frequencies.

If the stable zone strategy is undertaken as a rotation of insecticides, using two or more insecticides with different modes of action and different mechanisms of resistance in a planned time frame, the period of rotation of insecticides and the generation time, play an important role (Lenormand and Raymond 1998). The relative performance of mixtures and rotations for delaying resistance have been extensively compared in models under varying hypotheses as preferable to mosaics (Commins 1986; Curtis and Lines 1985; Mani 1985; Mani 1989; Roush 1989; Roush 1993; Tabashnik 1989).

Lenormand and Raymond (1998) suggested how the efficiency of disease control by insecticides increases with a lower period of rotation of insecticides per generation time (Figure 17), so that mixtures should be a favourable strategy. However he cautioned that the advantage of a mixture should be weighed against the economic or ecological cost of increased insecticide usage.

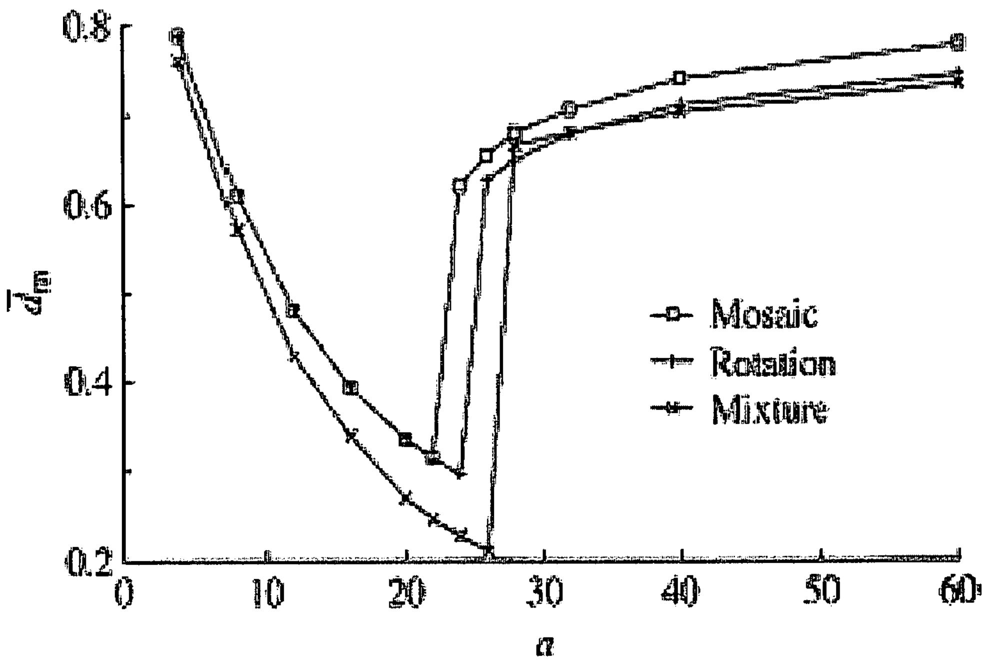


Figure 17. Shows average density ($\bar{d}_m$) after migration in a treated area of size α(km), at equilibrium. The treated area is sub-divided into two equal sub-areas where two different insecticides are applied. In the mosaic, each sub-area always receives the same insecticide, in a rotation each sub-area alternately receives each insecticide, and in a mixture each sub-area simultaneously receives both insecticides each generation. (Reproduced from Lenormand and Raymond 1987).

1.8.4 Field Based Resistance Management Strategies

Some computer simulation and mathematical models for resistance management strategies have been tested using a silverleaf whitefly field population (Prabhaker et al., 1998). They were selected for resistance by continuous applications of bifenthrin, endosulfan or chlorpyrifos and rates of resistance development were compared with the rotation of these insecticides or 1:2 bifenthrin-endosulfan mixtures. Their results showed a delay in the insecticide rotation and mixture treatments for an additional 10 generations over the continuous use treatments. Rotation of two insecticides did little to slow resistance development in the housefly (MacDonald et al., 1983). The requirement for activation of organophosphorus insecticides by monooxygenases led to the idea that rotation of a pyrethroid and organophosphorus insecticide could, in theory, slow the evolution of resistance (Scott 1999). This is based on the principle that the pyrethroid would select for increased levels of the monooxygenase cytochrome P^{450} CYP6D1, while an organophosphorus insecticide would select for reduced the levels of CYP6D1 and therefore retard or delay the evolution of this pyrethroid resistance mechanism. However, this has yet to be properly field tested.

The major field trial of insecticide resistance management in *Anopheles* is that of Penilla et al. (1998). This study consisted of twenty-four villages in Chiapas, Mexico. These villages were divided into eight groups of three villages. Each village group was physically separated and did not share common mosquito breeding sites. The total experimental site covered over 100 kilometres with villages from two to twenty kilometers apart. Three cycles of insecticides treatment were carried out per year, except for DDT, where only two cycles were applied annually due to the longer residual effect of this insecticide. The controls consisted of four groups of villages, two groups treated continuously with the pyrethroid deltamethrin and the other two groups treated with DDT throughout the seven years of intervention. The rotation strategy was based on the use of three unrelated insecticides, each one rotated annually, a carbamate, a pyrethroid and an organophosphorus insecticide. The organophosphorus and carbamate insecticides are different chemical classes, but share a common target site, hence they were always separated by a year of pyrethroid treatment within the rotation. The mosaic strategy involved two unrelated insecticides, an organophosphate and a pyrethroid, which were sprayed separately in half of the houses in each village during the seven years of the treatment.

Initial frequencies of resistance genes present in *An. albimanus* were obtained in a year of baseline studies before the treatment regimes began. In this Mexican study resistance in *An. albimanus* is a complex mix of numerous resistance mechanisms, some of which interact together to produce multiplicative effects on resistance levels.

After the first three years of the resistance management strategies in the Mexican study the *An. albimanus* population responded to continued DDT pressure and the gradual decline of agricultural insecticide pressure over several decades by producing a decline in P^{450} and non-specific esterase levels, both of which may confer resistance to pyrethroids. Altered AChE and GST activity were marginally reduced by continuous DDT use. However, although *An. albimanus* is highly resistant to DDT, DDT could still be actively selecting for kdr-based resistance, by a mechanism that will confer cross-resistance to pyrethroids.

The continuous use of a single pyrethriod produced increased levels of pyrethroid resistance, a decline in altered AChE gene frequency and GST activity levels, but did not affect the P^{450}s and esterase levels. The rotation strategy gave the best results reducing

GSTs levels and altered AChE frequencies and P^{450}s, and only selecting low levels of pyrethroid resistance compared to the pyrethroid treatment alone (Penilla et al., 2001). However, after three years of intervention the non-specific esterase levels declined most under the mosaic strategy. The mosaic was as good as the rotation strategy in decreasing the altered AChE frequencies and P^{450}s levels although it was not as good as the rotation in reducing GST activity levels. It selected less resistance than the single use of DDT. The mosaic strategy has caused much debate as a strategy for resistance management. It uses a spatial application of insecticides pieced together (Tabashnik 1990). The mosaic, however, worked well in the Mexican study.

1.8.5 Lessons Learned from the Models

Insecticide selection in a treated area should reduce the density of a pest (Lenormand and Raymond 1998) suggested that environmental manipulation to reduce the quality of the environment for the pest, reducing the carrying capacity of the area or the growth rate of the pest, should be viewed as a primary long-term strategy for population control. In the stable zone strategy, each resistance gene should only reach a low frequency and will therefore be present predominantly as heterozygotes. An immediate improvement of the strategy would therefore be to use a dose of a fast decaying insecticide that kills heterozygotes (RS).

Resistance can be selected at different rates between species and even between populations of the same species due to genetic, reproductive, behavioral, ecological and operational factors (Georghiou and Taylor 1977; Georghiou et al., 1980; Wood and Bishop 1981). Operational factors can be easily manipulated but other factors may be beyond our control, e.g. migration in and out of treated habitats. Croft (1984) attempted to manipulate this factor, by experimenting with pheromones to attract higher numbers of susceptible individuals into the treated habitats. The quantity of insecticide taken up by the insect may also be manipulated. The quantity of insecticide taken up by the insect may also be manipulated. Dominance of resistance was considered as an unchanging genetic property until Wood and Cook (1978), Curtis et al. (1978) and Taylor and Georghiou (1979) recognised that dominance might be modified according to the insecticide dose applied (effective dominance).

Influencing the reproductive rate of arthropods to reduce the number of offspring per generation or the number of generations per year may reduce the need for multiple insecticide applications, an example that illustrates that some genetic, reproductive and behavioural/ecological factors have operational components. Substitution of the term "agronomic/control" for "operational" was suggested by Leeper et al. (1986). Agronomic refers to the various cultural practices in cropping systems. The capture and documentation of these practices can only be achieved by detailed interactions with stakeholders. Such interactions were initiated during this PhD study.

1.8.6 Other Operational Influences

To investigate the factors responsible for resistance selection in *Anopheles* in southern Africa, this study looked closely at all operational factors that contribute to the problem of resistance management, and are thus expected to influence the implementation of planned resistance management strategies. The most important organizations likely to be involved are: extension services, pest-management consultants and farmers, regulatory agencies, the pesticide industry and international organizations.

The cooperative extension service needs to take a leadership role in developing educational programmes in the area of pesticide resistance management, coordinating input from state agricultural experimental stations, pest control advisors, the pesticide industry, commodity associations, regulatory agencies and end users. Regulatory agencies can influence decisions by addressing the problem of what alternative pesticides are available when insecticide resistance occurs, and what is the appropriate role in decisions on insecticide deployment of state and provincial agencies that currently regulate pesticide use. Directions for use or prohibitions against certain practices might be added to pesticide labels to prolong the useful life of a pesticide. Shortcomings of these agencies that inhibit integration of resistance management into regulatory agency decision-making are taken up in the discussion section.

International organizations like the Food Agricultural Organization (FAO) regard resistance problems and related strategies as an inherent part of an intergrated pest management (IPM) programme. WHO programmes for detecting and monitoring resistance, involve interpreting and analysing test results, feedback, periodic reporting and

follow-up advisement to member countries. The World Bank, which may affect resistance to pesticides through its investments in agricultural development or public health projects, that provide funds for pesticide purchase may also have a role to play in improved implementation of resistance management programmes. The envisaged IPM route of insecticide resistance management through the judicious use of pesticides is vital for long term effective insect control, which not only requires the continued use of existing insecticides, but also needs the continued search for availability of new insecticides. The decline in the rate of insecticide discovery and development is a result of the cost of insecticides discovery, increased costs of registration, increased costs of production and increased competition (Hammock and Soderlund 1986).

1.9 Specific Background to the Study Area

Malaria is one of southern Africa's most serious diseases, which affects the health and well-being of local communities and impacts on tourism, agriculture and industrial development in the region. The emergence of insecticide resistance poses a major problem to the efficient control of medical, veterinary and agricultural insect pests by insecticides. The region relies heavily on residual house spraying for malaria vector control and increasingly on insecticide treated bed nets (ITNs) (Tables 1 and 2). Prior to this study there was little or no expertise in southern Africa to assess possible insecticide resistance in natural populations.

South Africa, for example, is at the southern end of the sub-Saharan malaria distribution and experiences seasonal epidemic malaria (November to May). Malaria also occurs in agricultural areas due to high rainfall and mild temperatures. The highest malaria incidence in South Africa occurs in northern KNZ, which adjoins Mozambique to the north. The disease occurs in the low-lying areas of the country, which mainly border neighbouring countries. As a result of cross border movement for a variety of social, political and economic reasons, there is an increasing awareness of the need to address malaria as a regional and not a country-specific problem. This prompted the formation of the Regional Malaria Control Commission (RMCC), a body of scientists, public health specialists and control programme staff who are tasked with addressing the problems associated with malaria in the Lubombo Spatial Development Initiative (LSDI) region (see www.malaria.org.za). The LSDI is a three-country initiative involving Mozambique,

South Africa and Swaziland. Swaziland and South Africa have had a malaria control initiatives in place for many years, but control in Mozambique has, until recently, only been undertaken in the major urban areas.

1.10 Background to this and related studies.

This PhD came about as a result of the MIM/TDR funded project in southern Africa to establish expertise to assess insecticide resistance in natural populations of the malaria vectors. The first phase of the project was to identify collaborators from the southern African region. This was done by myself with assistance from Dr. Brian Sharp, Director of the National Malaria Research Programmeme, Medical Research Council, South Africa. The Project proposal was written by myself, Dr. Brian Sharp, Professor Janet Hemingway (Director, Liverpool School of Tropical Medicine) and Professor Maureen Coetzee (Head, Department of Entomology, University of the Witwatersrand, South Africa). The second phase of the project was:

1. To train two people from South Africa in insecticide resistance diagnosis techniques to enable a capacity for these techniques in the region (myself and Dr. Basil Brooke).
2. To transfer this capacity to the collaborating partners in southern Africa.

The collaborating partners included three malaria research institutes (Mozambique, Zambia and Zimbabwe) and the three malaria control programmes, Swaziland, Botswana and Namibia. Information from Namibia and Zimbabwe are not included in this PhD, as no collections were made during the study period. The initial training of Dr. B. Brooke and myself took place at Cardiff University in 1999 for six months. The subsequent transfer of skills to the other African partners by myself, assisted by Dr. Graham Small and Dr. B. Brooke, took place in February 2000. This allowed the collaborators to carry out collections in their respective countries during the malaria season. The skills transferred included the use of Geographical Position Systems (GPS), field collection methods, susceptibility testing with the standard WHO susceptibility tests and molecular and biochemical assays. Professor Janet Hemingway attended an initial workshop (Appendix 2). Dr. Graham Small then spent four weeks to further assist myself in implementing the training programme on laboratory techniques. A further training

workshop ran from the 17th June to the 17th July, with the assistance of Dr. Graham Small for the three collaborators from the three malaria research institutes (Mozambique, Zambia and Zimbabwe). This PhD together with Dr. Brooke's PhD (completed 2003) and Ms Sonia Casimiro's MSc dissertation (completed 2003) were the result of the above-mentioned MIM/TDR sponsored project in insecticide resistance in southern African malaria vectors.

1.11 Objectives

The objectives of this study were as follows:

1. To establish entomological baselines for operational resistance management programmes, first through a small-scale programmeme in Gabon.
2. To monitor the development and spread of insecticide resistance in southern African mosquito vectors.
3. To determine the relative importance of anti-malarial and agricultural pesticide usage in the selection of insecticide resistance.
4. To help establish a rational policy for insecticide choice and use for southern Africa, on the basis of the above.
5. To develop a national capacity in various southern African countries for utilization of molecular and biochemical tools for resistance monitoring.
6. To integrate the molecular resistance detection tools with molecular techniques for species identification, blood meal analysis and parasite infections in the vector within the African laboratories, to facilitate monitoring and evaluation within the regional LSDI programme.

CHAPTER 2

Monitoring the development and spread of insecticide resistance in southern African mosquito vectors with biochemical and molecular tools

2.1 Introduction

As already reviewed in Chapter 1, malaria transmission varies considerably between and within countries (see figures 1-3 and section 1.1.2). Botswana, South Africa, Swaziland are in unstable malarious areas with shorter malaria transmission seasons, while Mozambique, Zambia and Gabon are countries with stable malaria transmission. The three countries with unstable malaria transmission are prone to epidemics, which can result in high levels of morbidity and mortality but are usually prevented or contained by to their efficient malaria control programmes. Swaziland also has a risk of highland malaria (see section 1.3).

All the above countries are or have been involved in extensive malaria control measures that are influenced by the type of malaria transmission in the country (see section 1.4.2, Figure 7, Tables 1 & 2) and factors that could contribute to the success or failure of implemented malaria control strategies have also been outlined (section 1.8.6). Resistance to the insecticides used in public health and agricultural sectors is a major factor influencing the efficiency of the insecticide-based vector control. This Chapter describes a study of the resistance status of three major malaria vectors in southern Africa and how the data can be used to influence insecticide usage policy in Mozambique and South Africa. This approach, combined with the present and continuing molecular understanding of resistance (see section 1.7.1), could also help to develop novel strategies with which resistance could be efficiently managed in the southern African region. Presently the emphasis in research is in resistance, but this, coupled with the knowledge of the biology, ecology and behaviour of the disease vectors, should lead to a better understanding of the molecular basis of resistance and ultimately to implementation of better resistance management strategies, with a view to efficiently control the development and spread of resistant vector populations (see section1.8).

Using one insecticide until resistance becomes a limiting factor or changing insecticides without prior baseline knowledge of resistance on the ground, has serious implications for efficient vector control through insecticide usage. Resistance management strategies such as rotations, mosaics and mixtures (see section 1.8.4) have been proposed. These were based on numerous mathematical models produced to estimate how these strategies

should be used (see section 1.8.3, Table 9). However, these models have rarely been tested under field conditions for insect vectors, except for a large scale Mexican trial (see section 1.8.4 p. 64), due to the practical difficulties in estimating changes in resistance gene frequencies in large samples of insects. Prior baseline knowledge of resistance on the ground is of vital importance in changes planned for resistance management strategies.

Estimation of resistance gene frequencies has been made easier by the development of biochemical and molecular techniques for resistance gene frequency estimation (explained in detail in section 2.3.4 & 2.3.5) and have assisted in the Mexican field trials of resistance management strategies (see section 1.8.4). These techniques have now been fully implemented in southern Africa in order to implement efficient vector control with judicious use of insecticides.

2.2 Description of Field Sites

2.2.1 South Africa

Intensive adult and larval collections were undertaken in the three malaria endemic provinces of South Africa, namely Limpopo, Mpumalanga and (KZN). These macro- and micro-geographic collections were used to develop a robust baseline data set for determination and mapping of resistance gene frequencies throughout the malaria endemic areas of South Africa. Data collection sites were chosen to allow identification and mapping of permanent breeding sites in both high-risk malarious areas and intensive agricultural areas (Figure 18). The study sites included areas targeted for future agricultural developments. The most detailed spatial and temporal collection (micro-geographic) was undertaken in two districts, Ingwavuma and Ubombo in northeastern KZN (Figure 18).

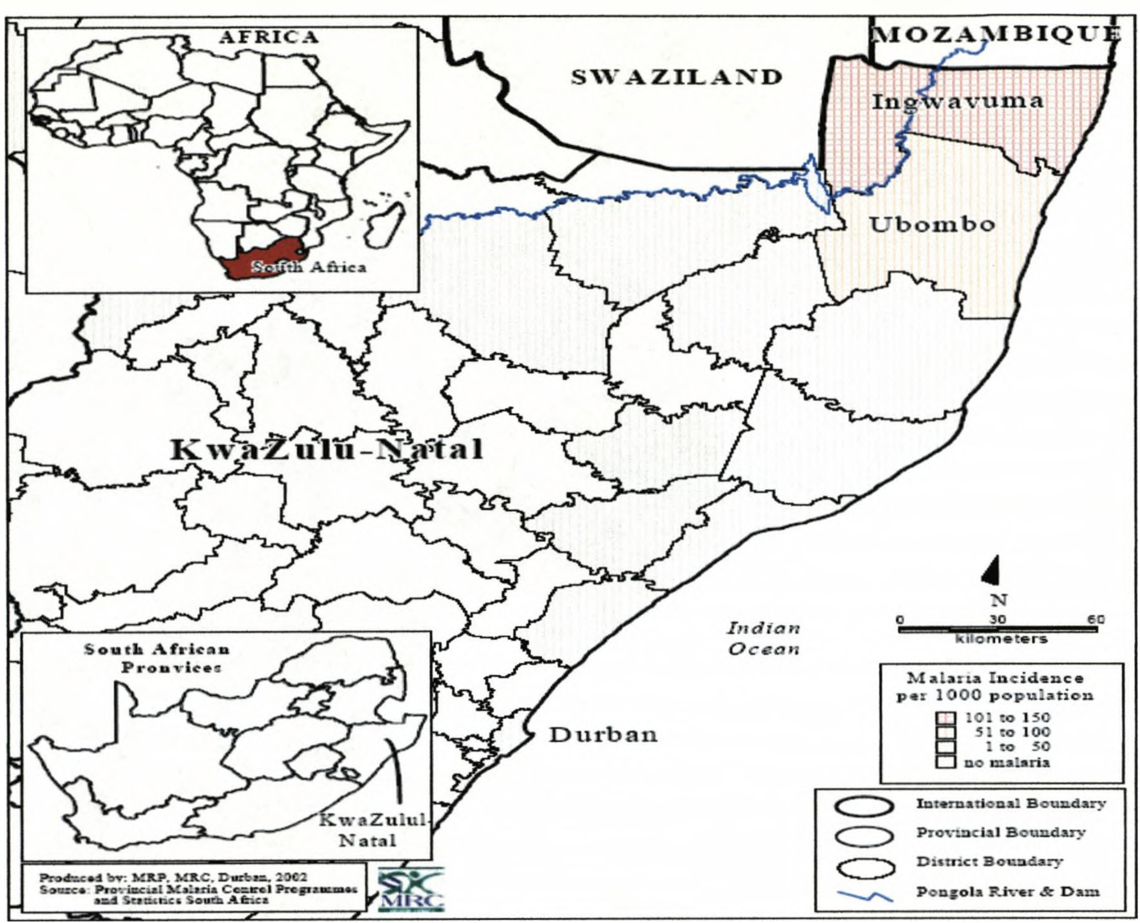


Figure 18. Shows the two study districts, Ingwavuma and Ubombo in north-eastern KZN. (Source: Malaria Research Programmeme, MRC, South Africa).

The KZN province is divided into administrative units, with the two northern districts of Ingwavuma and Ubombo experiencing the highest malaria incidence in the country. Sentinel sites were established in Kwa-Jobe, Makhathini, Pongola, Mamfene, Ndumo and Mzinyeni villages. The study sites are situated in the low-lying, semi-tropical malarious area of KZN. Within the area small and large-scale agriculture is practiced. The farming includes cattle as well as cotton, sugar cane, wheat and various vegetables crop production. This rural area has historically not experienced high levels of economic and infrastructural development, but is now being specifically targeted by the government for agricultural and tourism development. Subsistence farming by the local community is supplemented by the introduction of cash crops, such as sugar, cotton and vegetables, all of which use pesticides to enhance their productivity. Farming activities are predominantly carried out by women due to the migration of men for economic reasons. A Cotton Ginny is in the final stages of construction in anticipation of receiving large quantities of raw cotton from local farmers. This increase in cotton farming will also see a major increase in pesticide usage. Insecticide usage in South Africa is primarily

dependent on crop production, each farmer deciding on the amount, and type of insecticide used based on the crops produced.

2.2.2 Mozambique

Mozambique is located on the east coast of the southern Africa region (Figure 19). It is bordered by Tanzania to the north, Malawi, Zambia and Zimbabwe to the west and South Africa and Swaziland to the south. It is an agricultural country, mostly involved in subsistence farming. Farming in Mozambique includes maize, rice, cassava, sorghum and cash crops being mainly cashew nuts, coconut palms and cotton.

The country has two main seasons, the hot and wet season from November to April and the dry and cool season from May to October. Mozambique is very prone to floods. It experienced severe floods in February 2000, when heavy rains in southern Africa caused extensive flooding, especially in the Boane, Bela-Vista and Catuane districts, located in the southern part of the country where collections for this study were made. It shares thirteen river basins with the rest of the southern African region. Figure 19 (http://www.un.org/Depts/Cartographic/map/profile/mozambique.pdf) below shows the three most important river basins, being the Limpopo, Zambezi and Save. Sources for most of these rivers are in the higher elevations of Zambia and Zimbabwe, heading towards the Indian Ocean via the lower elevations of Mozambique.

Figure 19. Map of Mozambique. The geographical location of Mozambique in relation to South Africa, Zimbabwe, Zambia, Malawi and Tanzania.
(Source: http://www.un.org/Depts/Cartographic/map/profile/mozambique.pdf).

2.2.3 Swaziland

Swaziland, a former British protectorate, got its independence in 1968. It is a small country occupying a land area of 17.364 km^2, landlocked between South Africa and Mozambique (Figure 20). The country experiences a sub-tropical to near temperate climate. Larval collections were undertaken in the southern part of the malaria endemic eastern part of the Manzini province. Sentinel sites were established in the Shiselweni and Lubombo districts. Sampling was undertaken in Lavumisa in the Shiselweni district and Vuvulane, Lomahash and Big Bend in the Lubombo district. Detailed micro-geographic collections were made in Big Bend in Mahlabathini, Nasaline, Sitilo, Tamboti and Maplotini villages.

Tamboti is a major citrus commercial farming area, with Maplotini involved in small-scale cotton farming. Big Bend lies on a big bed in the Usuthu River where it meets the Lubombo Mountains. It is ideal for irrigation of a large area of low-lying bush, which has made it a centre for sugar production.

Figure 20. Map of Swaziland. The red arrows show areas where mosquito collections were made during this study in two discricts Shesilwene and Lubumbo.
(Source: http://go.hrw.com/atlas/norm_htm/swazilnd.htm).

2.2.4 Zambia

Malaria is endemic throughout Zambia and is the leading cause of morbidity and mortality in children under five. Prior to 1970, malaria in urban areas in Zambia was kept to a minimum by an effective prevention and control programme. In the late 1970s the Zambian Government could no longer support control efforts due to economic difficulties and the increases in malaria incidence and death rates from 1976 to 2000 reflect this breakdown in prevention and control measures.

The potential adverse economic effects of malaria prompted the Konkola Copper Mines to embark on a control programme covering Chililabombwe and Chingola towns on the Copperbelt, and Nampundwe in Lusaka West, Zambia.

Mosquito collections were made in collaboration with Ms. Violet Siachinji, being the WHO collaborator and myself during January and May 2001 and April 2003. The May 2001 collections collections were initially unsuccessful as these were done during the winter season when mosquito densities were low. Ms. Violet Siachinji undertook training in field collection techniques at the Medical Research Council in South Africa. Mosquitos were collected in district of Chingola, on the Copperbelt. Permanent breeding sites were mapped and documentation of pesticides usage documented in the small-scale farms around the mining area. Areas covered were Chililabombwe, Kapisha, Msinga, Lubengele-RBW section, Chabayama, Mimbula, Lulamba, Kapoto and Chiwamba (Figure 21).

Collections were also made in Kitwe and Fiwale in the Ndola district, which consisted mainly of adult blood fed female mosquitoes. Fiwale is involved in small scale vegetable farming activities, with the products sold on fruit and vegetable markets around Ndola town (Figure 22 a, b, c & d). Further collections were made in the Kabwe province, which is an important producer of most of the arable crops marketed in Zambia. Maize is the major staple crop accompanied by other cash crops like sunflower, cotton, soybeans, wheat, tobacco and groundnuts. Collections were made in Meliya's village, Wangwa farm (Lendor Agricultural Holdings, LTD, Rose farm) and Mulembwe farm – a tomato farm. Further collections were made in the northwestern district of Mwinilunga, which lies between Angola and the Democratic Republic of Congo (DRC).

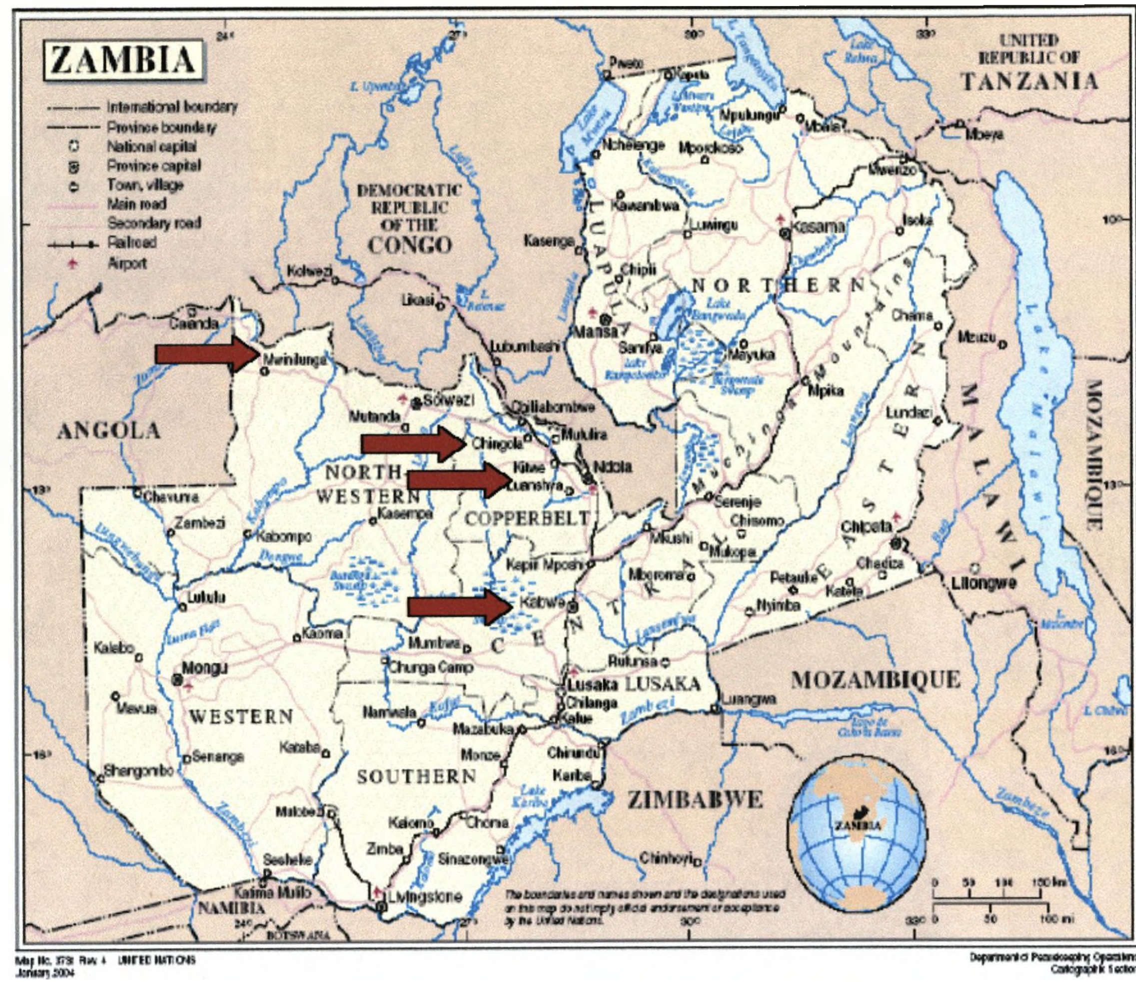


Figure 21. Map of Zambia. Red arrows show collections sites in the four districts, Mwinilunga, Chingola, Ndola and Kabwe.
(Source:http://www.un.org/Depts/Cartographic/map/profile/zambia.pdf)

A. An irrigated garden without mosquito breeding sites.

B. Cabbage garden with standing water bodies.

C. Overflowing cattle water troughs creating breeding sites

D. larval collections in between the cabbage garden

Figure 22. (A-D).*An. gambiae* s.s man-made breeding sites in Fiwale.The types of breeding sites found in Fiwale, Ndola, Zambia, where larval collection were made.

2.2.5 Botswana

Botswana is in south-central Africa, bounded by Namibia in the west, Zambia in the North, Zimbabwe in the east and South Africa in the south (Figure 23). Most of its land mass is desert with the Kalahari occupying the western part of the country. The eastern part is hilly, with salt lakes in the north. Botswana has four ethnic groups, with Batswana making the largest percentage followed by Bakalanga, Basarwa and Bakalgadi. Samples were collected in two areas, Shorobe and Samedupe, in the Maun district (Figure 23).

These areas are on the fringes of the Okavango Delta. Aerial spraying with endosulfan and deltamethrin for tsetse control was carried out in the delta from the 1971 to 1991. The two areas are involved in substantial cattle farming with very little cotton farming occurring in Shorobe. Mr. Mokgweetsinyana (Malaria Control Officer) was trained in field collection techniques at the Medical Research Council (Appendix 1). Mosquito

collections in Botswana were undertaken by myself and Mr. Mokgweetsinyana during the March 2000 malaria season.

Figure 23. Map of Botswana. The Red arrow shows Maun district where mosquito collections were made during this study.
(Source: http://www.infoplease.com/atlas/country/botswana.html).

2.3 Mosquito Sampling and Analysis Methods

2.3.1 Mosquito Collections

Collaborations were established with the control programme personnel in South Africa, Swaziland and Botswana as well as with two research institutes in Zambia and Mozambique that interfaced with their national control programmes. As most of these countries did not have trained manpower at the start of this study, it was necessary to provide training and guidance for the collaborators over a two-year period. Initial training was performed through a workshop held at the Medical Research Council in Durban, South Africa (Appendix 2). Country specific plans were established outlining the dates,

localities and parties responsible for mosquito field collections during the first workshop (Appendix 2). All the countries involved in the study were supplied with WHO insecticide susceptibility test kits in year one to enable rapid baseline susceptibility testing of the mosquitoes collected and to allow the collaborators to gain practical experience before a second workshop in year two. Standard WHO discriminating dosage papers treated with DDT, deltamethrin, permethrin, malathion and propoxur were supplied, as well as field data collection sheets and detailed methodologies.

Collections of both adult and larval stages of mosquitoes were undertaken. The exact timing of collections for each country was established according to the countries malaria transmission season. Practical Geographic Positioning System (GPS) points were taken at all collection sites.

Where this was not possible geo-referencing of localities were undertaken for later cross-referencing. The author took part in all the mosquito collections in southern Africa. All collected samples were transported to the Medical Research Council in South Africa for processing and analysis. Below is the description of collection methods employed during this PhD.

The collection methods used were as follows:

- Larval collections
- Indoor resting
- Window traps
- Outdoor resting (Pit-traps and natural refuges)
- Landing outdoor catches

Larval collections were done in all the study countries except in Mozambique. South Africa, Swaziland, Botswana all have *An. arabiensis* as the main malaria vector. Due to *An. arabiensis* exophilic behaviour, larval collections were deemed the best method of collection. Time during the collections in Botswana and Swaziland did not allow the implementation of window traps, pit-traps and collections at natural refuges. In South Africa, the collection methods included window traps, but no indoor resting and landing outdoor catches were performed. Only indoor resting catches were performed in

Mozambique due to the anthropophilic behaviour of the *An. funestus*. In Zambia, both larval and indoor catches were performed. In Fiwale, Ndola, and Mwinilunga in Zambia (Figure 21), only indoor catches were performed. In Chingola, Zambia no mosquitoes were found resting indoors due both to the newly implemented indoor insecticide residual spraying and the period during which the collections were made.

The range of collection techniques used in any country was dependent on the density of mosquitoes. The objective was to obtain as many mosquitoes per collection per site as possible for all the three major malaria vectors, *An. arabiensis*, *An. funestus* and *An. gambiae* s.s. Where GPS points could not be taken, geo-referencing of localities was undertaken for later cross-referencing. Documentation on insecticide usage for both agricultural and public health purposes in the collection areas were obtained at the time the mosquito collections were made. Initially suppliers and farmers were not prepared to supply information on temporal and spatial usage of pesticides. To overcome this, an interdisciplinary workshop was held in KZN, South Africa and a stakeholders' forum was established to look at pesticide usage issues in the agricultural areas. The forum involved Agriculture and Health Research Institutes and covered social issues such as male migration, women farmers and labourers, pesticide companies, public, private and traditional health workers, malaria control programmes and Non Governmental Organisations (NGOs) (Table 31).

As discussed in section 1.3, an understanding of the ecology of the vector informs the design of effective malaria control strategies. Larval abundance and distribution are important factors affecting successful control of adults or larvae. *An. gambiae* s.l larvae develop in freshwater habitats that are small, temporary, clean and exposed to sunlight.

Observation made by most of the collaborators in Appendix 1 showed that these *An. gambiae* s.l habitats were not the only types used by these species. The larval habitats were different from country to country and area to area and therefore a larval collection protocol had to be designed in order to capture most of these different habitats (Table 10). For example, a dew pond (Table 10) is a man-made pond placed on top of hills, built for watering livestock. It is used in areas where a natural supply of surface water may not be readily available. This pond system occurs in Namibia and is different from animal watering troughs used in other countries, e.g. Zambia (Figure 22C). Discussions were

held with all collaborators concerning the best way to record larval collections and a table was devised to record data (Table 10).

Protocols for feeding larvae to allow them to be successfully reared to adults were also discussed, based on the availability of different larval foods in different countries.

Country	Province	Area/ village	Section	Site	Type of breeding	Insecticide sprayed	No.of larvae	Long	Lat.	Date Coll.	Date Frozen

Table 10. Form for recording of larval mosquito field collections. Types of breeding sites were classified as follows:

1.Rice paddy 2. Stream 3. Rock pool 4. Pit 5. Riverbed 6.Irrigation canal 7. Swamp-like seepage 8. Natural pond 9. Man-made pond 10. Drain 11. Rain puddle 12. Artificial container 13. Dew pond 14. Temporary stream 15. Cattle/animal hoof prints 16. River fringes 17. Lake margin 18. Drying soil 19. cotton fields 20. Within vegetable gardens.

2.3.2 Mosquito Processing

All collections made in the study countries were transported to the Medical Research Council (MRC), Durban in South Africa for processing during this PhD. All larvae and adult bloodfed female mosquitoes were transported to malaria control stations in all study countries. Larvae were put in larval containers and fed with crushed cat food. Different possible larval food and schedules of feeding were outlined according to the availability of different cat food in study countries.

The adult blood fed female mosquitoes from South Africa, Mozambique and Zambia were transferred to labelled breeding tubes containing a wet filter paper placed on wet cotton wool as outlined in Figure 24. These were fed with a 10% sugar solution and induced to lay eggs. The eggs from a single female mosquito were then transferred to labelled larval containers until they hatched. F1 progeny emerging from the larvae were collected and transferred into new paper cups and fed with 10% sugar solution. These were then reared to one to three day old mosquitoes.

WHO susceptibility tests were not undertaken with samples obtained from larval collections. The F1 progeny from larval collections was only used for biochemical tests, as three members of the *An. gambiae* complex occur sympatrically in southern Africa,

being *An. merus*, *An. quadriannulatus* and *An. arabiensis*. These needed to be separated using molecular assays described by Scott et al. (1998). One to three day old adult mosquitoes were processed as shown in (Figure 24).

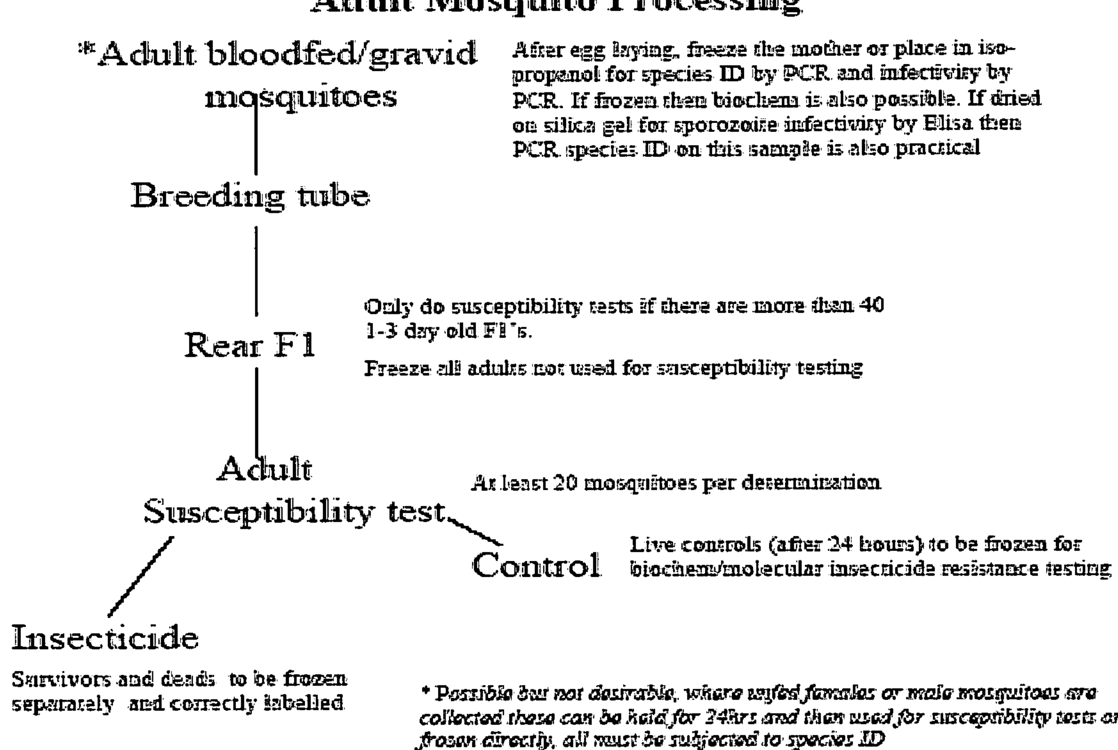


Figure 24. Mosquito processing chart. Flow chart of a general adult mosquito processing from egg laying to susceptibility testing. Survivors from insecticide treated papers were also used to establish a permethrin/DDT resistant *An. arabiensis* strain.

WHO susceptibility tests were performed on one to three day old male and female mosquitoes. The aim was to use representatives of all the major four insecticide groups, that is, pyrethroids, DDT, carbamates and organophosphates (Table 11). The choice also depended on the availability and use of insecticides in respective countries of study. Lambda cyhalothrin/deltamethrin were used with permethrin due to their wide usage in the study country in both insecticide indoor residual spraying (IRS) and treated nets (ITN).

Susceptibility testing

Insecticide	Use
Lambda cyhalothrin/Deltamethrin	Widely used in partner countries, first choice if in use or potentially to be used
DDT	First choice if used in country. Second choice if pyrethroid resistance found as this may indicate kdr.
Permethrin	Non cyano pyrethroid. Good comparative compound
Malathion	Representative of the organophosphates
Propoxur	Representative of the carbamates

Table 11. The insecticides used for susceptibility testing and the order of their use depending on the situation in country.

2.3.3 WHO Susceptibility Tests

All WHO susceptibility tests were performed on 1 - 3 day old adults of the first generation progeny of mosquitoes collected from Mozambique, Zambia and South Africa at the MRC in Durban, South Africa. Bloodfed female mosquitoes collected were kept under standard insectary conditions until oviposition. Larvae hatched from the eggs were reared through to one - three day old F1 adults for testing. Tests were performed by exposure to insecticide treated papers in a standard WHO test kit for one hour (or two hours for fenitrothion) with controls for each carrier oil used in the paper impregnation. Tests were carried out at 27.5 - 28°C and 75 - 85% Relative Humidity. Mosquitoes were subsequently transferred to holding tubes and maintained under same insectary conditions for 24 hours and provided with a 10% sugar solution before mortalities were scored. Survivors were stored in 100% isopropanol for species identification and molecular kdr detection, while controls were frozen, and stored at –20° degrees for later use in biochemical analysis. The above procedure was not undertaken for adults emerging from the larval collections.

2.3.4 Biochemical

Biochemical assays from individual 1-3 day old adult F1 progeny for altered acetylcholinesterase (AChE), glutathione S-transferases (GSTs), esterase and monooxygenase-based resistance mechanisms were performed with the bioassay control mosquitoes (Figure 24). Each family was tested for insecticide susceptibility from Zambia, Mozambique and South Africa as well as all one-three day old adult from larval collections from South Africa, Swaziland, Botswana and Zambia in microtitre plates and recorded as illustrated in Tables 12 and 13.

These biochemical assays were performed in South Africa (MRC), University of Cardiff and finally at the Liverpool School of Tropical Medicine during this PhD.

One to three day old F1 progeny were individually homogenized in 200µl of distilled water in flat-bottomed microtitre plates (Table 12). This was carried out on ice using a 96 pin Teflon homogeniser that matched with the 96 wells of the microtitre plate. Appropriated volumes of homogenate as detailed below were then transferred to a new microtitre plate as illustrated in table 13. In South Africa, the F1 progeny were individually homogenized in 1.5ml eppendorf tubes. Two replicates of 25µl of crude homogenate from each mosquito were transferred to a fresh microtitre plate for the AChE assays.

Plate code: PetBV03/11/04 X.

A	BV-1											
B	BV-1											
C												
D												Blank

Table 12. Representation of data recording from biochemical assays for 47 individual mosquitoes on a 96 well microtitre plate.This could be modified according to the number of individual mosquitoes processed. BV-1 (a) means female mosquito from adult female mosquito number 1 from area Bela-Vista. BV-1 (b) means male mosquito from adult female mosquito number 1 from area Bela-Vista. Plate code denotes the name of the researcher, area, day/month/year and X= the number of plates done on that date.

Plate code: PetBV03/11/04 X.

A	BV-1										
A	BV-1										
B	BV-1										
B	BV-1										
C											
C											
D											Blank
D											Blank

Table 13. Shows replicate homogenate from single mosquitoes for the respective biochemical assays. AA= replicate aliquots from the individual female BV-1 homogenate and BB = replicate aliquots from the individual male BV-1 homogenate (see table 13).

The remaining homogenate was spun at 1400 rpm for 5min in a microfuge. Initially in Cardiff and later at the Liverpool School of Tropical Medicine, the individual mosquitoes were homogenized in 96 well microtitre plates and spun at 3750 rpm for 30 in a GS-6R centrifuge (Beckman) at 5°C. Two 20µl replicate homogenates from each sample were transferred to fresh microtitre plates for the elevated esterase naphthyl acetate assays and monooxygenase assay as illustrated in table 13. Two replicate 10µl aliquots of homogenate were transferred to fresh microtitre plates for the GST, elevated esterase ρ-NPA and protein assays.

This procedure was done for *An. arabiensis* and *An. gambiae* s.s. The protocol was slightly modified for *An. funestus*, because of the small size of the mosquito compared to the other two vectors. In this case, only single 20µl aliquots for elevated esterase naphthyl acetate, monooxygenase assays and single 10µl aliquots of homogenate for the GST and, elevated esterase ρ-NPA were used. One 50µl aliquot of homogenate was used for the protein assays. Microtitre plates with aliquoted homogenate were kept on ice until analysis.

All the buffer solutions for all the biochemical assays were kept at room temperature, 0.01M propoxur, 30 mM α-naphthyl acetate (1-NA), 30 mM β-naphthyl acetate (2-NA) and 0.1M ρ-NPA were kept at 4°C. The rest of the solutions for the biochemical assays were prepared immediately before the assays were performed.

2.3.4.1 Acetylcholinesterase (AChE) Assay

The membrane bound AChE in the mosquito homogenate was solubilised by the addition of 145 μl of Triton phosphate buffer (1% Triton X-100 in 0.1M phosphate buffer pH 7.8) to each replicate of homogenate. Ten μl of DTNB solution (0.01M dithiobis 2-nitrobenzoic in 0.1M phosphate buffer pH 7.0) and 25 μl of the substrate ASCHI (0.01M acetylthiocholine iodide) were added to one replicate to initiate the reaction. The ASCHI solution was substituted with 25 μl ASCHI solution containing 0.2% of the inhibitor propoxur (0.1mM) for the second replicate. Identical reagents were added to 25μl of distilled water instead of insect homogenate in the control wells.

The kinetics of the enzyme reaction were monitored continuously at 405 nm for 5 minutes in a thermoMax microtitre plate reader (Molecular Devices) connected to a computer through the Wsoftmax software for samples analysed in Cardiff and Liverpool and a thermoMax microtitre plate reader (Molecular Devices), this time connected to a computer through the SoftProMax software for samples analysed in Durban, South Africa.

The percentage inhibition of AChE activity in the test well with propoxur compared to the uninhibited wells without propoxur for the same insect was calculated. The assay conditions were pre-set with insecticide susceptible *An. arabiensis* (susceptible strain) so that the activity in homozygous susceptible (SS) individuals without an altered AChE-based resistance mechanism was inhibited by >80%. Resistance gene frequencies were then calculated from the numbers of SS individuals observed assuming the population was in Hardy-Weinberg equilibrium.

2.3.4.2 Esterase Assays

Two hundred μl of α-naphthyl acetate (1-NA) acetate solution was added to one replicate and the same amount of β-naphthyl acetate (2-NA) acetate solution to the other replicate. The enzyme reaction was left for 30 minutes at room temperature before the addition of fast blue stain solution (22.5mg fast blue in 2.25 ml distilled water, plus 5.25ml of 5% sodium lauryl sulphate diluted in 0.1M phosphate buffer, pH 7.0) to stop the reaction. Blanks contained 20μl of distilled water, 200 μl of 1-NA or 2-NA solution and 50μl of

stain. The amount of product from the enzyme reaction was calculated by reading absorbance at 570nm in the thermoMax plate reader as an end point after 30 minutes. Optical densities for individual mosquitoes were compared with standard curves of optical densities for known concentrations of the products 1-naphthol and 2-naphthol respectively. The results were reported as nmol of product formed/min/mg protein.

2.3.4.3 ρ-Nitrophenyl Acetate (ρNPA) Assay

200μl of ρNPA working solution (100 mM ρNPA in acetonitrile: 50 mM sodium phosphate buffer pH 7.4, 1:100) were added to each replicate. Two blanks were prepared for each plate with 10μl of distilled water and 200μl of working solution. The enzyme reaction rate was measured at 405 nm for 2 minutes. The ρNPA activity per individual was reported as μmol of the product formed/min/mg protein, using the published extinction co-efficient corrected for the path length of the solution in the microplate well.

2.3.4.4 Glutathione S-transferase Assay

Two hundred μl of GSH/CDNB working solution (10 mM reduced glutathione prepared in 0.1M phosphate buffer pH 6.5 and 63 mM chlorodinitrobenzene diluted in methanol) were added to each replicate. Two blanks were prepared for each plate with 10μl of distilled water and 200μl of working solution. Rates for the enzyme reaction were measured at 340 nm for 5 minutes. The GST activity per individual was reported as mmol CDNB conjugated/min/mg protein, using the published extinction co-efficient corrected for the path length of the solution in the microtitre plate well.

2.3.4.5 Monooxygenase Assay

The haem-peroxidase assay, as modified by (Brogdon et al., 1997), was used to titrate the total amount of haem containing protein in each mosquito. Eighty μl of 0.0625 M potassium phosphate buffer pH 7.2 and 200 μl of TMBZ solution (0.01 gm of 3,3',5,5'-tetramethyl benzidine in 5 ml of absolute methanol mixed with 15 ml of 0.25 M sodium acetate buffer pH 5.0) were added to 20μl aliquots of insect homogenate. 25μl of 3% hydrogen peroxide was added and the mixture was left for 2 hours at room temperature. Two controls per plate were prepared with 20μl of distilled water, plus the working solution. Samples were read at 650 nm and values were compared with a standard curve

of known concentrations of cytochrome C. The values were reported as equivalent units of cytochrome P^{450}/mg protein corrected for the know haem content of cytochrome C and P^{450}.

2.3.4.6 Protein Assay

Three hundred µl of BIO Rad protein reagent solution, prepared as a 1:4 dilution in distilled water, were added to 10µl of the crude insect homogenate. Two blanks were prepared for each plate with 10 µl of distilled water and 300 µl of BIO Rad solution. The reaction was read at 570 nm after 5 minutes at room temperature.

2.3.5 Molecular Assays

2.3.5.1 Species Identification

Mosquitoes were identified to species level using the PCR method of (Scott et al., 1993) (Table 14) for the *An. gambiae* complex and Koekemoer et al. (2002) for the *An. funestus*. The PCR analysis was further optimised for identification using different body parts (e.g. leg). Species identification was essential for all collections as three members of the *An. gambiae* complex, being *An. merus*, *An. quadriannulatus* and *An. arabiensis* occur sympatrically in southern Africa and their spatial and temporal distribution is relevant to disease transmission vector control and the management of potential resistance. The *An. funestus* complex is composed of nine morphologically similar members (Table 15). The different species of the *Anopheles funestus* complex were recognised initially in Zanzibar (Sobti 1968) and South Africa (Gillies 1962; De Meillon et al., 1977) by their behaviour after successful indoor residual spraying (Hemingway 1980) Two PCR species identification methods for *An. funestus* complex have been designed in South Africa. One uses the Ha11 endonuclease to distinguishe between *An. funestus* and *An. vaneedeni* (Koekemoer et al., 2002).

The primers are developed from the D3 region in the 28s ribosomal gene, and amplified products digested with the restriction endonuclease Hpa11 and visualised on agarose gel. The second used in this study, is also based on the use of primers from the D3 region in

the 28s gene but the products are electrophoresed on single-strand conformation polymorphism (SSCP) gels.

Primer name	Primer sequence (5' to 3')	Product size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	
GA	CTG GTT TGG TCG GCA CGT TT	390
ME	TGA CCA ACC CAC TCC CTT TA	466
AR	AAG TGT CCT TCT CCA TCC TA	315
QD	CAG ACC AAG ATG GTT AGT AT	153

Table 14. Primer sequence of species-diagnostic of *Anopheles gambiae* complex, with expected PCR product size. UN = Universal band, GA = *An. gambiae* s.s., ME = *An. merus*, AR = *An. arabiensis* and QD = *An. quadriannulatus*. *An. merus* and *An. melas* are allopatric but both are identified by ME primers. An. melas does not occur in southern Africa.
(Reproduced from Scott et al., 1993).

Primer name	Primer sequence (5' to 3')	Product size (bp)
UV	TGT GAA CTG CAG GAC ACAT	
FUN	GCA TCG ATG GGT TAA TCA TG	505
VAN	TGT CGA CTT GGT AGC CGA AC	587
RIV	CAA GCC GTT CGA CCC TGA TT	411
PAR	TGC GGT CCC AAG CTA GGT TC	252
LEES	TAC ACG GGC GCC ATG TAG TT	146

Table 15. Primer sequence of species-diagnostic *An. funestus* complex, with expected PCR product size. FUN = *An. funestus* s.s, VAN = *An. vaneedeni* Gillies & Coetzee, RIV = *An. rivulorum Leeson*, PAR = *An. parensis* Gillies, LEES = An. leesoni Evans.
(Reproduced from Koekemoer et al., 2002).

2.3.5.2 Polymerase Chain Reaction for kdr Detection

The diagnostic PCR developed by (Martinez-Torres et al., 1998) to distinguish between 'resistant' and 'susceptible' kdr alleles was employed. This allowed detection of resistant homozygotes and heterozygotes from the field populations. The assay for the West African mutation used primers AgD1 and AgD2 that flank the region containing the kdr mutation and amplify a 293 bp product from genomic DNA. Primers AgD3 and AgD4, internal to this region, were allele specific. Primer AgD3 binds only to the resistant kdr allele and, when paired with AgD1, will amplify a 195 bp fragment if this allele is present in the individual. AgD4 binds only to the susceptible allele and will pair with AgD2 to produce a 137 bp band if the susceptible allele is present (Martinez-Torres et al., 1998). Ranson et al. (2000) adapted this PCR to detect the novel mutation found in the RSP-ST strain by substituting primer AgD3 for AgD5 (Table 16).

Primer name	Primer sequence (5' to 3')
AGD 1	ATA GAT TCC CCC GAC CAT G
AGD 2	AGA CAA GGA TGA TGA AAC C
AGD 3	AGA CAA GGA TGA TGA ACC
AGD 4	AAT TTG CAT TAC TTA CGA CA
AGD 5	CTG TAG TGA TAG GAA ATT TA

Table 16. Primer sequence for the kdr diagnostic PCR. (Reproduced from Martinez-Torres et al., 1998; Ranson et al. 2000).

2.3.5.3 The Heated Oligonucleotide Ligation Assay (HOLA)

Single nucleotide polymorphisms (SNPs) are DNA sequence variations that occur when a single nucleotide (A, T, C or G) in the genome sequence is altered. For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs make up 90% of all the human genetic variation and occur every 100 to 300 bases along the 3-billion-base human genome. Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding and non-coding regions of the genome.

In mosquitoes, insecticide resistance is often due to a single mutation that results in a structural change of the gene product, as occurs for example with kdr. The insect sodium channel is the site of action of DDT and pyrethroid insecticides. Resistance to pyrethroids due to changes in this target-site is produced by a single Leu – Phe substitution in the S6 segment of domain II of the sodium channel gene in *D. melanogster* (Jackson et al., 1984), *M. domestica* (Williamson 1996), and *Blattella germanica* (Hemingway et al., 1993; Dong and Scott 1994). Resistance to pyrethroids due to the voltage-gated sodium channel type of mechanism (kdr) in the main malaria vector *An. gambiae s.s.* results from a single point mutation (leucine TTA phenylalanine TTT) at position 312 in the sodium channel gene (Martinez-Torres et al., 1998) and a (Leucine TTA to Serine TCA substitution at position 311 in East African *An. gambiae.*

The kdr HOLA method was designed based on a technique developed by Black and Walker (in press). The technique consists of a PCR to amplify across the site of the mutation using primers AgD1 and AgD2 from the traditional multiplex PCR screening technique (Martinez-Torres et al., 1998). The PCR product is then combined with a fluorescein labelled reporter oligo, a biotin labelled detector oligo and the enzyme "Ampligase". The oligos are designed to complement the DNA exactly and when

annealed are directly adjacent to each other on the DNA strand. The 3' end of the detectors are designed to complement either the susceptible or resistant point mutation, hence two detectors are needed and therefore two reactions are set up.

If the detectors match the point mutation of the allele the Ampligase enzyme ligates them to the reporter to form a single oligo labelled with both fluorescein and biotin. Biotin labelled oligos will bind to a streptavidin coated plate. Anti-fluorescein antibody and a colour substrate are added to the wells and in a positive reaction adheres to the fluorescein of the ligated oligo resulting in a vivid blue colouration (Figure 25). In a negative reaction the fluorescein labelled oligo is not ligated to the biotin labelled oligo and so is washed away and the reaction mix remains colourless. When the resistant and susceptible assays are set up side by side, homozygotes (resistant and susceptible) and heterozygotes resistant can be easily visualised.

2.3.5.3.1 Streptavidin Plate Preparation

One mg of streptavidin was prepared in 1ml ddH_2O to make a 1mg/ml stock solution. 50 μl of streptavidin stock solution was added to 10ml ddH_2O to make the final 5μg/ml working solution. One hundred μl of streptavidin working solution was added to each well of the 96 well microtitre plate. The plates were then placed for 24 hours at room temperature to allow the streptavidin solution to evaporate to dryness. The dried plates were subsequently washed three times with PBS containing 0.1% Tween 20 by adding 250 μl of 1X PBS to each well. The solution was removed each time by vigorously flicking the plate over the sink, with traces of excess buffer removed by banging the plate (well-side down) on a paper towel, on the bench top.

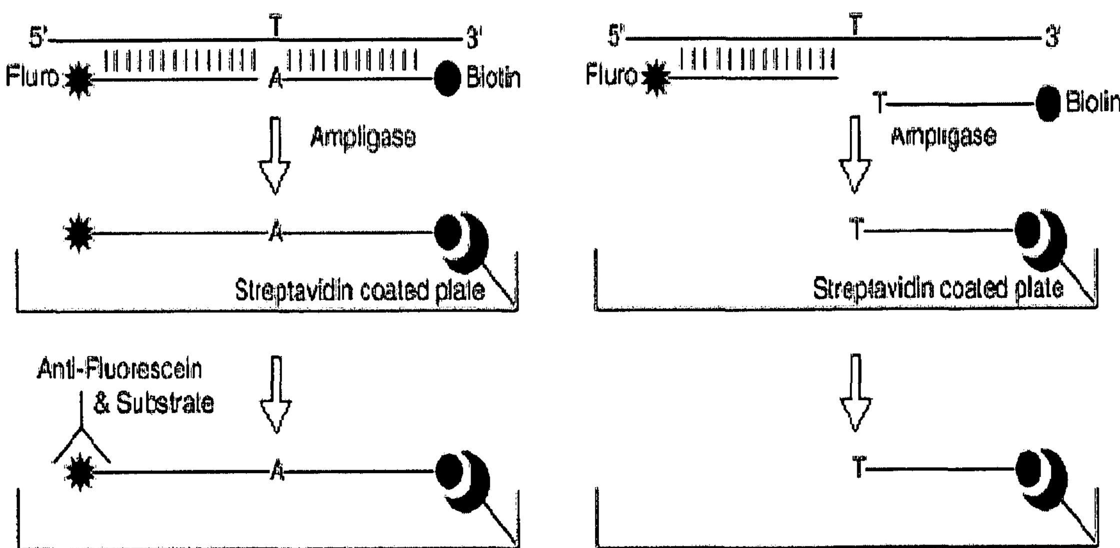


Figure 25. The HOLA detection system with resistant & susceptible kdr samples. A = Adenosine, T = Thymine (Source: Wondji pers. Comm.).

Description	Oligo Name	bp Position[a]	Oligo sequence 5' – 3'	Modifications
Susceptible East *kdr* detector	Kdr104L-DTe	311-15*i*	ATTTGCATTACTTACGACT A	5' Biotin
Resistant East *kdr* detector	Kdr104S-DTe	311-15*i*	ATTTGCATTACTTACGACT G	5' Biotin
East *kdr* reporter	Kdr104-RTe	291-310	AATTTCCTATCACTACAGT G	5' Phosphorylation 3' Fluorescein
Susceptible West *kdr* detector	Kdr104L-DTw	312-16*i*	AATTTGCATTACTTACGAC T	5' Biotin
Resistant West *kdr* detector	Kdr104F-DTw	312-16*i*	AATTTGCATTACTTACGAC A	5' Biotin
West *kdr* reporter	Kdr104-RTw	292-311	AAATTTCCTATCACTACAG T	5' Phosphorylation 3' Fluorescein

Table 17. The sequence of the detector (susceptible) and reporter (resistant) biotin labelled oligo sequence 5'-3' used for the HOLA assay. (Source: Amy Lynd pers. comm.).

Two hundred µl of the blocking solution (PBS + 2% BSA) was added to each well and the plate incubated at room temperature for an hour to block non-specific absorption. The plates were then washed four times with 1X PBS containing 0.1% Tween 20 as previously. After that plates could be stored for a week at 4°C before use. Plates could not be stored longer as background signal started to increase.

2.3.5.3.2 Hot Ligation

All the primers (detector and reporter) (Table 17), were resuspended at 500 pmol/μl in TE pH 8.0. The working primer stocks were prepared by adding 2μl of each detector (500 pmol/μl) and 2 μl of the corresponding reporter (500 pmol/μl) to 996 μl TE pH8.0. This gave a solution that contained reporter and detector at concentrations of 1 pmol/μl. The primer stock was stored at -20°C and did not need to be freshly prepared for each assay.

2.3.5.3.3 Detection of SNPs for kdr

Twenty μl of TNE followed by 20μl of the HOLA reaction product were pipetted into each well of the streptavidin coated plate. This was then incubated at room temperature (in a dark place) for 30 minutes. The TNE + HOLA reactions were removed from the wells of the plate using a multi-channel pipette to avoid contamination of other wells. Washing of the TNE + HOLA reactions was done by adding 250μl of freshly made wash buffer 1 to all the wells in the plate. The contents were then mixed vigorously before the contents discarded into the sink. This was repeated three times. Next the plate was washed twice with wash buffer 2 and washing was again repeated 3 times. The antibody was prepared by adding 0.5 μl of HSP-conjugated anti-fluorescein Ab (150 u/μl) to 1 ml of (wash solution 2 + 1% BSA) final concentration of 75u/ml. 40μl of the Ab solution was added to each well and incubated at room temperature for 30 minutes. The antibody solution was then removed from the wells by using a multi-channel pipette and the plate washed by adding 250 μl of wash buffer 2. The washing was repeated two times and the plates tapped on a paper towel to remove any excess wash buffer. Hundred μl of pre-warmed TMB solution was added to each well and the plates left for 5 minutes before absorbance was read at 650nm.

2.4 Metabolism Experiments

These assays were undertaken to confirm the results from both WHO susceptibility tests and basic biochemical assays on *An. arabiensis* from South Africa, showing cross-resistance between permethrin and DDT. The metabolic products for DDT were measured by high performance liquid chromatography (HPLC).

2.4.1 DDT Metabolism

Two methods were performed on homogenates of 4th instar larvae, adult F1 and F15 females and males mosquitoes. Fourty adult mosquitoes or fourty 4th instar larvae were separately homogenised in 300µl of distilled water. GST activity and protein concentration were measured as described earlier using two replicates of 10µl homogenate from each batch. Analysis of DDT metabolites was performed by HPLC.

The reaction mixture for the DDT metabolite analysis contained an appropriate volume of the enzyme supernatant (determined from the GST and protein assays), 50µl DDT (2 mM in ethanol) and 20 mM GSH in 100mM sodium phosphate buffer pH 6.5 to make a 1ml volume. The reactions were incubated at 28°C for two hours before adding 1.5ml $CHCl_3$ and gently mixing. Extraction of DDT and DDE from the reaction mixture was performed by first gently shaking the reaction mixtures for two minutes continuously, followed by spinning at 3700 rmp for three minutes to allow the solution to separate into the $CHCl_3$ and aqueous layers. The aqueous layer was removed and the $CHCl_3$ layer transferred to a new glass tube. The extraction of the aqueous phase with chloroform was repeated three times. The chloroform extracts were then air dried at room temperature overnight and stored at -70°C unit HPLC analysis.

The dried extracts were resuspended in 100µl of isopropanol and 100µl of mobile phase solution (the isocratic mobile phase contained methanol:acetonitrile:water (72.5:12.5:15 v/v). One hundred µl of the extracts containing 1:1 isopropanol:mobile phase was transferred into an HPLC vial for HPLC analysis. HPLC was performed on a machine with a Waters Model 510 HPLC pump, Waters 490 Programmemable Multi Wavelength detector and Waters Data Module 745 integrator. The reverse phase chromatographic analysis of DDT metabolites was performed by injecting 50µl sample of the extract into a Waters Radial-PAK cartridge column using a Rheodyne model 7215 manual injector with 50µl loop. The flow rate was 0.8 ml/minute.

The detector sensitivity was set at 0.5 absorbance units full scale (AUFS) at a wavelength of 236 nm. The amount of DDE produced (nmole/mg protein) was calculated by comparison with integrated values for reference standards of known concentrations of ρ,ρ'-DDE injected into the same HPLC column.

2.5 RESULTS AND DISCUSSION

2.5.1 Susceptible baselines

Two laboratory colonies, the *An. arabiensis* (Durban, South Africa) strain and the *An. albimanus* (Panama) strain were used as susceptible standards for biochemical assays (Figures 26 – 31). The percentage inhibition of AChE activity by propoxur in both laboratory susceptible strains (Figure 26) ranged from 80% - 100% for *An. arabiensis* and 60% - 100% for *An. albimanus*, indicating that neither of these populations carry an insecticide insensitive AChE gene. These strains were subsequently used as controls to show that the assay was correctly calibrated for the field collected material. A further calibration of the AChE assay was done with *An. gambiae* s.s from Gamba, Gabon, an area with no active vector control and almost no commercial agricultural activities, apart from small scale vegetable farming (Figure 77).

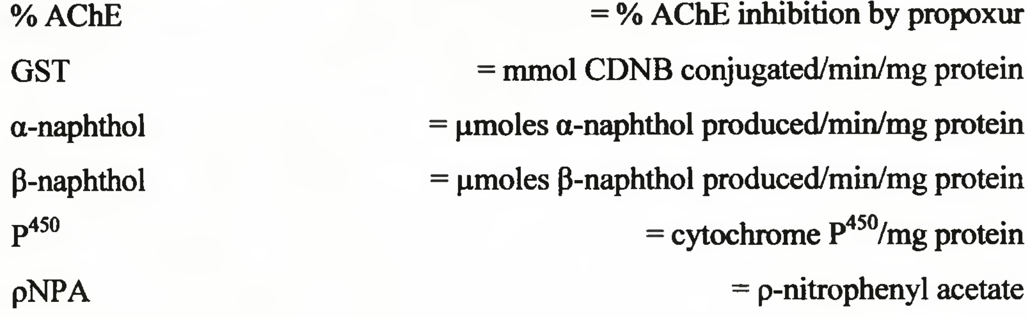
A minority of individuals of *An. arabiensis* (susceptible strain-Durban) also had elevated levels of glutathione S-transferase activity (Figure 27) which can confer resistance to DDT and pyrethroids. The *An. arabiensis*, Durban laboratory strain, which has been kept in the insectary for 15 years without selection pressure, still had a low frequency of individuals with elevated esterase activity which were particularly evident with the substrate p-nitrophenyl acetate (Figures 28, 29 and 30). These elevated esterases will confer resistance to most OPs. The Panama strain was fully susceptible for all resistance mechanisms, with the exception of the P^{450} assay where equivalent units of P^{450} were higher than anticipated for a susceptible strain (Figure 31). Table 18 shows the established cut off points for the different assays for susceptible mosquitoes as demonstrated by the assays on the two susceptible strains and for AChE for the *An. gambiae* s.s from Gabon.

Once accurate baselines had been set for the full range of biochemical assays, results from field collected mosquitoes could be compared to this baseline. Initially, a small scale pilot study was established in Gabon (Chapter 3) to trial the systems before large scale monitoring was undertaken in southern Africa.

Species	% AChE	GST	α-Naphthol	β-Naphthol	ρNPA	P450
An. arabiensis	<80%	>0.750	>0.001875	>0.0015	>0.1875	>0.000875
An. albimanus	<60%	>0.375	>0.00125	>0.001125	>0.0625	>0.005
An.gambiae s.s.	<80%					

Table 18. Established cut off points of biochemical assays using two susceptible laboratory strains. *An. arabiensis* (Durban), *An. albimanus* (Panama) and an insecticide susceptible field collected *An. gambiae* s.s (Gabon) strain for AChE.

The units of each assay were:



% AChE = % AChE inhibition by propoxur

GST = mmol CDNB conjugated/min/mg protein

α-naphthol = μmoles α-naphthol produced/min/mg protein

β-naphthol = μmoles β-naphthol produced/min/mg protein

P^{450} = cytochrome P^{450}/mg protein

ρNPA = ρ-nitrophenyl acetate

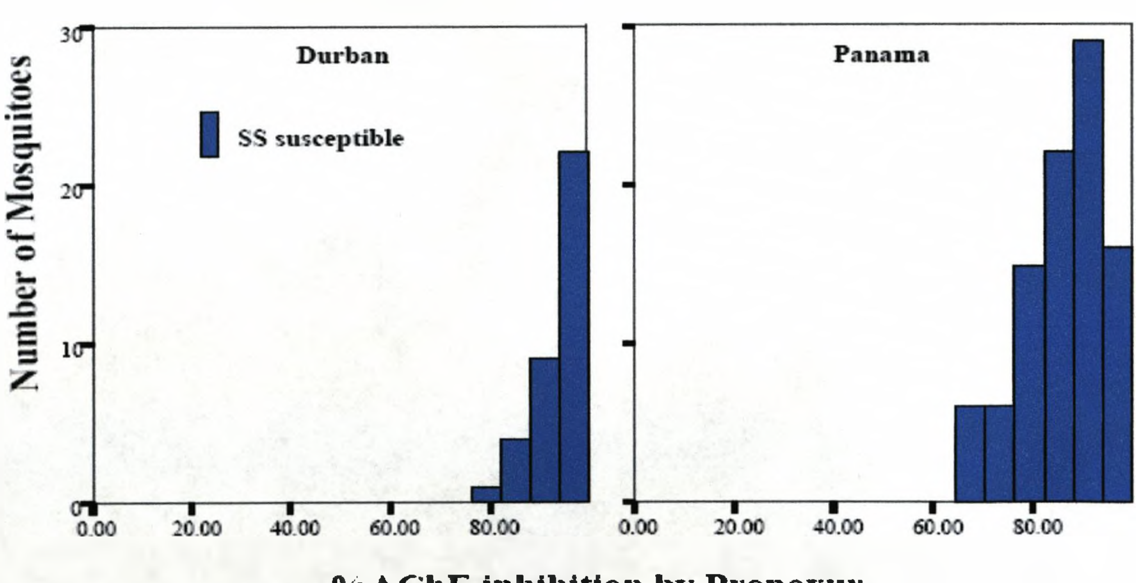


Figure 26. Percentage acetycholinesterase (AChE) inhibition ranges with susceptible laboratory strains *An. arabiensis* (Durban, South Africa), *An. albimunus* (Panama) susceptible strains. The SS (75%-100%), RS (25%-75%) and RR (0%-25%) ranges denote the levels of expected remaining activity for these three genotypes under the assay conditions used.

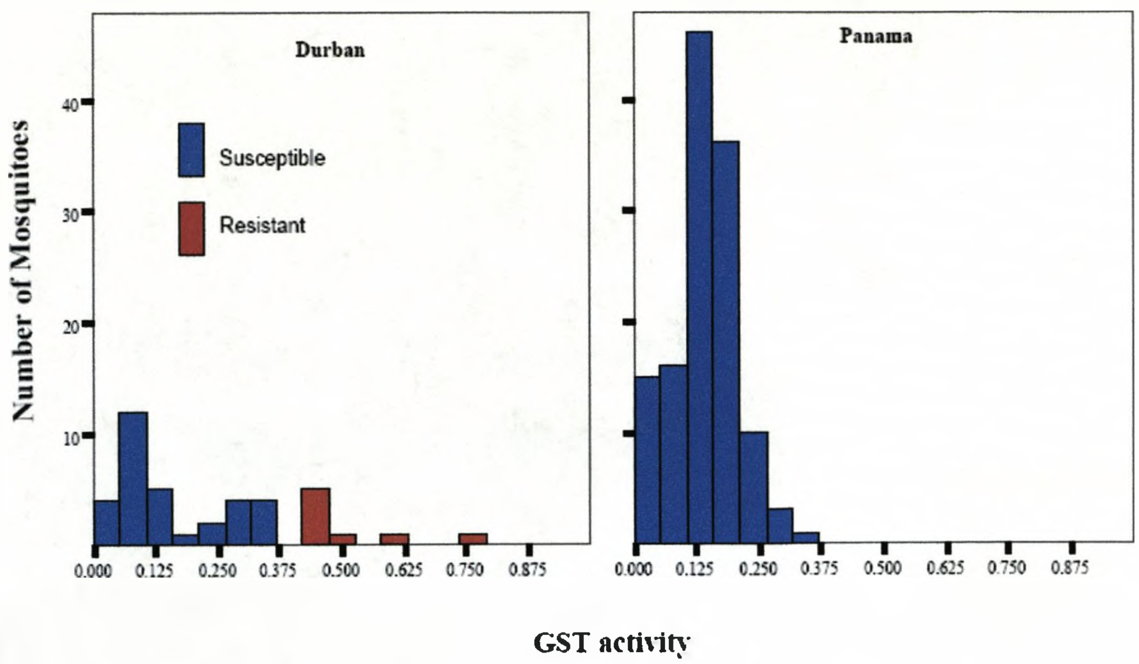


Figure 27. Glutathione S-transferase (GST) activity with the substrate chlorodinitrobenzene in the *An. arabiensis* (Durban, South Africa) and *An. albimanus* (Panama) susceptible strains. Ranges above 0.375 mmol CDNB conjugated/min/mg protein cut off point indicate resistance (coloured red).

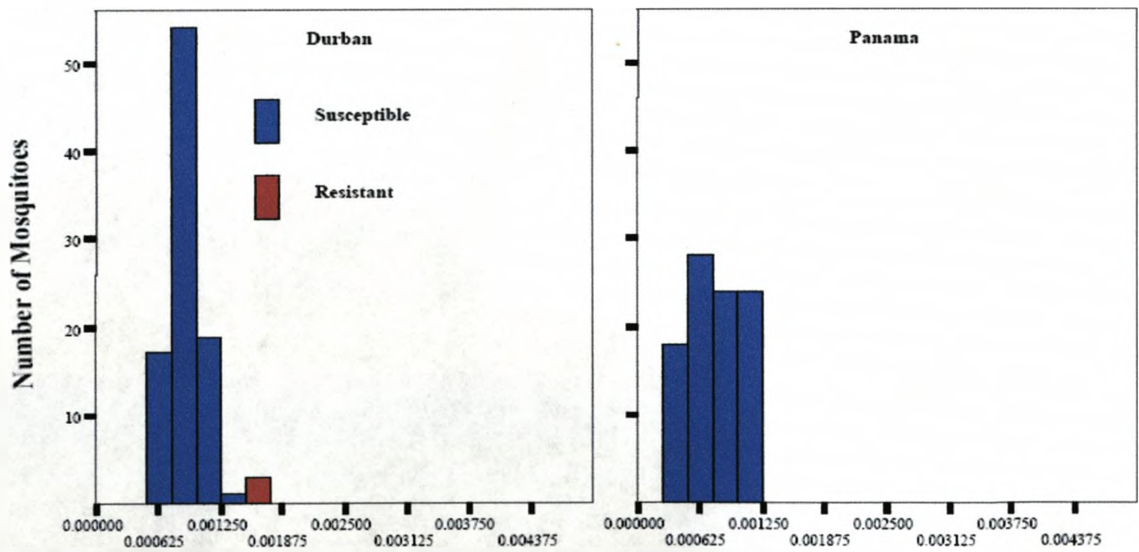


Figure 28. Esterase activity with the substrate α-naphthyl acetate in the *An. arabiensis* (Durban, South Africa) and *An.albimanus* (Panama) susceptible strains. Ranges above 0.00125 µMoles α-naphthol produced/min/mg protein indicate resistance (coloured red)

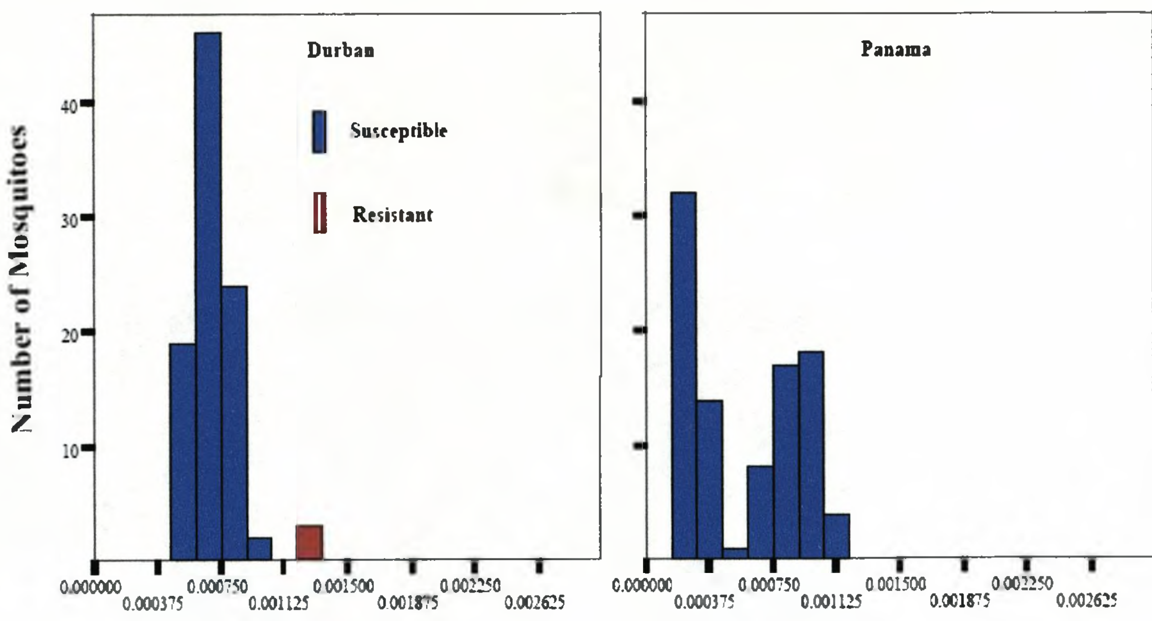


Figure 29. Esterase activity with the substrate β-naphthyl acetate in the *An. arabiensis* (Durban, South Africa) and *An. albimanus* (Panama) susceptible strains. Ranges above 0.001125 µMoles β-naphthol produced/min/mg protein indicate resistance.

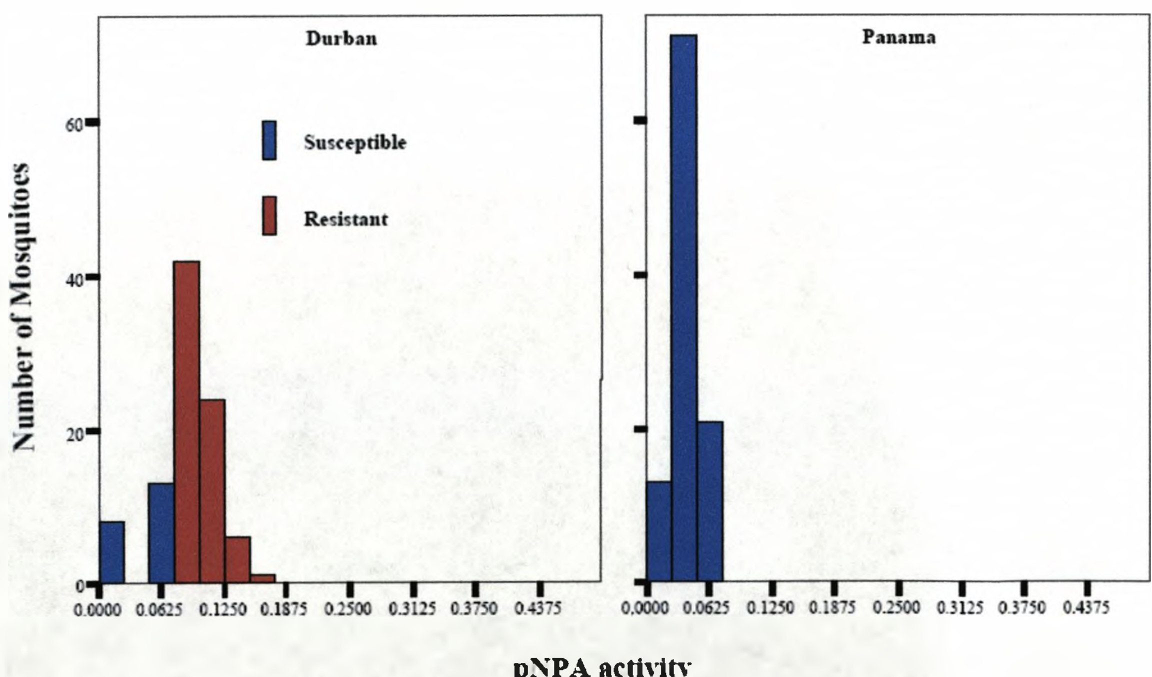
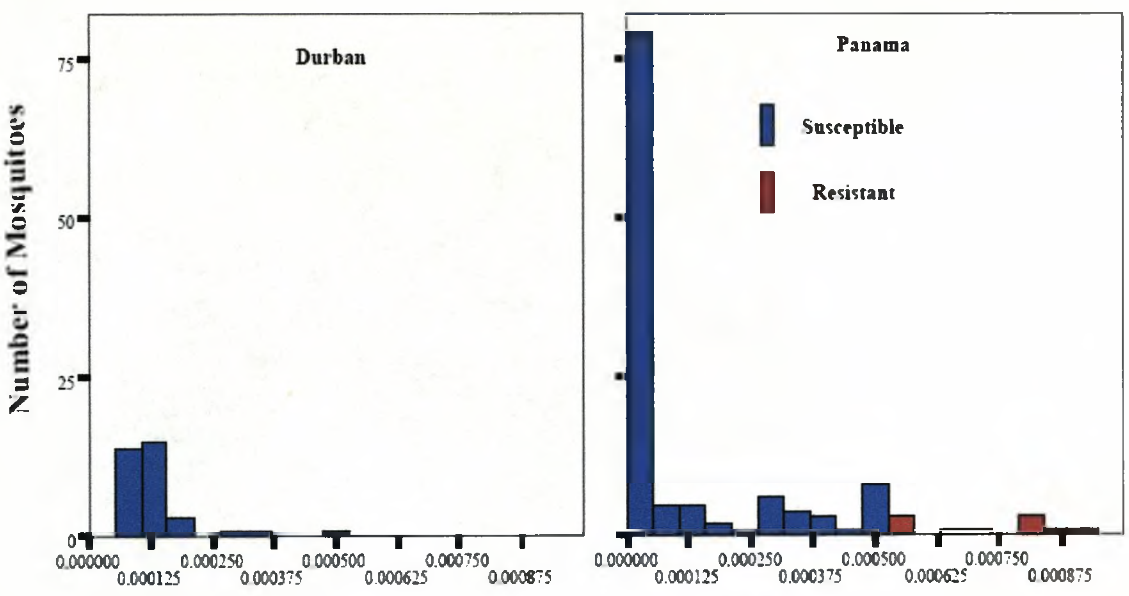


Figure 30. Esterase activity with ρ-nitrophenyl acetate *An. arabiensis* (Durban, South Africa) and *An.albimanus* (Panama) susceptible strains. Ranges above 0.0625 levels of esterase activity indicate resistance (coloured red).



Equivalent units of cytochrome P^{450}/mg protein

Figure 31. Equivalent units of cytochrome P^{450}/mg protein representing monooxygenase activity in the *An. arabiensis* (Durban, South Africa) and *An. albimanus* (Panama) susceptible strains. Ranges above 0.005 equivalent units of cytochrome P^{450}/mg protein indicate resistance.

2.6 South Africa

2.6.1 Collections

Permanent mosquito winter breeding sites were identified and mapped in three provinces (KZN, Limpopo and Mpumalanga) (Figure 32). In Limpopo and Mpumalanga provinces, collections included areas that were targeted for future agricultural developments. Four districts were covered in the Limpopo province, Phalaborowa, Messina, Venda and Tzannen (Table 19, Figure 32). Phalaborowa surrounds the Kruger National Park. Collections inside the park were not undertaken. Training of malaria control personnel in mosquito processing was undertaken in two provinces, Limpopo and Mpumalanga to ensure that mosquitoes collected from these provinces could be used later for biochemical and molecular analysis. Discussions were held with farmers in the Phalaborowa, Messina and Venda areas, which are far from the malaria control station in Tzaneen, to request that we establish trained workers on their farms for temporal mosquito collections in and around their farms to facilitate collections that could be used for further analysis.

2.6.2 Results

In two provinces, Limpopo and Mpumalanga produced *Anopheles coustani* a non vector, was collected. In Messina collections were made in an area of 1km radius within a bend in the river during the winter period and this included the adjacent Klein River Farm which was established as a potential collaborating partner to facilitate future collections. In Venda and Tzaneen, the collections were made from six rose farms and eight villages (Table 19). Breeding sites along the three rivers Letsilele, Letaba and Lerwatlou in Venda and Tzaneen were mapped. Dzimauli Agricultural Development area was mapped to facilitate future sample collections. *An. arabiensis* historically is the main malaria vector in the Limpopo province but less than five specimens have been found within a period of five years (Dr. P. Kruger, pers. comm.). The decision to identify and map many breeding sites in Limpopo was to address the problem of scarcity of *An. arabiensis*. In the Mpumalanga province, permanent breeding sites were mapped in seven villages, five farms and the Basil Read Dam (Table 19). The collections also produced mostly *An. coustani* and few *An. merus*. High percentage mortality was also encountered in this province transporting mosquitoes to the Medical Research Council, Durban. In KZN province, collections were made in the two districts, Ngwavuma and Ubombo (Figure 32 insert). In the Ngwavuma district, the work involved mapping of permanent breeding sites, documentation of pesticide usage and larval collections from two areas, Makanis and Sihangwane. In the Ubombo districts micro-geographic collections, mapping of permanent breeding sites and pesticide usage were undertaken at Mamfene.

Tables 20 a and b show the different pesticide groups used in the agricultural sector in the three malaria provinces, Limpopo, Mpumalanga and KZN. Data on choice, quantity and calendar usage of these pesticides was not well documented. To obtain this data and set up a collaborative working framework the multi-sectoral forum was established (Chapter 4). No analysis on samples collected in the Limpopo and Mpumalanga provinces were undertaken.

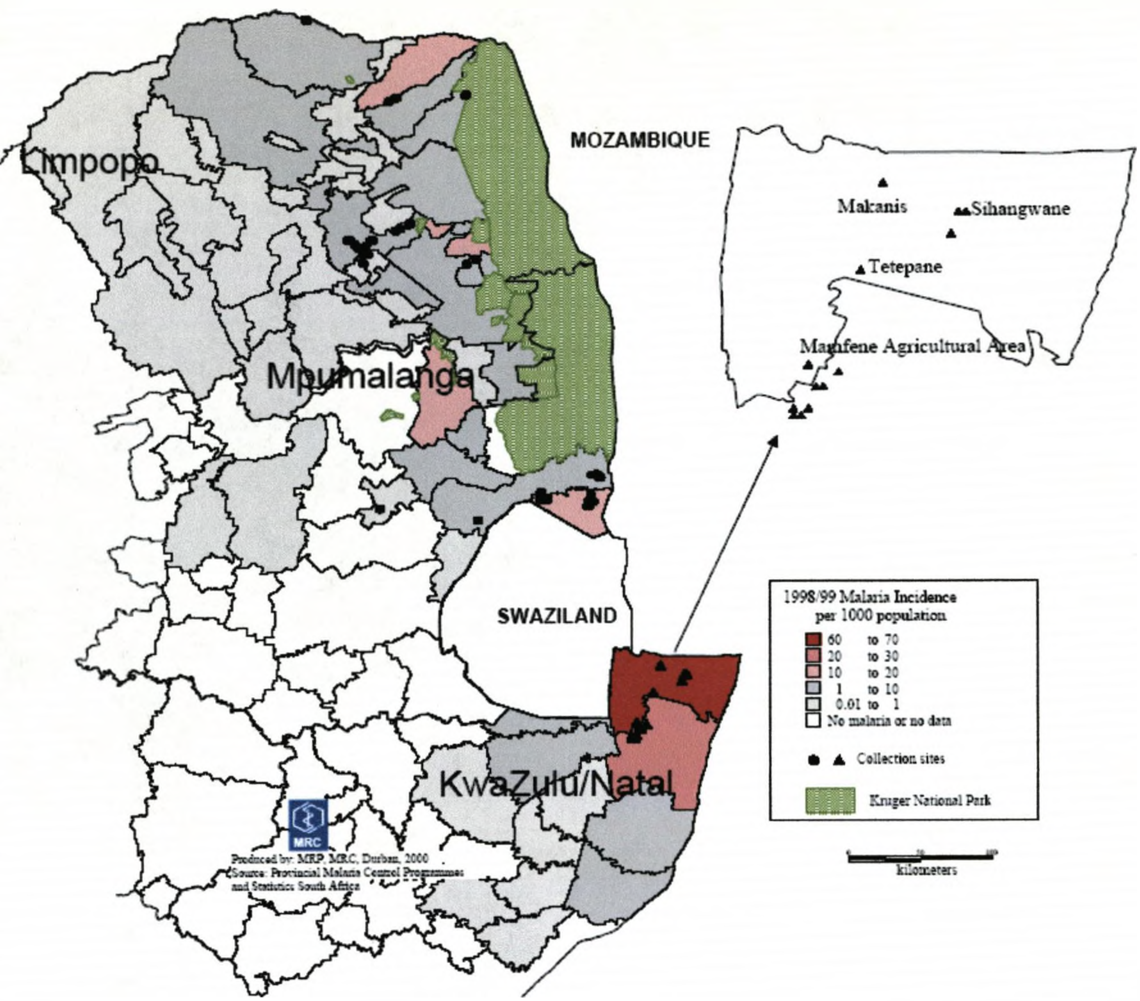


Figure 32. Collections sites in the three malaria provinces in South Africa (Limpopo, Mpumalanga & KwaZulu/Natal) with an insert showing study sites where cross-resistance to DDT/permethrin was detected. Altered AChE resistance mechanism conferring resistance to OPs and carbamate was detected at the Mamfene Agricultural Area, in the Ubombo district, KZN.

The collections of mosquitoes and mapping of their permanent breeding sites was also accompanied by the documentation of pesticides used for agricultural pests in all the three provinces (Table 20 a and b).

2.6.2.1 KwaZulu/Natal

WHO susceptibility tests were performed on 1-3 day old adult *An. arabiensis* (Table 21) from Mamfene agricultural area, one of the study sites in this PhD (Figure 32). Four insecticides, permethrin, DDT, propoxur and deltamethrin were used. Survivors from DDT and permethrin treatments were pooled together to establish a laboratory based permethrin/DDT resistant strain of *An. arabiensis*. The results from bioassay tests

indicated DDT/permethrin cross-resistance. Cross resistance between pyrethroids and DDT suggests the presence of a kdr-type based resistance mechanism. The kdr diagnostic PCR and HOLA methods were undertaken but produced negative results. More work with DDT/permethrin bioassays survivors is still being undertaken at the Liverpool School of Tropical Medicine, which includes the sequencing of the sodium channel gene with the DDT/permethrin survivors.

Limpopo Province	**District**	**Site**
	Phalaborowa	Selati River Namakhale Lulekani
	Messina	Limpopo River Klein Rivier Farm
	Venda	Dzimauli for Agricultural Developement Thengwe Village Minga Village
	Tzaneen	Lenyenye Village Letsilele River Dan Village Tshidjumba Mkgolobotho Morokolotsi Quarry Letaba River Letsebe Agric. Station Lerwatlou River Gert & Cecillia Farm Nkamboko Dam Ashream Farm James Tolmay Farm Tarantalrand Farm De la Rose Farm Tzaneen Dam
Mpumalanga Province	Mpumalanga	Driecopies Village Besenyama Village Midplas Village Nyetani Village Cotton farm Manqwene Village Basil Read Dam Petalteen Block C Mnyoni Village Landlou Farm Tomato Farm Sommereg Sugarcane Komati Port Agric. Area

Table 19. Shows all the mapped study sites (black dots in Figure 32) in five districts in the other two malaria endemic provinces in South Africa.

A. Limpopo and Mpumalanga Provinces

Pyrethroids	Organophosphate insecticides	carbamates	Organo-chlorine	Other (Trade names)
cypermethrin	profenos	decarzol	endosulfan	Abemectin
alphacypermethrin	malathion	aldicarb		Ortus
deltamethrin	prothiofos			Cordless
lambda-cyhalothrin				Lannate
bifenthrin				Thionix
pymetrozine				Mospilan
				Benlate
				Acorol
				Celecron
				Dithane
				Nemasys

B. Mamfene Agricultural Research Station Northeastern KZN Province

Organophosphate insecticides	Carbamates:	Pyrethroids	Other (trade names)
malathion	carbofuran	cypermethrin	Mineral oil
parathion		deltamethrin	Pyrine 480 EC
fenthion		citowet	Brovo
fenamiphos			Marinure
demeton-S-methyl			Aliette(fosetyl) Fungicide
sulprofos			Enthrel
disulfoton			Chloronil
chlorpirifos			Sewcor
			Semivin FS
			Peripel
			Oftanol
			Score 250 EC
			Pruming Paste
			Malason
			Ultracide
			Punche
			Seradix B No.1.
			Sencor
			Herbicide - 1,2,4-triazinone
			Nomol 3:2
			Weedmaster Alsystin
			Kelpak
			Buminal CB2
			Weedmaster 3.2
			Expose 70 WP
			Monsanto
			Karbaspray 850
			Demildex
			Ridomil Hygrotech
			Pravit cupravit

Table 20 a and b. The agricultural insecticides used in the three malaria endemic provinces in South Africa.

In Mamfene, three sentinel houses were chosen, which were sprayed with DDT and mosquito collections were undertaken using window traps for a period of three days. A total number of 130 adult female mosquitoes were caught. The majority (70.8%) of the adult female mosquitoes exiting the houses were not blood fed, revealing the repellency effect of DDT.

Insecticide	Concentration	N	% Mortality
DDT	4%	112	5.35%
Permethrin	0.75%	113	45.13%
Propoxur	0.01%	108	100%
Deltamethrin	0.05%	70	100%
Total		**403**	**14.14%**

Table 21. Mortalities from WHO susceptibility tests with permethrin, DDT, propoxur and deltamethrin with 1-3 day old adults *An. arabiensis* from Mamfene, KZN.

The results of DDT metabolism in mosquitoes from the F1 and F15 progeny of females collected from Mamfene, KZN are shown in Table 22 with their respective levels of GST activity. DDT and its metabolites were quantified against standard curves of reference metabolites after HPLC separation (Figure 33). Pooled adults and fourth instar larvae were initially checked for GST activity levels (Table 22). The primary DDT metabolite DDE, produced by the action of GSTs was found in significant quantities in mosquitoes with high GST activity (>0.375) (^{5}F selected) as compared to low GST activity (<0.375) (^{1}F susceptible). The one – three day old DDT/permethrin resistance F1 progeny (^{2}F, ^{3}M, ^{4}M and ^{6}M) produced low DDE % as compared to F15 (^{5}F selected).

The percentage recovery rate of fourth instar larvae was lower than that of one-three day old adults due to the high fat content in larvae. GST activity was not significantly different between one – three day old males F1 progeny (M) and females (F). Biochemical assays for the monooxygenase and specific esterase assays were not performed due to the lack of material after completing the metabolic assays. Synergist studies are to be undertaken with the established permethrin/DDT resistance strain to further elucidate the resistance mechanisms operating in this strain.

Biochemical tests were performed on one to three day old F1 progeny as described in section 2.4.4 from four study sites; Makhathini, Pongola River, Pongola village and Mjindi in Mamfene, Ubombo district, KZN. The same number of mosquitoes were used per assay, Makhathini (N=164), Mjindi (N=142), Pongola village (N=133) and Pongola River (N=45). The altered AChE resistance mechanism was detected at different frequencies in all four study sites in Mamfene (Figure 34).

	[1]F (Suc.)	[2]F	[3]M	[4]M	[5]F(Sel)	[6]F	[1]L	[2]L
GST activity	**0.178**	0.345	**0.459**	0.363	0.792	**0.606**	0.247	0.340
DDT %	97%	96.9%	98.5%	99.1%	93.3%	98.7%	88.3%	98.1%
Metabolites								
DDE %	1.06%	1.27%	0.07%	0.6%	5.12%	0.9%	8.2%	0.6%
Dicofol %	0.60%	1.45%	1.32%	-	1.33%	-	3.2%	1.1%
DDD %	0.94%	0.38%	0.11%	0.3%	0.25%	0.4%	0.3%	0.2%
Recovery rate %	**95.8%**	**88.4%**	**101%**	**98.3%**	**82.9%**	**84.4%**	**68.9%**	**79.4%**

Table 182. The percentage of DDT metabolised by fourth instar larvae (L) and one to three day old adult females (F) and males (M) *An. arabiensis* from Mamfene, KZN with their respective GST activities. The numbers F&M[1,2,3,4,5,6] and L[1,2] indicate different replicates.

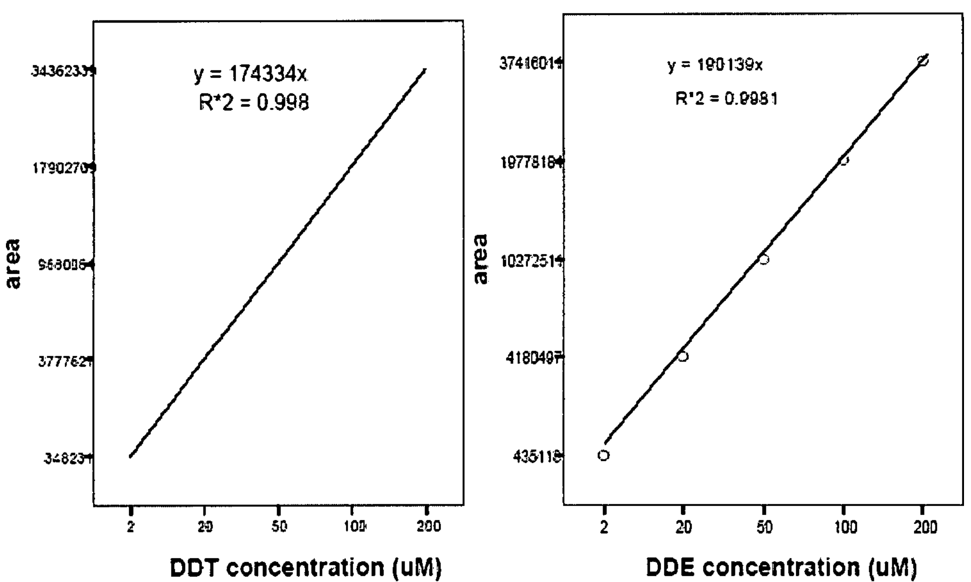


Figure 33. Standard curves for the insecticide DDT and its metabolites DDE in µmoles.

Mjindi, Makhathini Agricultural Research Station and Pongola village registered all the three genotypes, homozygote resistant (RR), heterozygote resistant (RS) and homozygote susceptible (SS), while the Pongola river only registered heterozygotes resistant and homozygotes susceptible. Figure 50 shows the percentage distribution of the altered AChE genes in Mamfene, Ubombo district. This mechanism produces resistance to both OPs and carbamates but resistance levels varies within the two groups of insecticides as described in detail in Chapter 5.

Organophosphate and carbamates insecticides are intensively used within the Makhathini Agricultural Research Station and the surrounding areas including Mamfene during cattle dipping. The association of the presence of altered AChE resistance mechanism with agricultural pesticide usage has been established in this study area and further work is being undertaken through the multi-sectoral forum established (Chapter 4) to further study these linkages. The range of propoxur inhibition values for the *An. arabiensis* populations tested showed a broad presence of the AChE genotypes (19.52% - 100%) from the four study sites which fall within what is called the Makhathini flats (Figure 34). The altered AChE gene is codominant to dominant and the three genotypes RR, RS and SS were established as ranging from 0% - 25%, 25% - 75% and 75% - 100% respectively (Figure 34). Based on the sample size used in this PhD, the results suggest that the altered AChE gene is present at low frequencies in these study sites.

Biochemical assays were performed to detect general esterase activity. Ranges of α-naphthol with the laboratory susceptible *An. arabiensis* were from 0–0.001875 μmoles α-naphthol produced/min/mg protein and 0–0.00125 μmoles α-naphthol produced/min/mg protein with *An. albimanus* susceptible strain. The 0.00125 cut off point was used as a baseline to determine the numbers of susceptible and resistance mosquitoes (Table 18 and Figure 28).

The ranges of field collected one to three day old F1 progeny were from 0.000 – 0.003125 μmoles α-naphthol produced/min/mg protein, showing the presence of elevated esterase activity with the substrate α-naphthyl acetate in *An. arabiensis* from the three study sites Makhathini, Pongola river and Pongola Village (Figure 35). Results for Mjindi were negative and were therefore excluded.

The baseline to determine the esterase activity levels for susceptible and resistant with the substrate β-naphthyl acetate was set at 0.001125 μmoles β-naphthol produced/min/mg protein (Table 18 and Figure 29). Ranges obtained were from 0.00 – 0.00469 μmoles β-naphthol produced/min/mg protein, showing the presence of elevated esterase activity with the substrate β-naphthyl acetate in *An. arabiensis*. The three study areas Makhathini, Pongola River and Pongola village showed no elevated esterase activity with the substrate β-naphthyl acetate but only Mjindi registered elevated esterases (Figure 36).

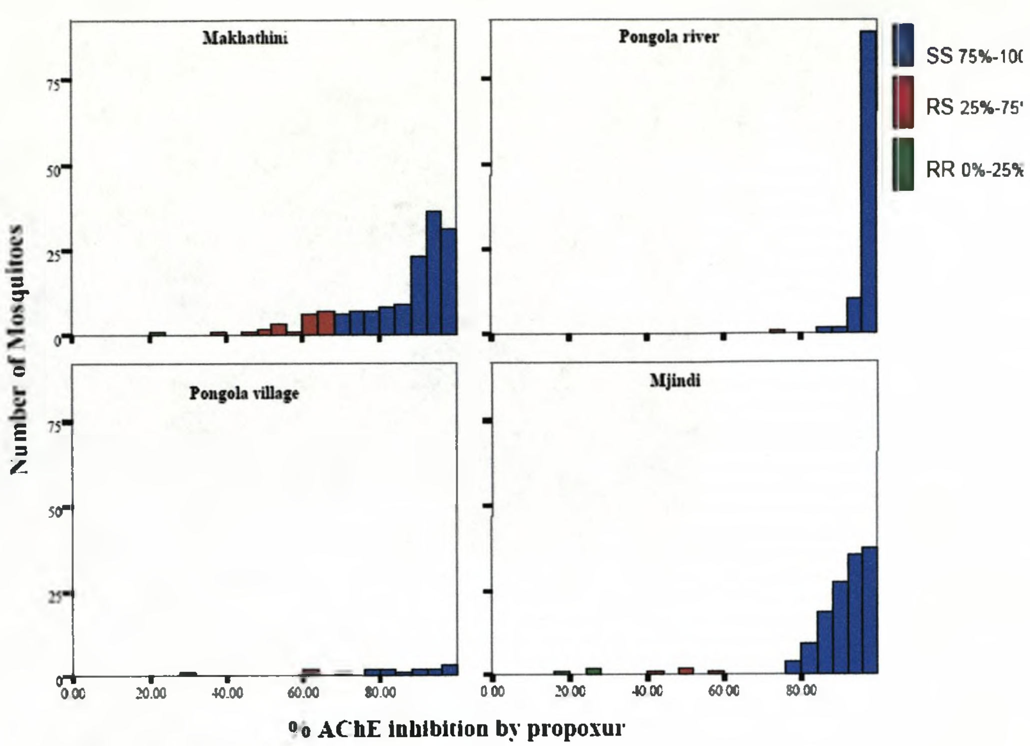


Figure 34. The range of acetylcholinesterase inhibition by propoxur in *An. arabiensis* in five study sites from KZN, South Africa.

Monooxygenase content in the laboratory strains ranged from 0 to 0.005 (*An. albimanus*) and 0 to 0.000875 (*An. arabiensis*) equivalent units of cytochrome P^{450}/mg protein. Ranges of monooxygenase content in the field collected samples from the four study sites in KZN were from 0 to 0.0017 equivalent units of cytochrome P^{450}/mg protein (Figure 37), which suggests that the baseline is likely different for *An. arabiensis* and *An. albimanus* laboratory strains.

GST activity in the Panama laboratory strain (*An. albimanus*) ranged from 0 to 0.375 mmol CDNB conjugated/min/mg protein indicating the activity range in a susceptible population. However, the Durban laboratory strain (*An. arabiensis*) had activity ranges from 0 to 0.750 indicating that a small number of resistance individuals are still segregating within this strain. This was also the case with the Mexican laboratory strain (*An. albimanus*) which had activity ranges from 0 to 1.402 mmol CDNB conjugated/min/mg protein, having been without insecticide selection pressure for four years (Penilla 2001). Raised levels of GST activity were detected from field collected *An. arabiensis* from all the four study sites with Mjindi registering the highest range 0 to 5.2 mmol CDNB conjugated/min/mg protein (Figure 38).

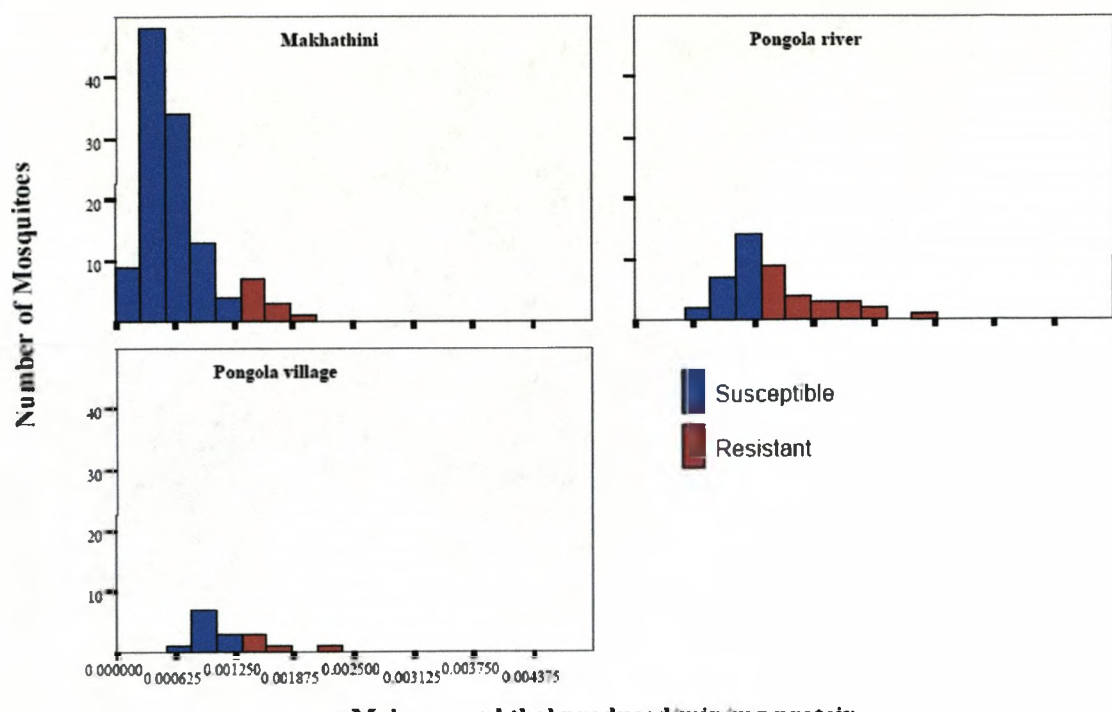


Figure 35. The range of esterase activity with α-naphthyl acetate in *An. arabiensis* in three study sites from KZN.

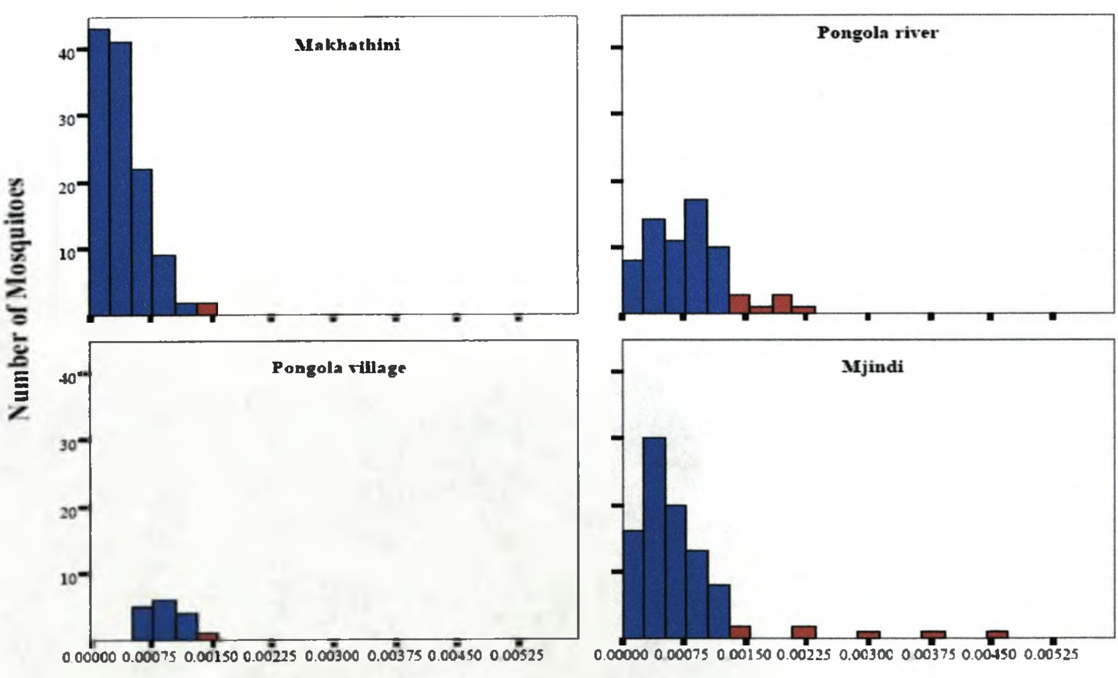


Figure 36. The ranges of esterase activity with β-naphthyl acetate in *An. arabiensis* in four study sites from KZN. Blue = susceptible and Red = resistant.

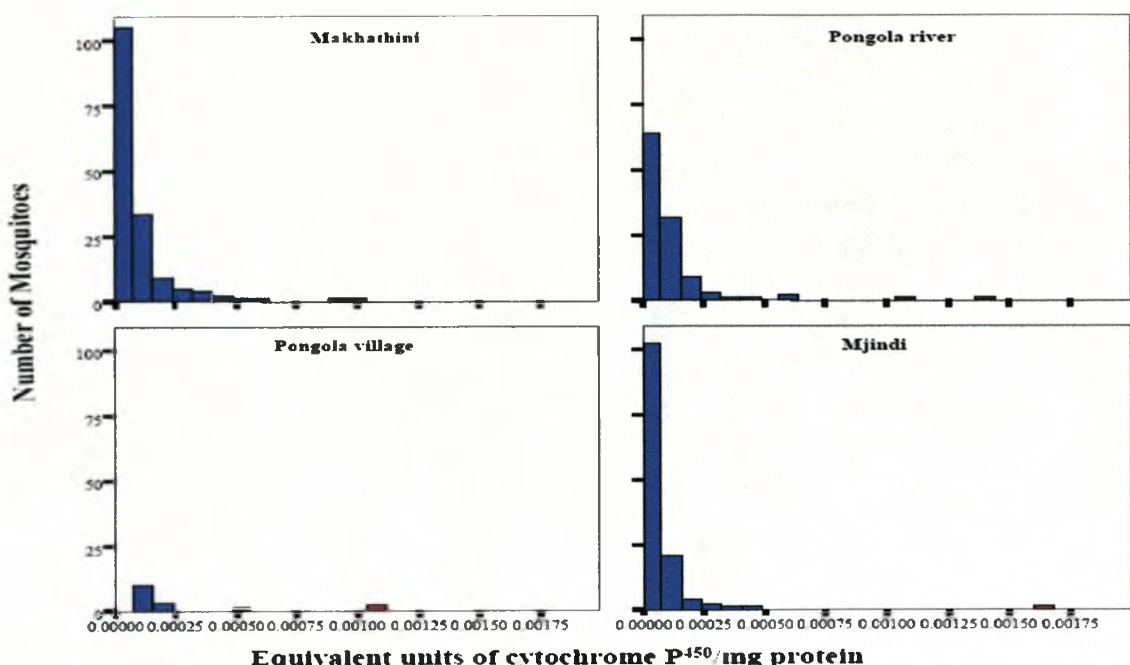


Figure 37. The range of equivalent units of cytochrome P^{450}/mg protein in *An. arabiensis* in four study sites from KZN, South Africa. Blue = susceptible and Red = resistant.

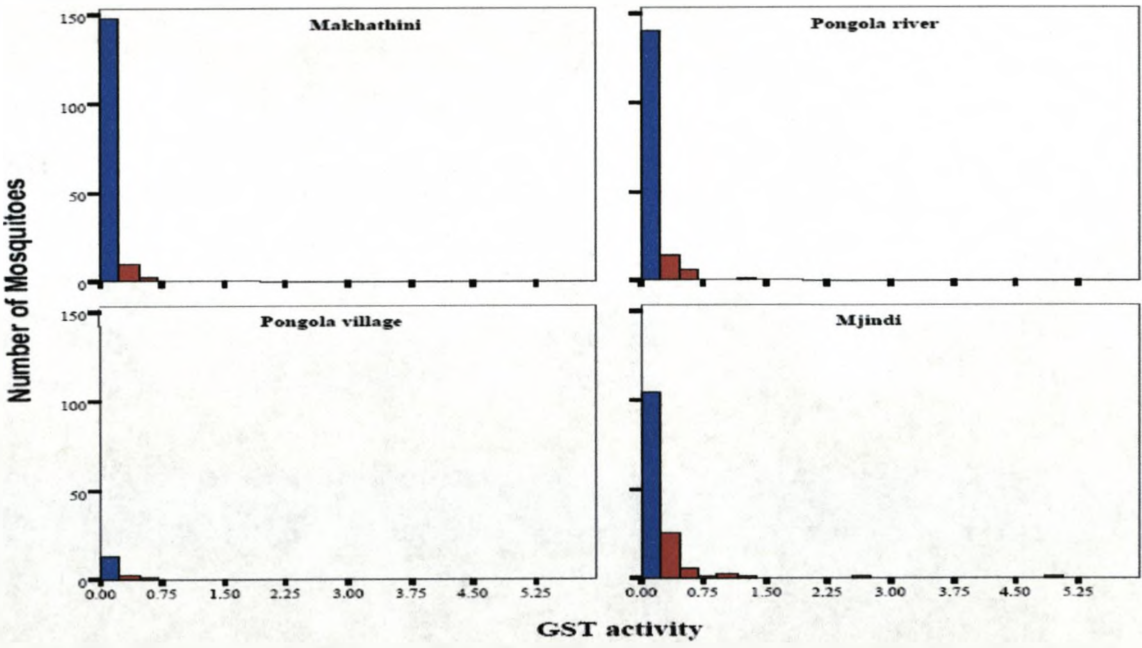


Figure 38. The range of GST activity (mmol CDNB conjugated/min/mg protein) in *An. arabiensis* in four study sites from KZN, South Africa. Blue = susceptible and Red = resistant.

The overall distribution of activity for GST, esterases, and ρNPA (Figure 39) for the field collected samples shows a similar pattern with the *An. arabiensis* (Durban) susceptible laboratory strain, while a similar distribution of monooxygenases with the field samples and *An. albimanus* (Panama) susceptible laboratory strain was observed. Figure 40 shows

the percentage distribution of the detected resistance mechanisms in South Africa and the neighbouring Swaziland.

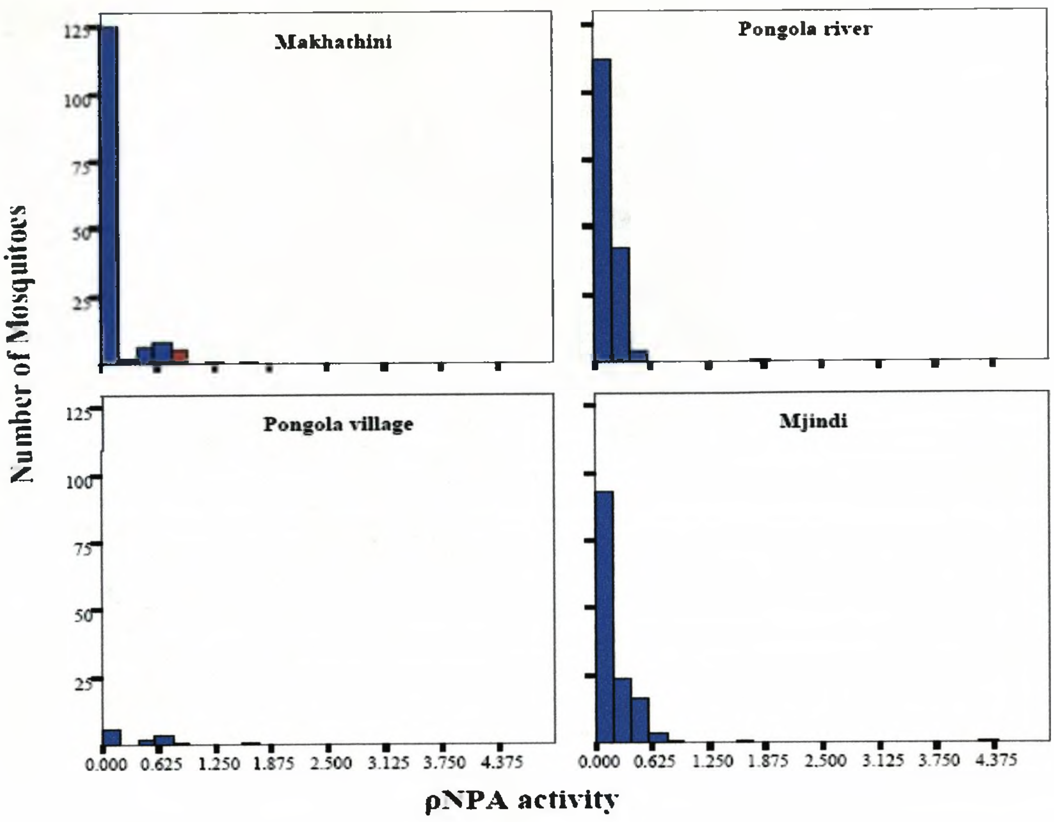


Figure 39. The ranges of ρ-nitrophenyl acetate (ρNPA) in *An. arabiensis* in four study sites from KZN, South Africa.

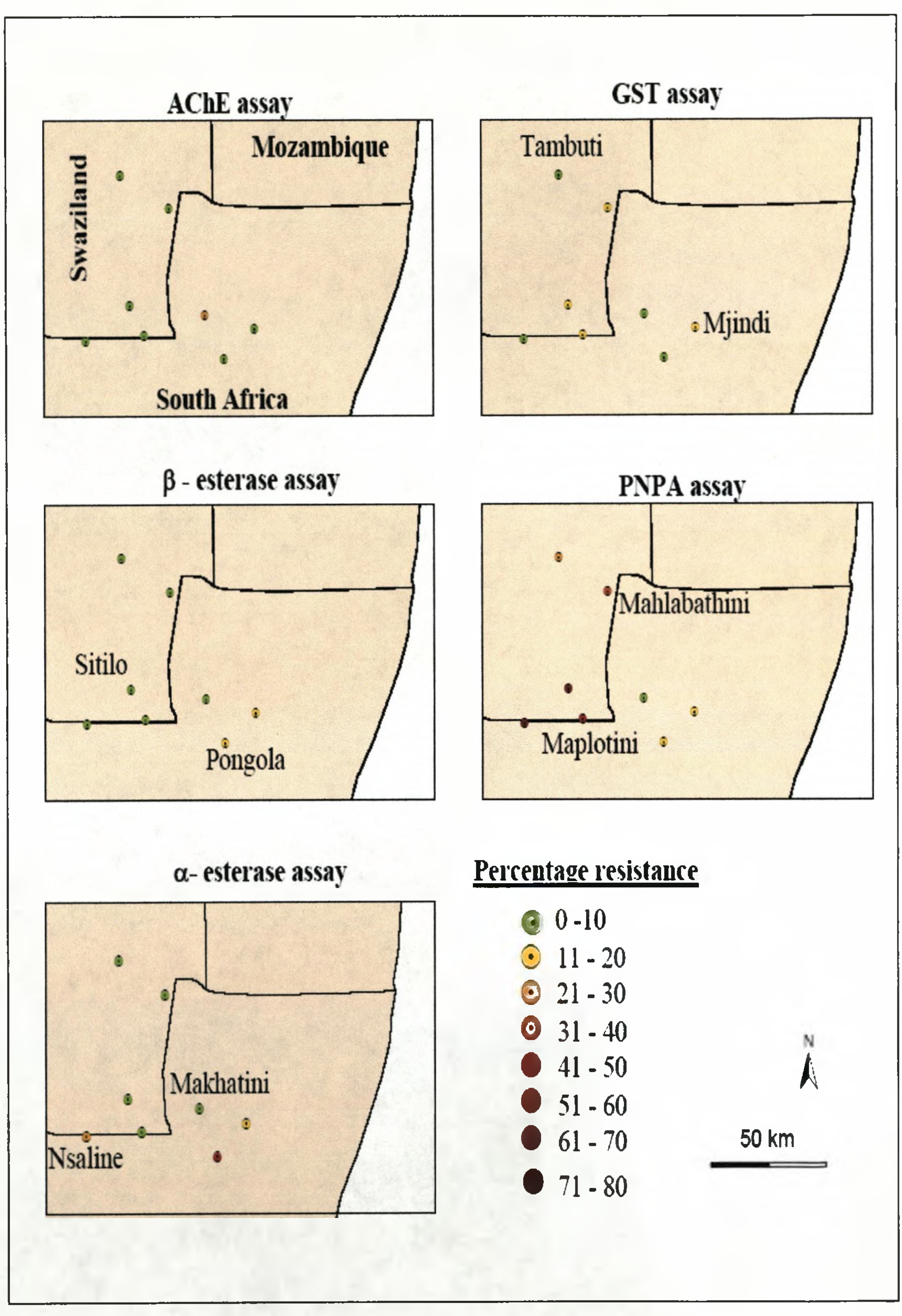


Figure 40. Percentage representation of altered AChE, elevated esterases, GSTs & ρNPA in four sites at Mamfene, Ingwavuma, KZN.

2.7 Mozambique

2.7.1 Collections

All samples from Mozambique were F1 progeny from adult blood fed female mosquitoes collected resting on indoor house walls. Mosquitoes were collected with aspirators and a torch. These were then transferred into paper cups which were in turn transferred to cool boxes with wet cotton to protect the mosquitoes from the heat while they were transported to the insectary. Individual mosquitoes were later transferred to breeding tubes fitted with a wet filter paper and fed with 10% sugar solution. All collections were transported to the Medical Research Council, Durban for processing.

2.7.2 Results

The mosquitoes collected were all *An. funestus*. WHO insecticide susceptibility tests showed that pyrethroid, and to a lesser extent, carbamate insecticide resistance was present in *An. funestus* from Mozambique (Table 23). WHO susceptibility tests showed high resistance to lambda cyhalothrin, with 57.7% and 62.8% survival in Boane and Bela-Vista respectively. Low resistance to deltamethrin was registered with 1.72% and 1.2% survival in Boane and Catuana respectively (Figure 41). No resistance was detected with the two carbamates – bendiocarb and propoxur, or the two organosphosphates – malathion and fenitrothion (at a 2 hour exposure for the latter), apart from Bela-Vista where there were some survivors on bendiocarb. DDT also showed 100% mortality in Boane and Bela-Vista.

Synergist studies undertaken with lambda cyhalothrin survivors tested on papers impregnated with deltamethrin and piperonyl butoxide (PB), gave 7.59 % survival, while on deltamethrin alone samples from the same batches of mosquitoes gave 39.7% survival (Table 24). This synergist data suggests that a monooxygenase resistance mechanism was present in *An. funestus*. PB alone is supposed to give 100% survival but the high mortality rate with the results is possibly due to the fact that the mosquitoes had already been exposed to Lambda-cyhalothrin and therefore had been stressed before being exposed to PB. However, there are problems with interpretation of synergist data alone to

conclusively identify the type of resistance mechanism involved, as will be outlined in full in the discussion session of this chapter.

Biochemical assays indicated the presence of an altered acetycholinesterase mechanism, which should confer resistance to both carbamates and organophosphates (OPs) (Figure 57). This resistance mechanism was detected in Boane with homozygotes susceptible (78%), heterozygotes resistant (20%) and homozygote resistant (2%) genotypes. Other areas with this resistance mechanism were Beluloane with homozygotes susceptible (84%), heterozygotes resistant (15.6%) and homozygote resistant (0.4%). Bela-Vista and Catuane registered heterozygote resistant (7% & 19%) and homozygote susceptible (93% & 81%) respectively. The detection of acetylcholinesterase-based resistance contrasts with the WHO susceptibility data suggesting no OP or carbamate resistance. However, the susceptibility assays almost by definition should give an underestimate of resistance as they are set at a sufficiently high level so as not to give false reports of resistance. In Mexican *An. albimanus*, it was shown that bioassays with propoxur only detected the homozygous altered AChE resistant insects (Penilla 2001).

A small proportion of individuals had elevated esterases registered in all the areas (Figures 42, 43 & 45), indicating a probable role in OP resistance. Bela-Vista and Beluloane had 27% and 21.5% of individuals respectively with elevated GSTs (Figure 44) and low levels at Boane and Catuane at 3.5% and 8.6% respectively which could indicate resistance to DDT or pyrethroids. Elevated GST activity has been detected in housefly strains resistant to organophosphates (Fournier and Hammock 1992) and *Aedes aegypti* strains resistant to organochlorines (Grant et al., 1992) and has been implicated in resistance to pyrethroid insecticides (Vontas et al., 2001). DDT and pyrethroid metabolic studies are being undertaken with *An. funestus* to confirm the role of these elevated enzymes in insecticide resistance in this species.

High levels of cytochrome P^{450} monooxygenases were registered in all the areas in Mozambique. Beluloane registered the highest levels of cytochrome P^{450} with 14.3% of population having elevated P^{450} levels, with Boane and Bela-Vista at 5.8% and 5.3% respectively (Figures 46 & 48). Monooxygenase are likely to produce pyrethroid or carbamate resistance and may give positive or negative cross-resistance to organophosphates.

Locality	Species	Insecticide	N Mosquitoes	% Survival	N Bioassays
Boane	*An. funestus*	Lambda cyhalothrin	689	57.5%	74
	An. funestus	Propoxur	193	0	20
	An. funestus	Bendiocarb	245	0	26
	An. funestus	DDT	233	0	22
	An. funestus	Deltamethrin	174	1.72%	20
	An. funestus	Malathion	31	0	2
	An. funestus	Fenitrothion	296	0	32
	An. funestus	Permethrin	121	0	8
Bela-Vista	*An. funestus*	Lambda cyhalothrin	223	62.80%	22
	An. funestus	Bendiocarb	242	0.41%	23
	An. funestus	DDT	206	0	20
	An. funestus	Malathion	112	0	10
	An. funestus	Fenitrothion	67	0	6
Catuana	*An. funestus*	Deltamethrin	31	6.45%	3
	An. funestus	Permethrin	83	1.20%	6
Beluloane	*An. funestus*	Bendiocarb	459	0	55

Table 23. WHO susceptibility data for one-three day old F1 *An. funestus* from 4 localities in Mozambique.

Locality	Insecticide	N mosquitoes	N survivors	% Survival	N tests
Boane	Deltamethrin + P. butoxide	79	6	7.59%	7
Boane	Deltamethrin	73	29	39.70%	7
Boane	P. butoxide	66	15	22.70%	6
	Total	218	50		20

Table 24. Synergist data for bioassays with deltamethrin and the monooxygenase inhibitor piperonyl butoxide as an indicator of a possible monooxygenase-based pyrethroid resistance mechanism in *An. funestus* from Boane.

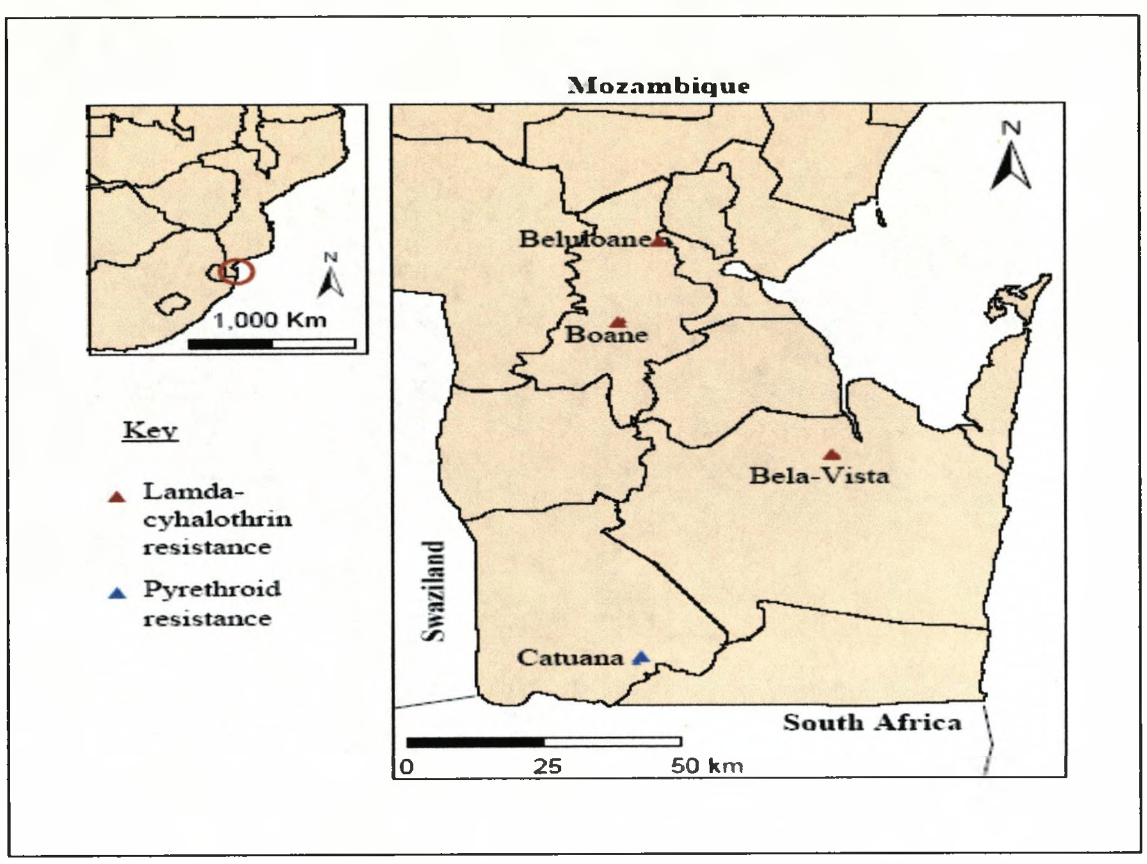


Figure 41. Representation of resistance due to pyrethroids with standard WHO susceptibility tests in Mozambique.

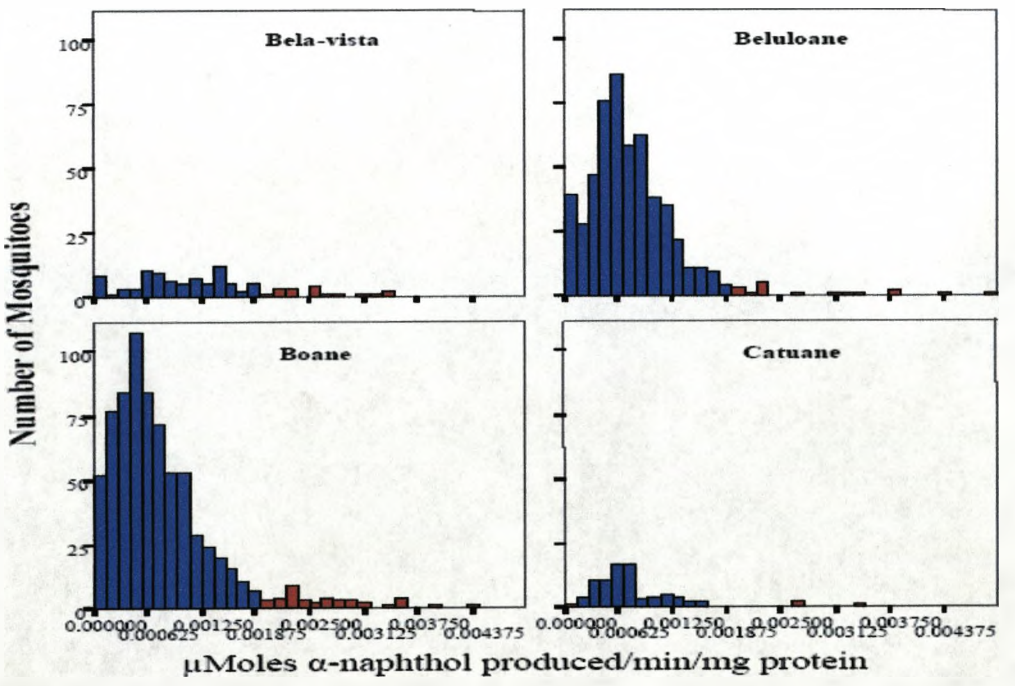


Figure 42. The range of esterase activity with α- naphthyl acetate in *An. funestus* Boane, Bela-Vista, Catuane and Beluloane (N=704, 127, 79 &551 respectively) in Mozambique. Red = resistance, Blue = susceptible.

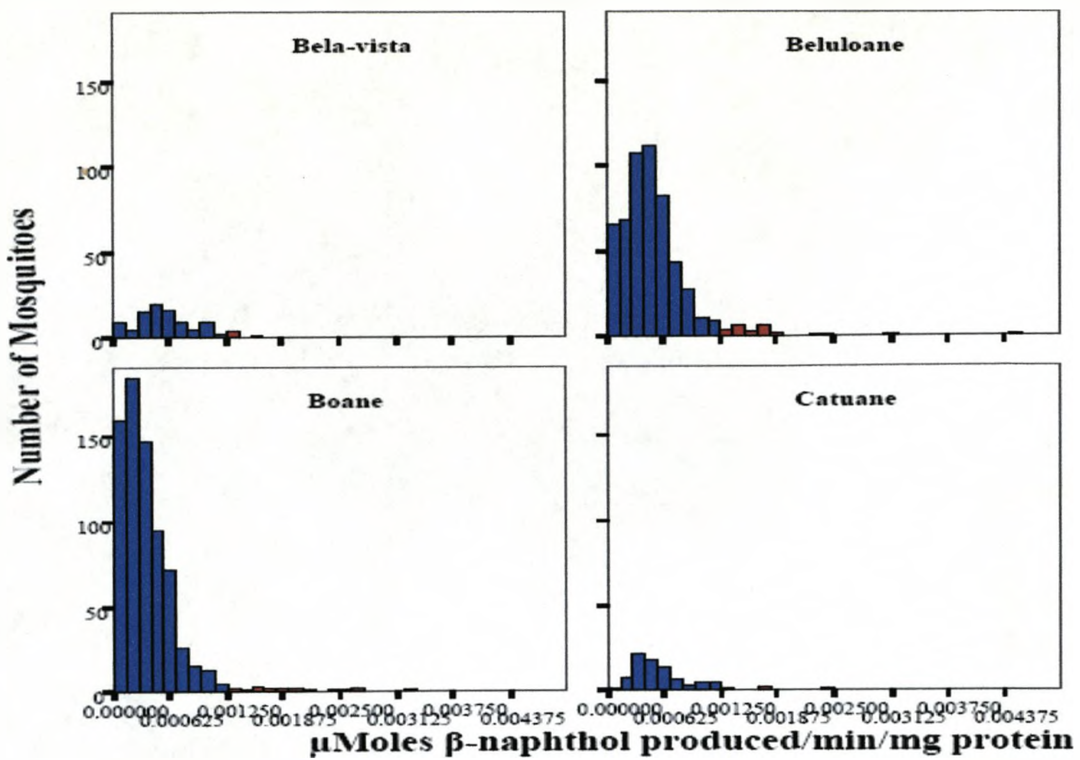


Figure 43. The range of esterase activity with ρ-naphthyl acetate in *An. funestus* Boane, Bela-Vista, Catuane and Beluloane (N= 702, 125, 78, & 560 respectively) in Mozambique. Red = resistance, Blue = susceptible.

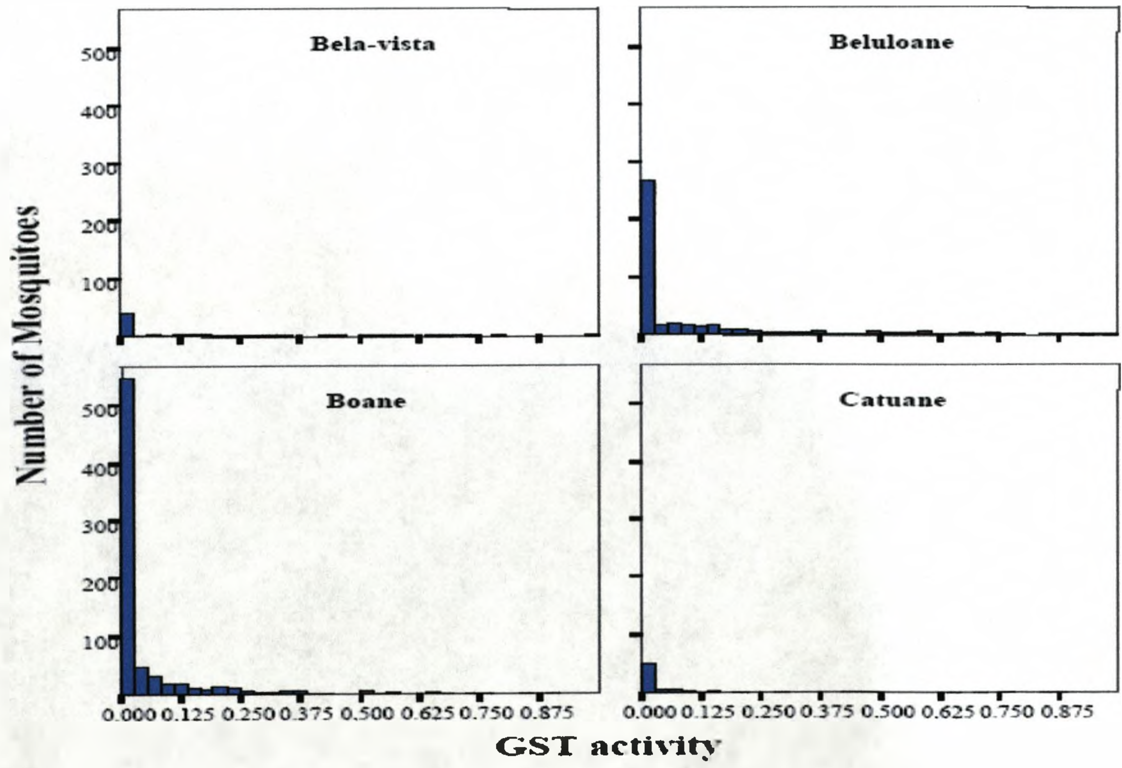


Figure 44. The ranges of GST activity in *An. funestus* from four study sites in Mozambique. Red = resistance, Blue = susceptible.

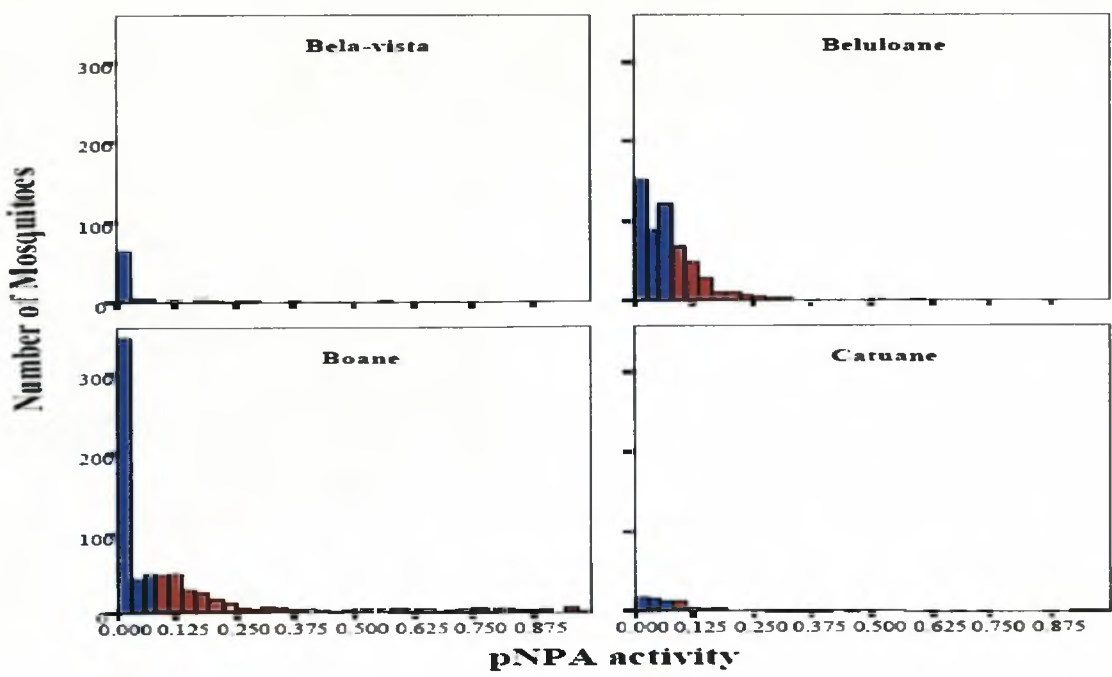


Figure 45. Esterase activity with ρ-nitrophenyl acetate in *An. funestus* in four sites from Mozambique. Red = resistance, Blue = susceptible.

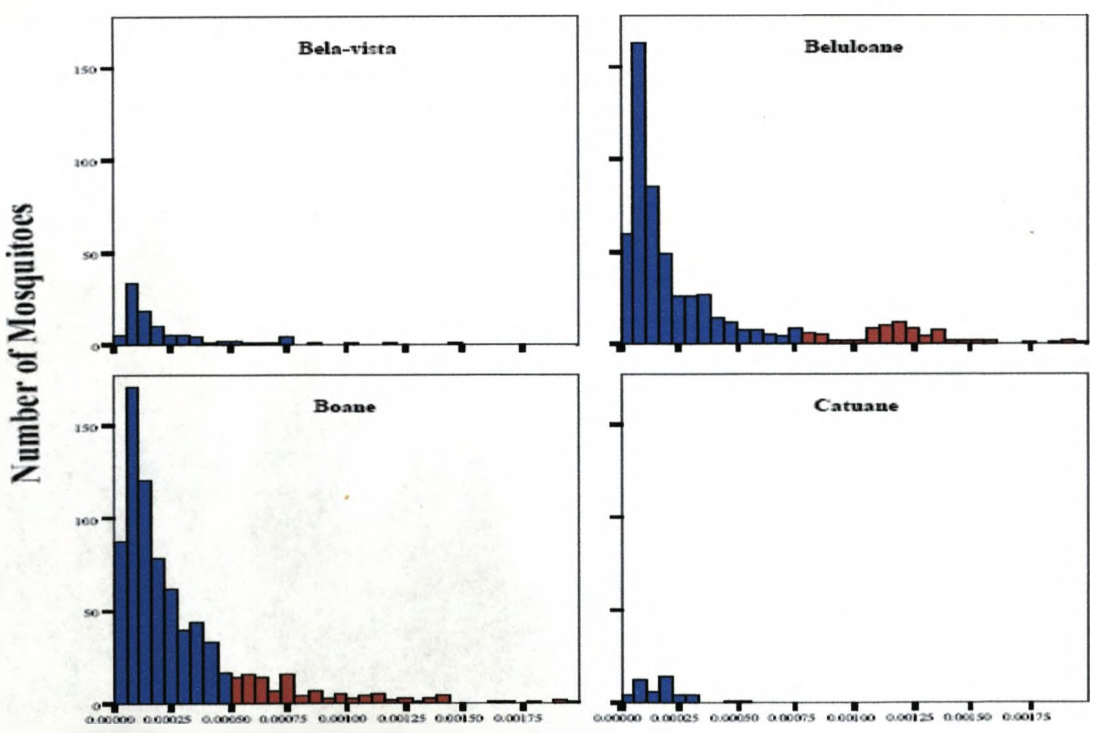


Figure 46. The range of equivalent units of cytochrome P^{450}/mg protein in *An. funestus* from Boane, Bela-Vista, Catuane and Beluloane (N= 743, 131, 42, & 602 respectively) in Mozambique. Red = resistance, Blue = susceptible

Figures 47 and 48 shows the percentage distribution of resistance mechanisms detected from the four study sites (Beluloane, Bela-Vista, Boane and Catuana) in Mozambique.

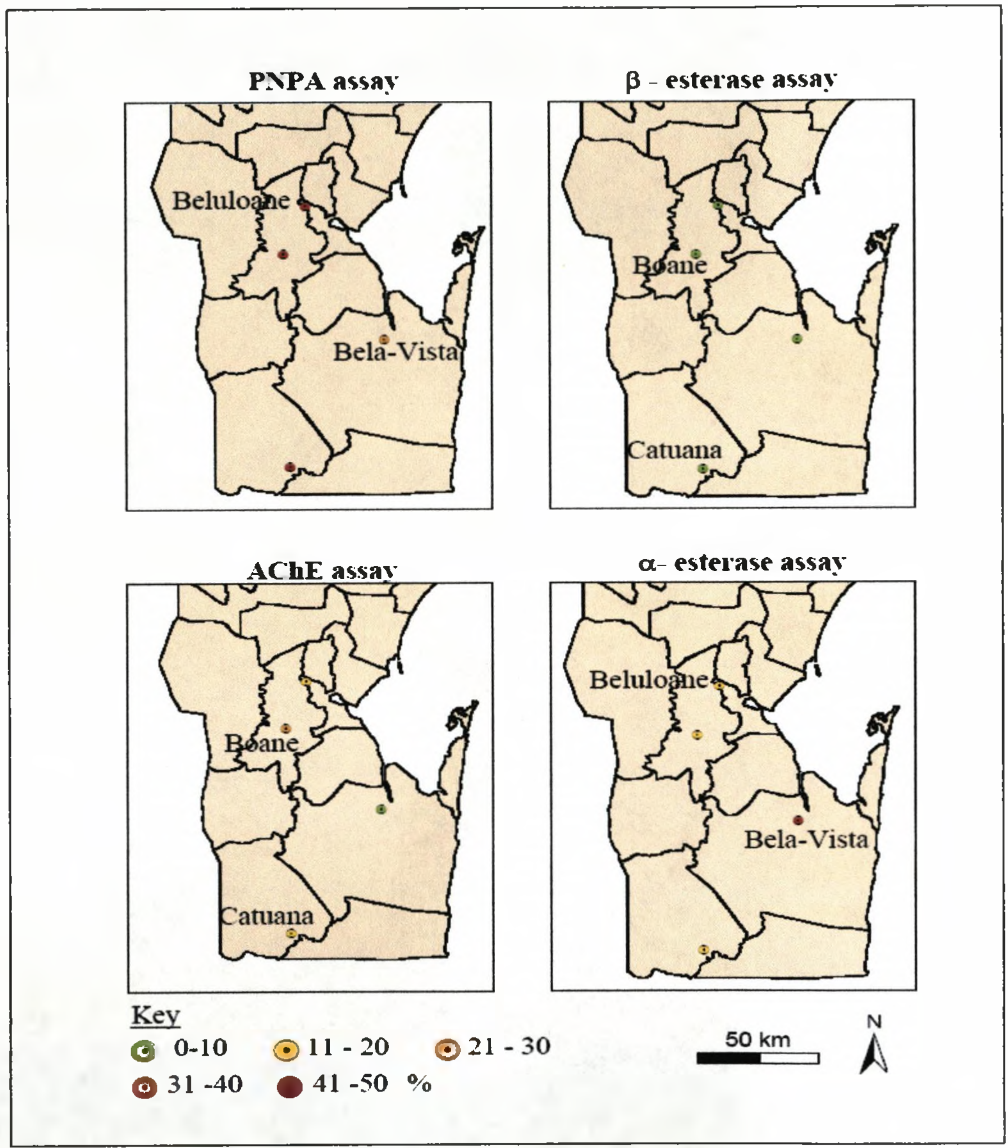


Figure 47. Percentage representation of insensitive AChE and general esterases type of resistance in Mozambique

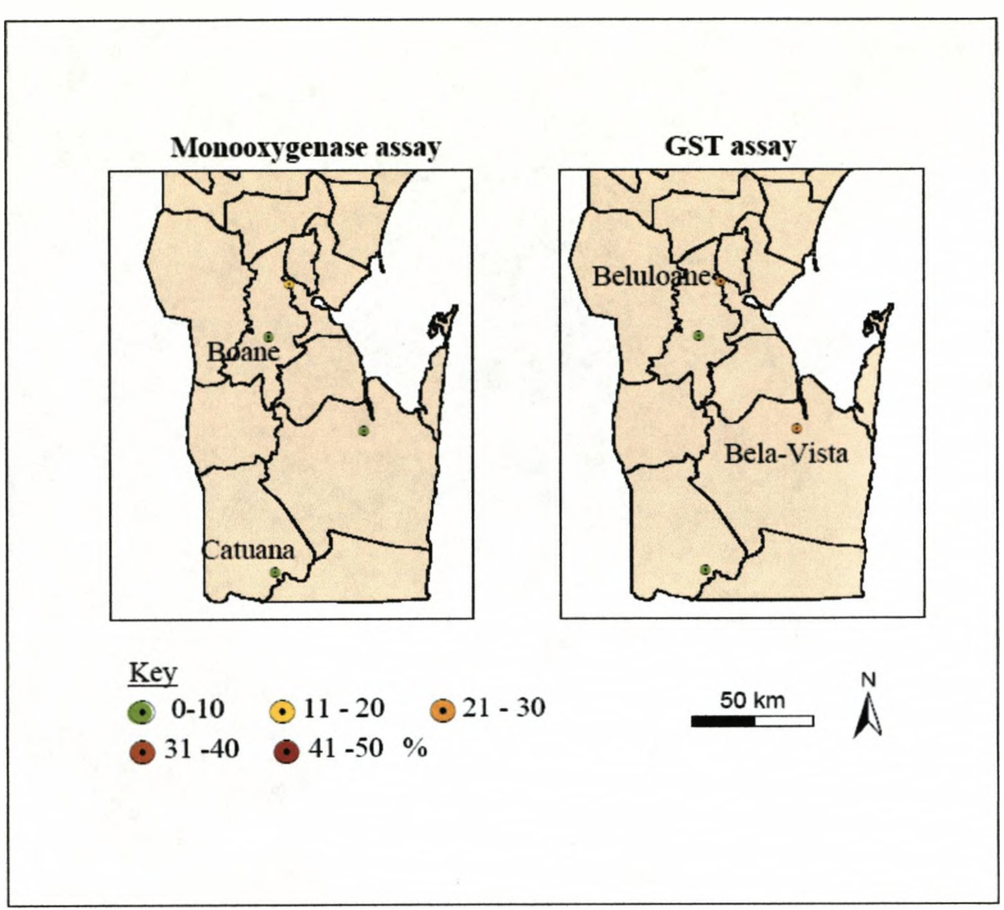


Figure 48. Percentage presentation of monooxygenase mediated resistance and elevated glutathione S-transferases from samples in Mozambique.

2.8 Swaziland

2.8.1 Collections

Documentation of pesticides used in agriculture was undertaken in one of the study sites, Stilo (Table 25) which is the highest citrus fruit producing area in Swaziland. Problems were experienced in Swaziland with co-operation of farmers on the issue of pesticide calendar usage. The farmers suggested that changes in their pesticide choice were based on 'Hindsight', meaning that a reduction in agricultural productivity resulted in a change in the pesticide applications.

Mosquito collections were undertaken in five study sites in the Manzini province. Vector control personnel were trained in sampling methods as explained earlier. Malaria control in Swaziland was launched in 1945 and residual indoor spraying with DDT on a limited scale started in 1947. DDT was temporarily abandoned from 1951-52 and replaced by the

organochlorine BHC. Indoor residual spraying of all households with DDT recommenced in the 1970s to the present and pyrethroids (cyfluthrin) were used only in houses with painted walls. Two methods of mosquito collections were employed in Swaziland; larval and cattle enclosure catches. The latter did not produce any *An. arabiensis* and therefore the analysis was based only on larval collections.

2.8.2 Results

No WHO susceptibility tests were performed on adult F0 progeny from larval collections. It was difficult to establish the vector species within these mosquitoes' collections due to the sympatric occurrence of the vector with non-vectors in this region. Hence the results for Swaziland for the biochemical assays are recorded as *An. gambiae* s.l, *An. arabiensis,* and *An.quadriannulatus*. Swaziland is below the southern boundary of the *An. gambiae* s.s and hence it is probable that all samples recorded as *An. gambiae* s.l were *An. arabiensis*. All mosquitoes were subjected to biochemical assays and body parts of single mosquitoes were used for species identification. The negative results from the species identification PCR were recorded as *An. gambiae* s.l, which could have been due PCR inhibition by either too much DNA content from lots of body parts used per reaction. The results showed very low levels of the vector *An. arabiensis* from all the sites.

The collections of mosquitoes including documentation of pesticides used in agriculture were undertaken during the rainy season towards the end of the malaria transmission season, which may have favoured collection of non-vectors. An attempt was made to undertake further collections, but this was unsuccessful for reasons discussed later. Biochemical assays were performed on one to three day old adult F0 progeny for the detection of, elevated esterases, glutathione S-transferases, AChE and esterases with the substrate ρ-nitrophenyl acetate and monooxygenases (Figures 49 – 54).

Organophosphates	Pyrethroids	Carbamates
Tokuthion	Cypermethrin	Lannate
Curacron		Temik
Ultracicle		
Mesurol		
Dicarzol		
Malathion		
Dipterex		
Methamidophos		

Table 25. The insecticides used in Citrus fruit production in Stilo, Swaziland.

Mahlabathini, Nasaline and Stilo had a few individuals with elevated esterase activity with α and β-naphthyl acetate (Figures 49 and 50). Elevated glutathione S-transferase (GST) activity was detected in sites Mahlabathini, Stilo and Maplotini in *An. arabiensis*, *An. quadrianulatus* and in the *An. gambiae* s.l collections (Figure 51). Altered acetycholinesterase (AChE) based resistance to carbamates and organophosphates was registered at very low frequencies at sites Mahlabathini and Nasaline (Figure 52). The elevated esterase based-mechanism detected with ρ-nitrophenyl acetate and the naphthyl acetates will confer some resistance to OPs. Elevated esterases with the substrate p-nitrophenyl acetate were detected in Mahlabathini, Nasaline, Stilo and Maplotini (Figure 53). The actual numbers of mosquitoes from each area used for the biochemical assays is given in Table 26.

Name	Number of mosquitoes
Mahlabathini	61
Nasaline	50
Stilo	53
Tamboti	5
Maplotini	17
Total	**186**

Table 26. Shows names of study areas and respective mosquitoes numbers used for biochemical assays in Swaziland.

Graphical presentations of biochemical assays performed on 1-3 day old F0 adult mosquitoes from five study areas in Swaziland as explained in the material and methods section are shown below.

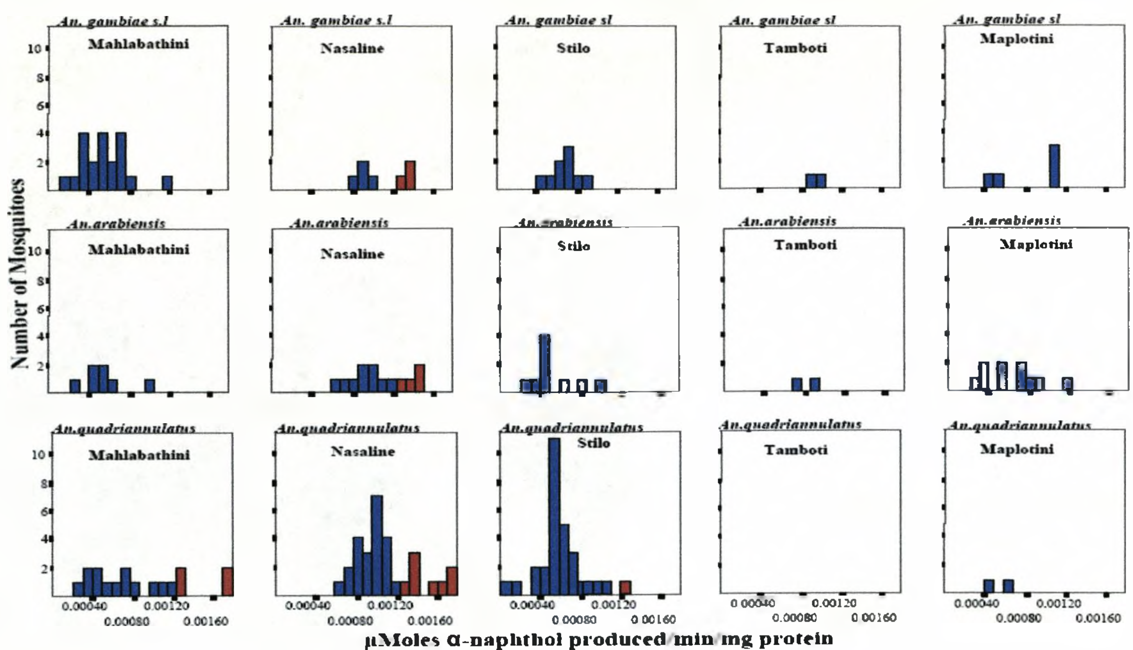


Figure 49. The range of esterase activity with α-naphthyl acetate in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Red = resistance, Blue = susceptible.

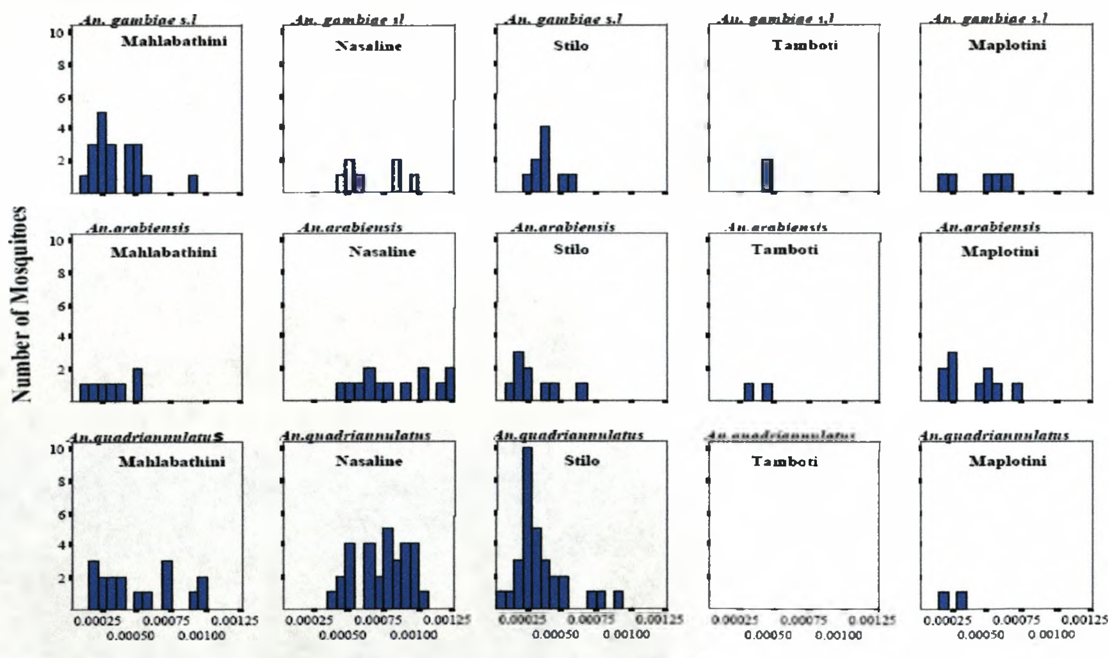


Figure 50. The ranges of esterase activity with β-naphthyl acetate in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Blue = susceptible.

No raised levels of esterases with the substrate β-naphthol acetate were registered from all the five study (Figure 50).

Very low levels of altered AChE resistance mechanism were detected from Mahlabathini and Nasaline indicating the probable segregation of this gene in the population from these two sites (Figure 52).

Raised levels of esterase activity with the substrate ρ-nitrophenyl acetate were registered from Mahlabathini, Nasaline, Stilo and Maplotini (Figure 53). Mosquitoes from Tamboti were susceptible but the sample size form this area was very low.

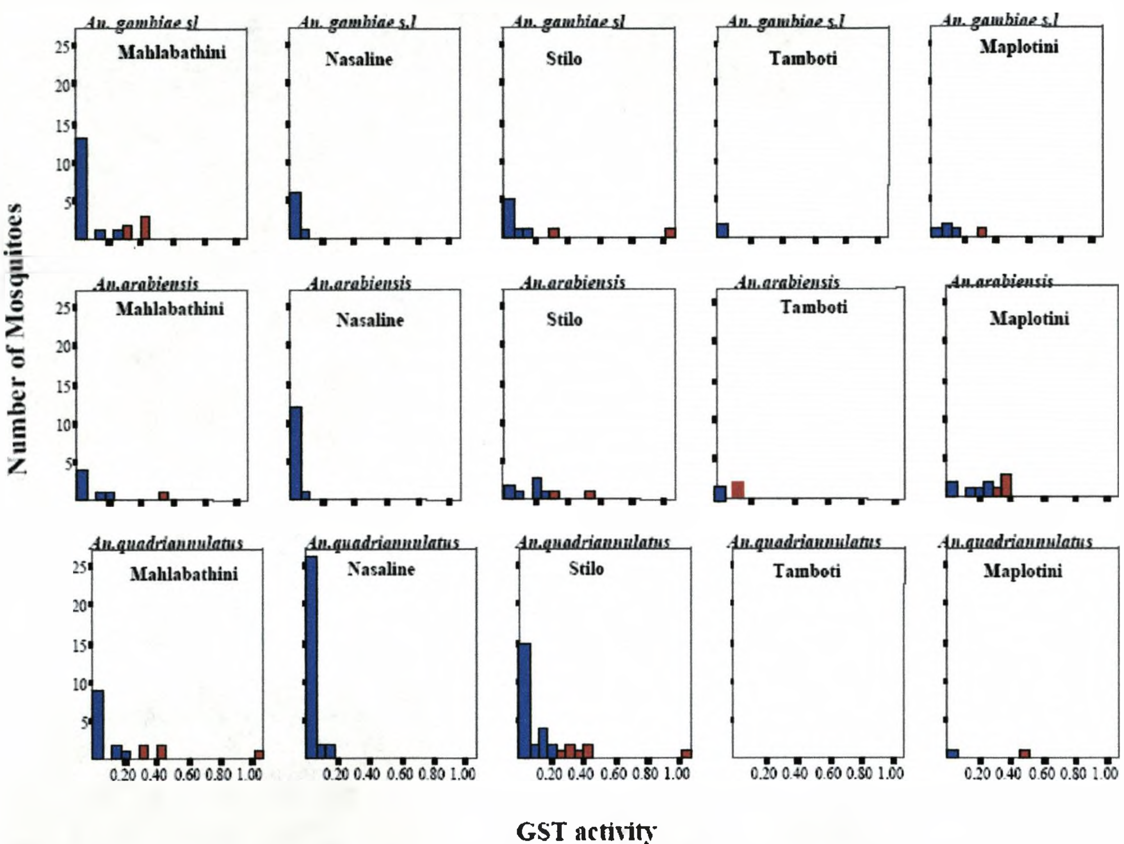


Figure 51. The range of GST activity (mmol CDNB conjugated/min/mg protein) in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Red = resistance, Blue = susceptible.

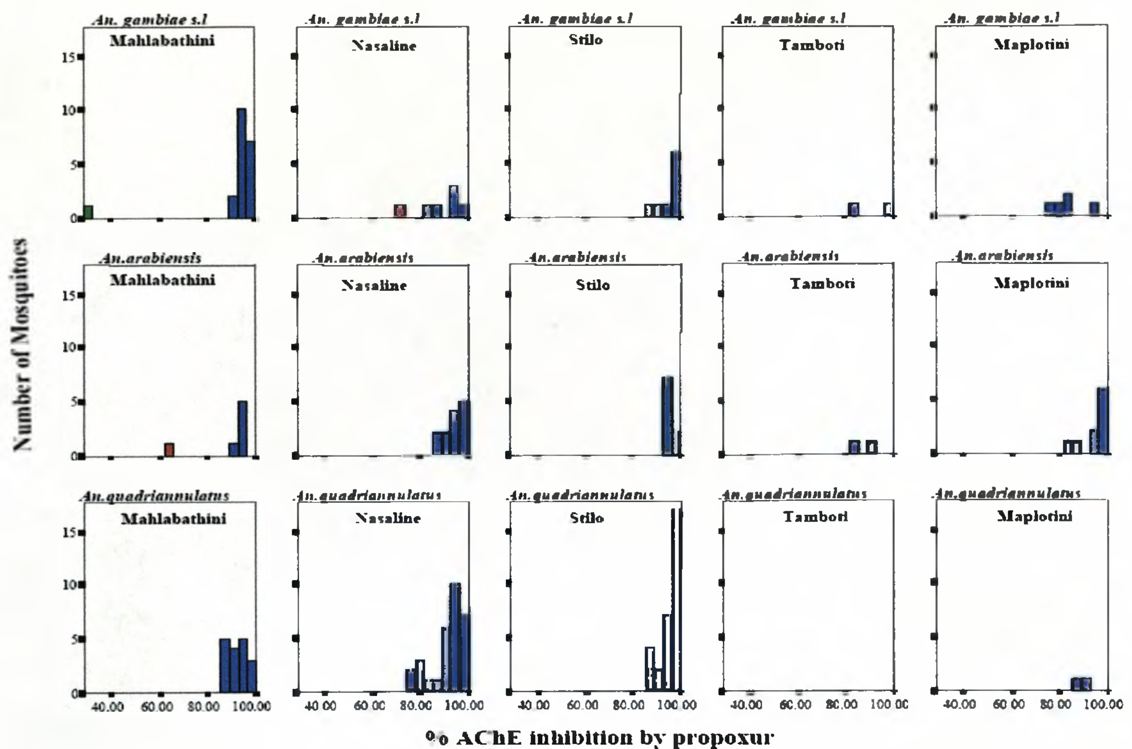


Figure 52. The range of acetylcholinesterase inhibition by propoxur in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Red = resistance, Blue = susceptible.

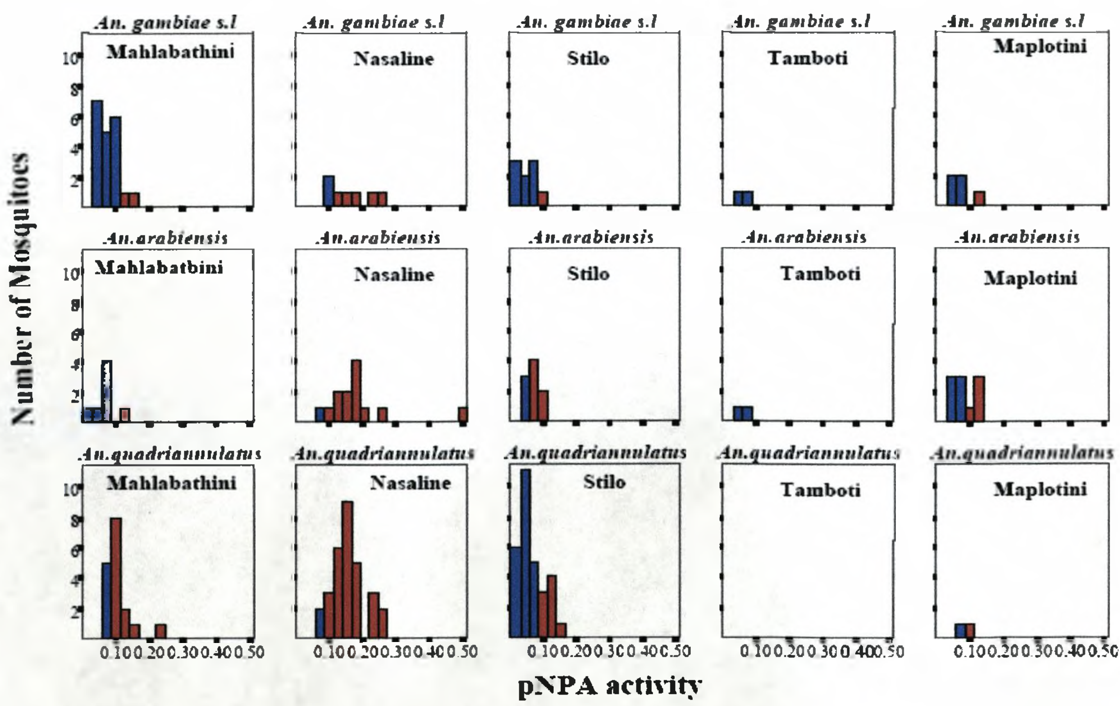


Figure 53. The ranges of activity with ρ-nitrophenyl acetate (ρNPA) in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Red = resistance, Blue = susceptible.

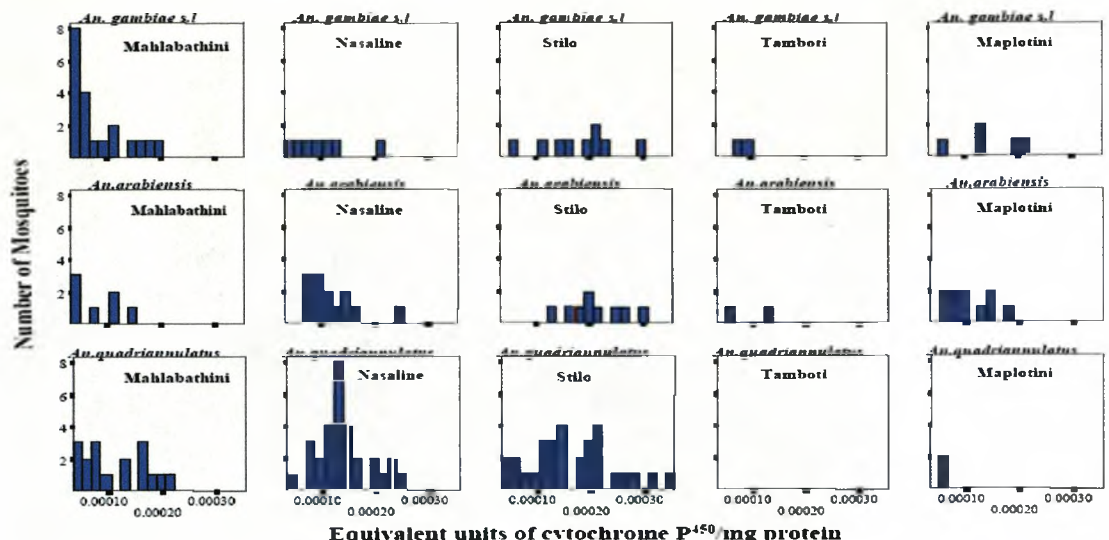


Figure 54. The range of equivalent units of cytochrome P^{450}/mg protein in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland. Red = resistance, Blue = susceptible.

Figure 55 below shows resistance mechanisms detected in Swaziland with biochemical assays as described in the material and methods section.

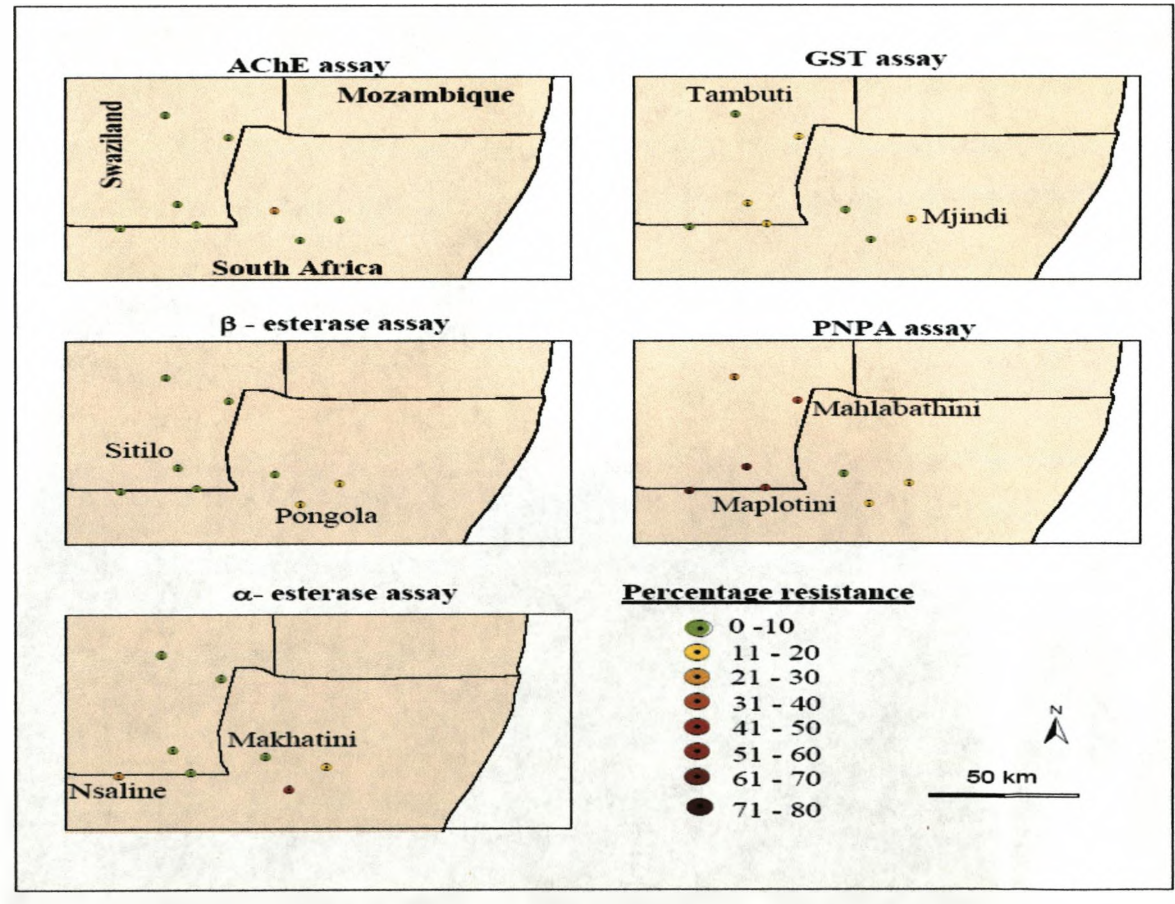


Figure 55. Map of Swaziland showing the percentage of the different resistance mechanisms detected in Sitilo, Nsaline, Mahlabathini, Maplotini and Tamboti.

Collections sites in Swaziland are close to study sites in KZN, South Africa and therefore single Swaziland map could not be produced, hence the appearance of study sites in Figure 55.

2.9 Zambia

2.9.1 Collections

Study sites in Zambia covered three districts, Chililabombwe, Chingola and Kabwe in Ndola province and Mwinilunga in the north western province (Figure 21). Figure 56 shows micro geographical collections in Isomeka, Kasembe and Fiwale. In Chililabombwe and Chingola, an intersectorial 'Roll Back Malaria'-partnership was set up to oversee the planning, implementation, monitoring and evaluation of a malaria control programme. The Konkola Copper Mines is a partner in this project and has embarked on a control programme covering Chililabombwe and Chingola towns on the Copperbelt. An indoor residual spraying campaign was commenced at the end of 2000 that reduced the malaria incidence rates to 33/1000 for Chingola and 58/1000 for Chililabombwe respectively (Konkola Copper Mines plc technical report 2001). The indoor residual spraying programme implemented in the Copperbelt area has also resulted in the reduction of the vector as is seen by the low number of samples collected for analysis, compared to Fiwale in Ndola province where there was no control programme.

Fiwale is a rural area, dependent on subsistence farming in the form of vegetables. The Kabwe district forms the major agricultural part of the Ndola province. Collections were made in May during the dry cold season, and did not yield any samples. However, the insecticide monitoring and pesticide documentation programme was established at this time with the communities, farmers and the Tropical Disease Research Institute in Zambia. The north Western province of Mwinilunga lies between Angola and the Democratic Republic of Congo (formerly Zaire) (Figure 21). This province is involved in very little subsistence farming as the majority of income is being raised from diamond smuggling.

2.9.2 Results

Table 27 shows the number of samples used for biochemical assays with their respective study sites. Documentation of pesticide usage was undertaken in all the areas visited in this study with only Fiwale being cooperative in this regard (Table 28). WHO susceptibility tests were performed on samples from Fiwale in Ndola province and Mwinilunga (Table 29). Chililabombwe and Chingola did not produce any adult blood fed mosquitoes due to the DDT indoor residual spraying implemented in these areas. Only larval collections were analysed from Chililabombwe/Chingola province. WHO tests were performed on one to three day adult F1 progeny from Fiwale, in Ndola province and Isomeka, Mwinilunga province.

WHO susceptibility tests with deltamethrin (0.05%) and DDT (4%) on one to three day old F1 progeny from samples collected in Fiwale, Ndola gave 93.55% and 98.5% mortality respectively. Resistance to both to DDT and deltamethrin was detected at low levels suggesting the presence of a kdr-based type of resistance mechanism at very low frequencies (Table 30). Biochemical assays (Figures 57 to 71) showed no altered acetylcholinesterase (AChE) based resistance mechanism in all areas except for very low levels at Fiwale where AChE inhibition levels ranged from 41% - 100%. This area also had slight levels of elevated esterases, which could indicate resistance to organophosphorus insecticides.

Problems were encountered with the kdr PCR method for detection of the kdr mutation. A new method called HOLA was therefore employed (see Materials and Methods section). The failure of the test to establish the kdr status on some of the samples could be due to the poor condition of the sample DNA conditions. Initial results with the standard kdr PCR assay (Martinez-Torres et al., 1998) at MRC, Durban, South Africa during this study showed that the West African mutation was present in *An. gambiae* s.s from Fiwale, Zambia. Problems with recording of the initial results were encountered at the MRC as the laboratory lacked bio imaging instruments. Sequencing of the DNA with more samples from Fiwale is being undertaken at the Liverpool School of Tropical Medicine, in order to validate the initial results.

Name	Number of mosquitoes
Kasembe	18
Lulamba	7
Chabayama	4
Lubengele-RBW section	20
Chililabombwe	10
Meliya	10
Kapoto	7
Fiwale	168

Table 27. Shows names of study sites in Zambia and the respective numbers of samples from these sites analysed by biochemical assays.

Table 28 below shows insecticides used in Fiwale vegetable gardens with their calendar period.

Trade name	Calender usage
Cypermethrin – EC 40%	All year
Thiodan 35%	All year
Fenkil 40%	All year
Thiokil 35%	All year
Fenkil 40%	All year
Phoskil 40%	All year
Diathane M45	Winter to dry season
Urathane M45	Winter to dry season
Brewe	Winter to dry season
Soluber	Winter to dry season
Colbox copper chloride	Winter to dry season
Actellic super	Winter to dry season
Marathon	Winter to dry season

Table 28. Pesticides used in Fiwale on vegetable gardens.

Locality	Insecticide	N	% Survival	N bioassays
Fiwale	Deltamethrin	31	6.45%	3
Fiwale	DDT	67	1.49%	8
Isomeka	Deltamethrin	153	0	24
Isomeka	DDT	9	0	1
Isomeka	Propoxur	7	0	1

Table 29. WHO susceptibility tests for three sites (Fiwale, Isomeka and Kanyi) in Zambia

Figures 57 – 61 below show ranges of resistance mechanisms detected with biochemical assays performed on 1-3 day old adult F1 progeny (*An. gambiae* s.s) as described in the materials and methods section from eight study sites in Ndola and Chingola provinces,

Zambia. Ranges of esterase activity with the substrate α-naphthyl acetate in *An. gambiae* s.s from Fiwale were from 0.00024 - 0.00563 μMoles α-naphthol produced/min/mg protein (Figure 57). The distribution of esterase activity from the other seven sites showed a similar pattern to that of *An. albimanus* susceptible strain indicating that the populations from these areas are susceptible. The low numbers of mosquitoes collected from these sites could also be a contributing factor. The lack of adult blood fed female mosquitoes resting indoors also indicates that the population is susceptible to DDT used for IRS programmeme.

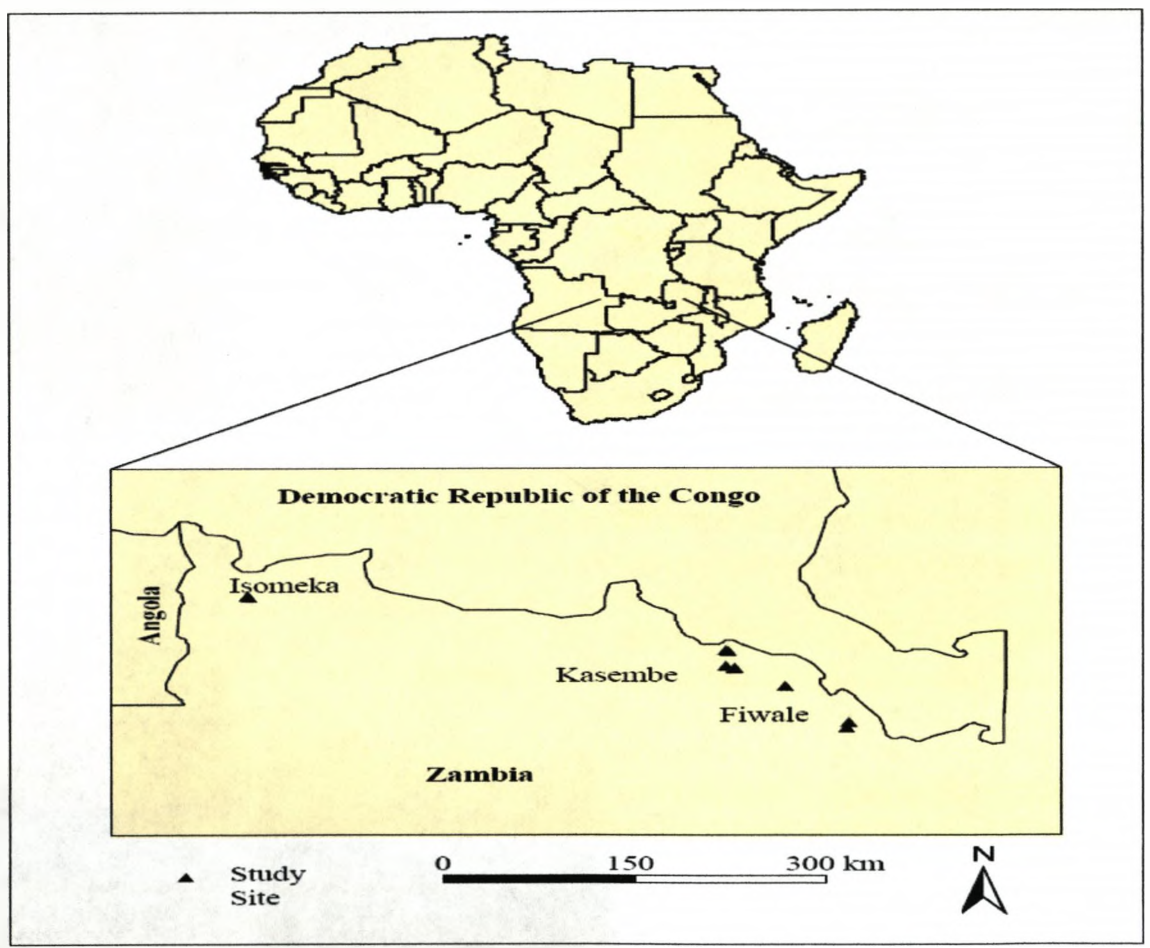


Figure 56. Shows selected mosquito collections sites in Fiwale, Kasembe and Isomeka in Zambia.

Figure 58 below also shows a similar pattern of the distribution of esterase activity with the substrate β-naphthol acetate as observed in Figure 56. The ranges of esterase activity with this substrate in *An. gambiae* s.s from Fiwale was 0.0017 – 0.00421 β-naphthol produced/min/mg protein.

Raised levels of GST activity were detected in Chililabombwe at very low frequencies and in Fiwale at significant levels, while the other six study sites produced similar pattern of the distribution of GST activity as observed with *An. albimanus* susceptible strain. As already stated earlier the GST results correlate well with WHO susceptible tests from Fiwale (Table 30) and with the observation made with mosquito population density in these IRS programmeme areas. The GST ranges for Fiwale *An. gambiae* were from 0.007 - 0.75 mmol CDNB conjugated/min/mg protein (Figure 59), suggesting that the GST are conferring to DDT and possibly pyrethroid resistance in combination with the kdr detected in these population from Fiwale, Ndola, Zambia.

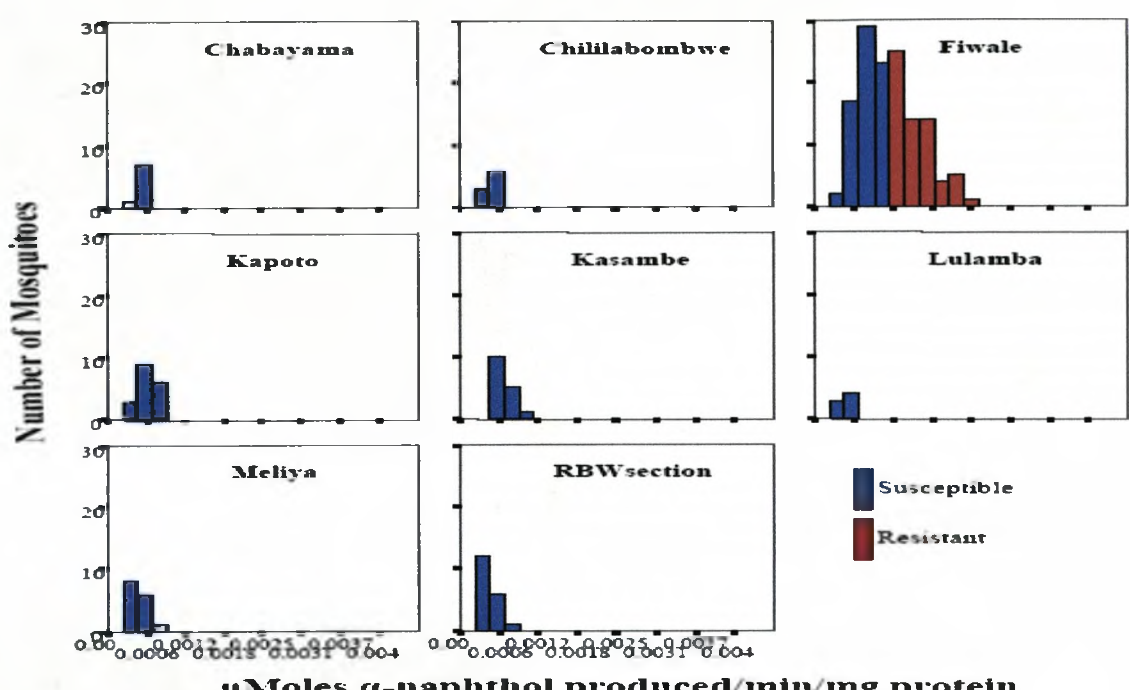


Figure 57. The range of esterase activity with α-naphthyl acetate in *An. gambiae* s.s in eight study sites from Zambia.

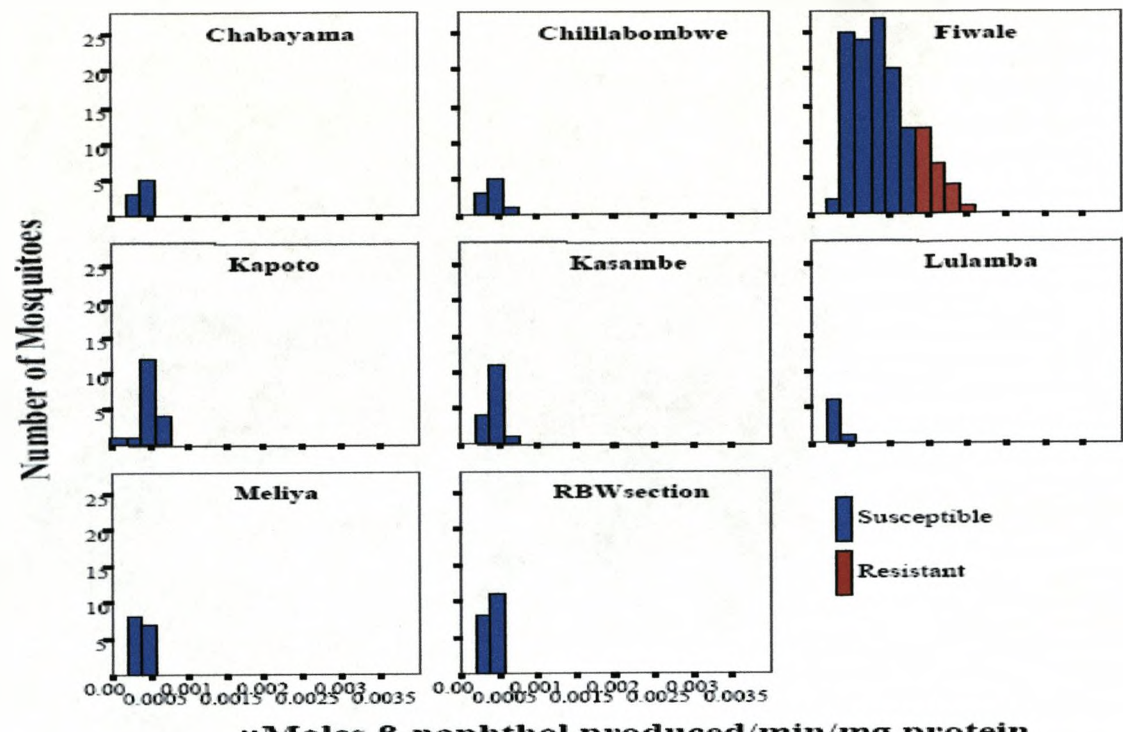


Figure 58. The ranges of esterase activity with β-naphthyl acetate in *An. gambiae* s.s in eight study sites from Zambia.

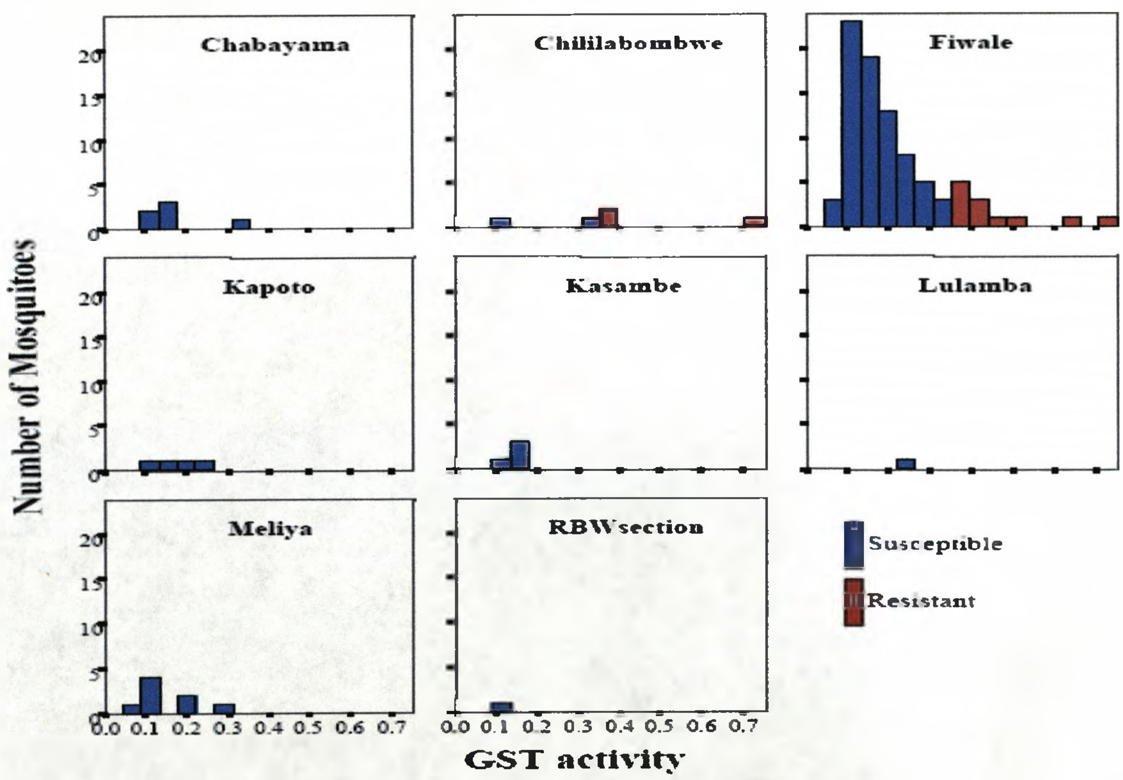


Figure 59. The range of GST activity (mmol CDNB conjugated/min/mg protein) in *An. gambiae* s.s in eight study sites from Zambia.

An altered AChE gene was only detected in Fiwale at very low frequencies. The % AChE activity inhibition by propoxur ranged from 41% to 100%, which indicates the presence of some heterozygote genotypes segregating in the population (Figure 60). OP and carbamate use was documented from this area and this could be selecting for the altered AChE genes in this *An. gambiae* s.s population.

Elevated esterases were detected with the substrate ρ-nitrophenyl acetate in all the study sites. Activity levels ranged from 0.00 – 0.52, 5 fold higher than susceptible baseline cut off points for the *An. arabiensis* (Durban) strain.

Distribution of levels of monooxygenases followed a similar pattern to that observed with *An. arabiensis* susceptible strain, indicating the normal quantity of the enzyme in a susceptible population.

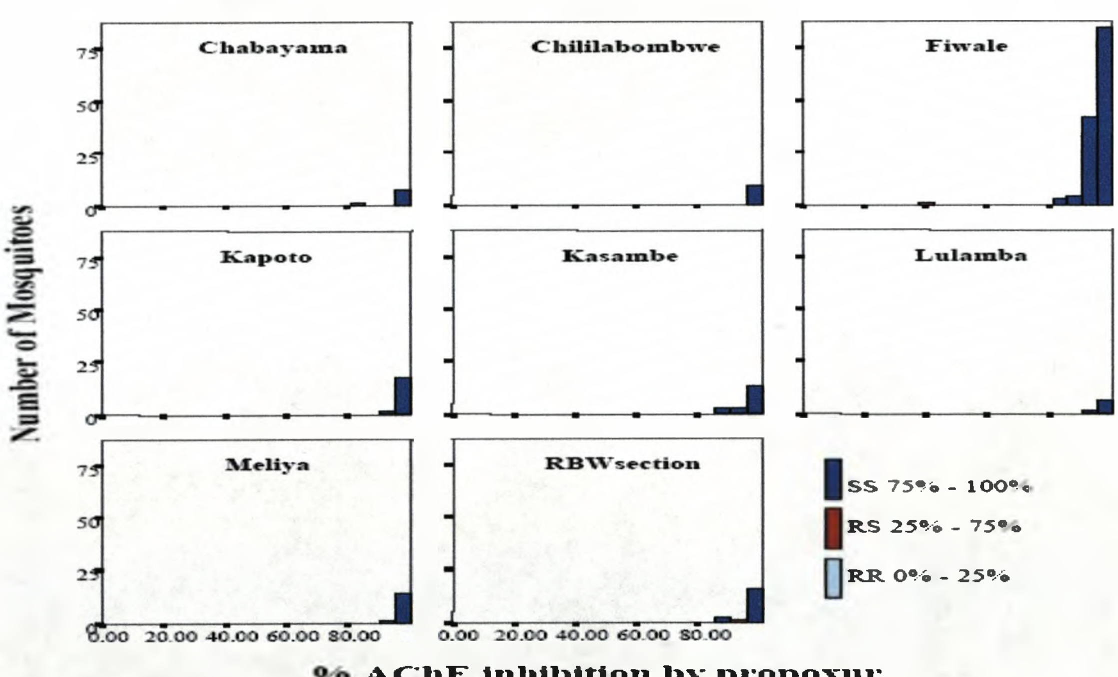


Figure 60. The range of acetylcholinesterase inhibition by propoxur in *An. gambiae* s.s in eight study sites from Zambia.

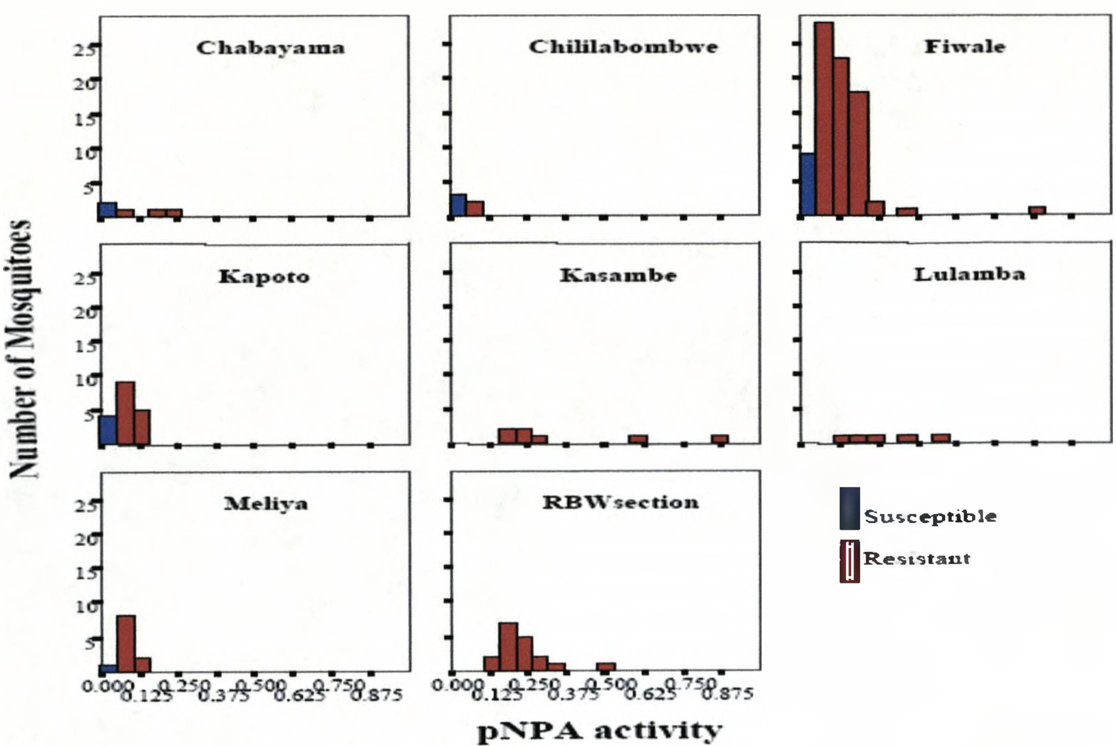


Figure 61. The ranges of ρ-nitrophenyl aceteta (ρNPA) in *An. gambiae* s.s. in eight study sites from Zambia.

Fiwale registered very low elevated levels of monooxygenases (This suggests that this mechanism of resistance is not responsible to the low levels of resistance to deltamethrin from this area). The range of monooxyganase content in the population from Fiwale was from 0.00001 – 0.00053 equivalent units of cytochrome P^{450}/mg protein, similar to the established baseline cut off point 0.0005 equivalent units of cytochrome P^{450}/mg protein with the *An. arabiensis* susceptible strain (Figure 62).

Figure 63 below shows the percentage distribution of resistance mechanisms detected with biochemical assays with *An. gambiae* s.s from Zambia.

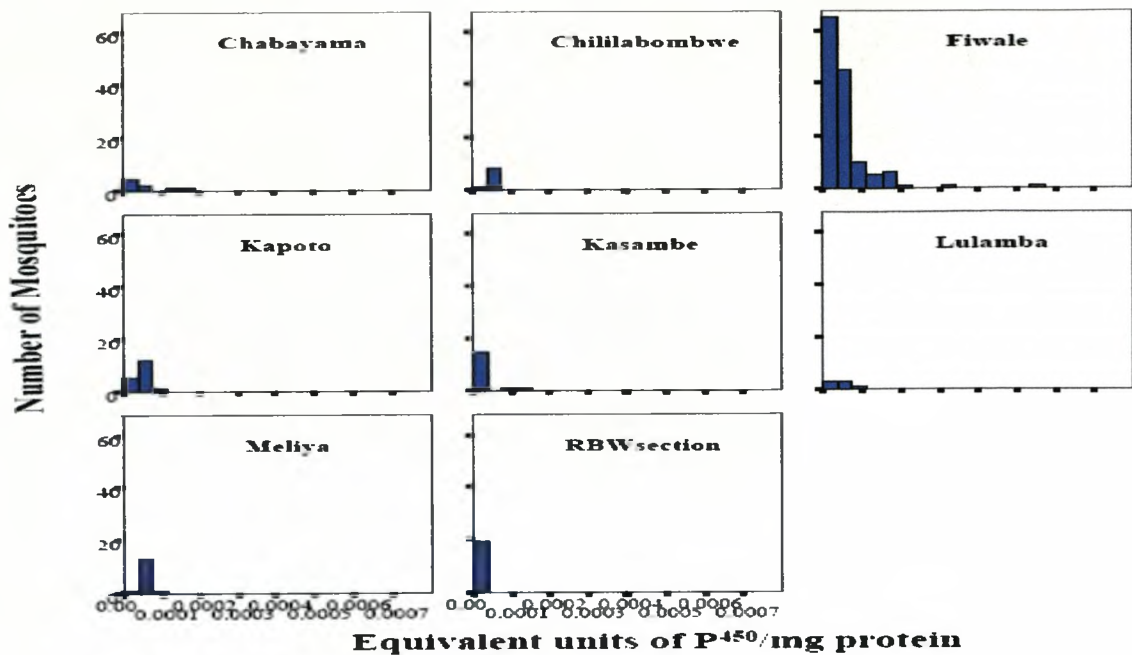


Figure 62. The range of equivalent units of cytochrome P^{450}/mg protein in *An. gambiae* s.s in eight study sites from Zambia.

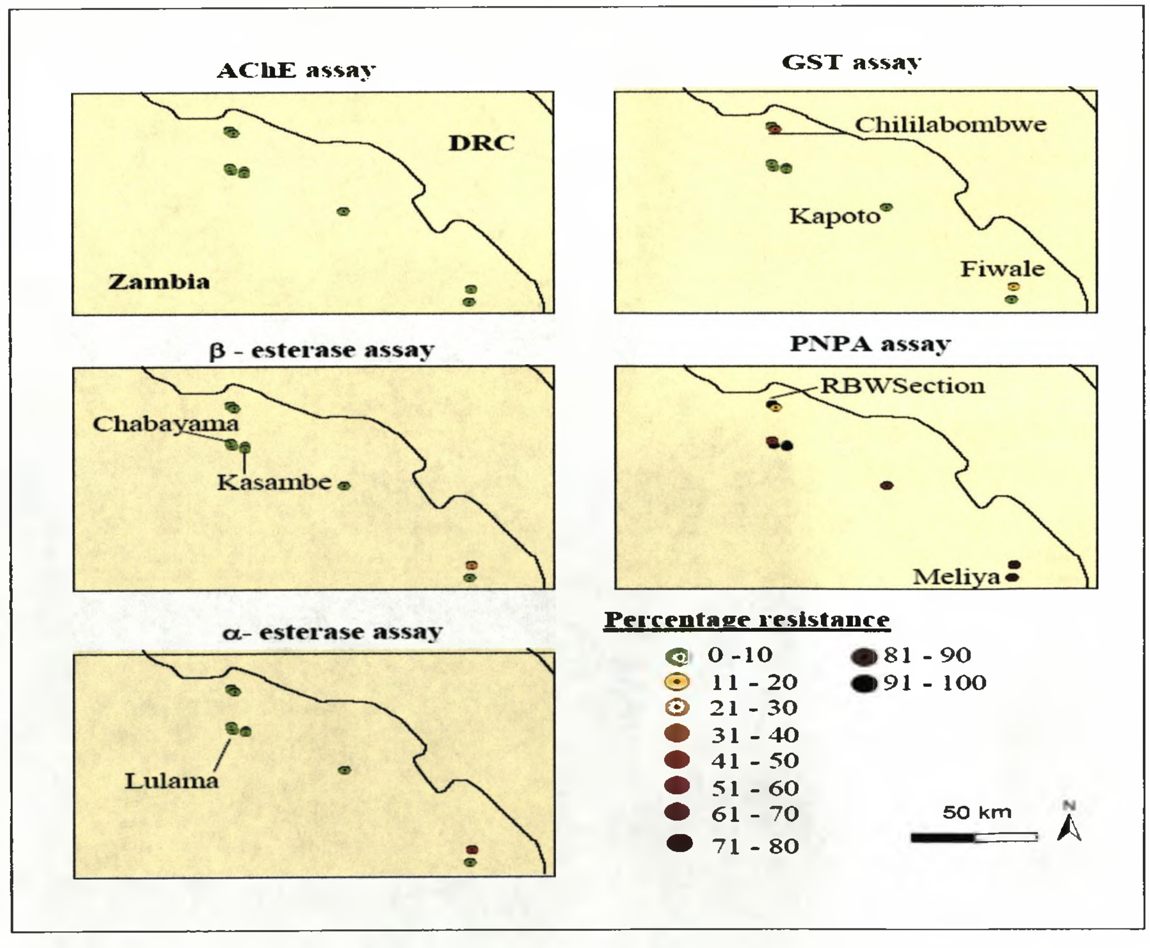


Figure 63. Shows percentage distribution of resistance mechanisms from Zambia.

2.10 Botswana

2.10.1 Collections

Malaria transmission in Botswana is unstable and closely related to the level of rainfall. Major epidemics occur in years of heavy rainfall with transmission taking place in the rainy season between November and May, mainly in the northern part of the country. Botswana is divided into three main epidemiological belts; the northern belt of regular high transmission, the central belt of intermediate transmission and the southern belt where little or no malaria transmission occurs. The five major malaria transmission districts are Chobe, Okavango, Ngamiland, Tutume and Boteti (Malaria, 1999 – a manual for health workers in Botswana). All samples analysed so far represent larval collections made in Shorobe, Maun, along the fringes of the Okavango delta, Samedupe and Talisiding (Figure 23).

2.10.2 Results

As in Swaziland, only larval collections were possible in Botswana. No WHO susceptibility tests were performed on these samples. Biochemical assays were performed on one to three day old mosquitoes reared from larval collections including the non-vector *An. quadriannulatus*. Species identification was performed with body parts from the debris left after aliquoting homogenates for biochemical assays. Below are graphical representations of biochemical results for the three study sites in Botswana.

Esterase activity with the substrate α-naphthol acetate showed similar distribution to that *An. arabiensis* (Durban) and *An. albimanus* (Panama) susceptible strains (Figure 64). Shorobe registered low levels of elevated esterases with *An. arabiensis* and *An. quadriannulatus*. The ranges of esterase activity were from 0.00001 – 0.00139 μmoles α-naphthol produced/min/mg protein, slightly higher than susceptible baseline cut off point for *An. albimanus* (Panama). No *An. arabiensis* was found in Samedupe. Talisiding showed a similar distribution with *An. albimanus* (Panama) suggesting a susceptible population.

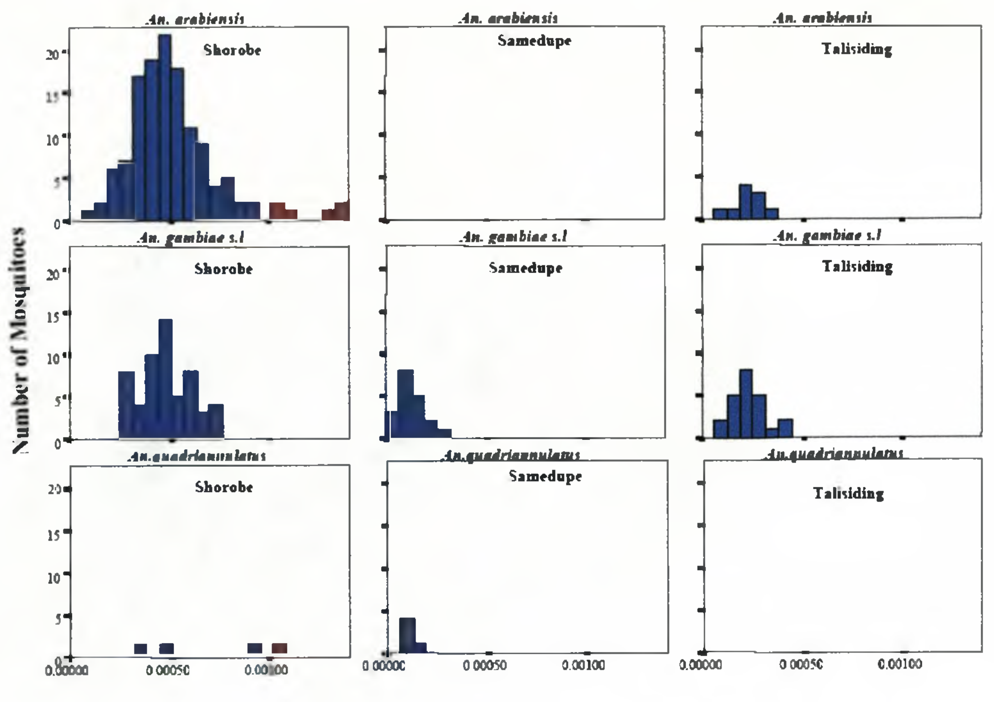


Figure 64. The range of esterase activity with α-naphthyl acetate in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in three study sites from Botswana.

The same distribution of esterase activity with the substrate β-naphthol acetate as α-naphthol acetate above was observed with *An. arabiensis* from Shorobe (Figure 65). The ranges of esterase activity were from 0.00003 – 0.00138 µmoles β-naphthol produced/min/mg protein. Talisiding also showed a susceptible population as compared to *An. arabiensis* (Durban) and *An. albimanus* (Panama) susceptible strains.

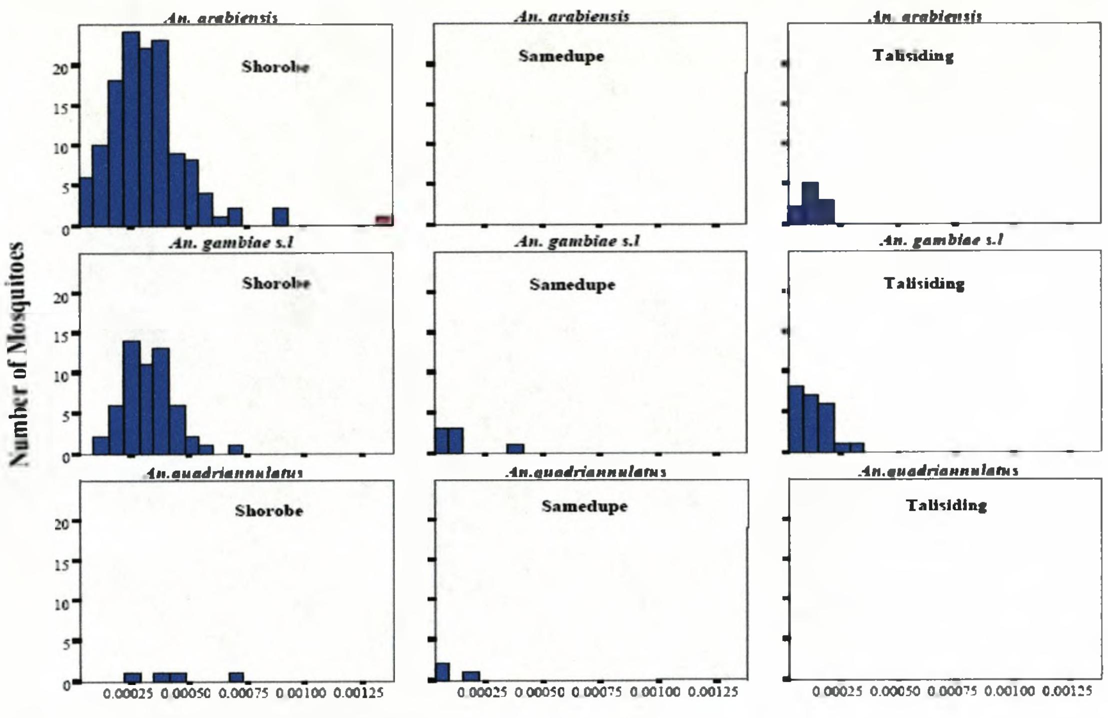


Figure 65. The ranges of esterase activity with β-naphthyl acetate in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in three study sites from Botswana.

Elevated GSTs were registered with *An. arabiensis* from Shorobe (Figure 66). Samedupe showed low levels of elevated GSTs with *An. gambiae* s.l and *An. quadriannulatus*.

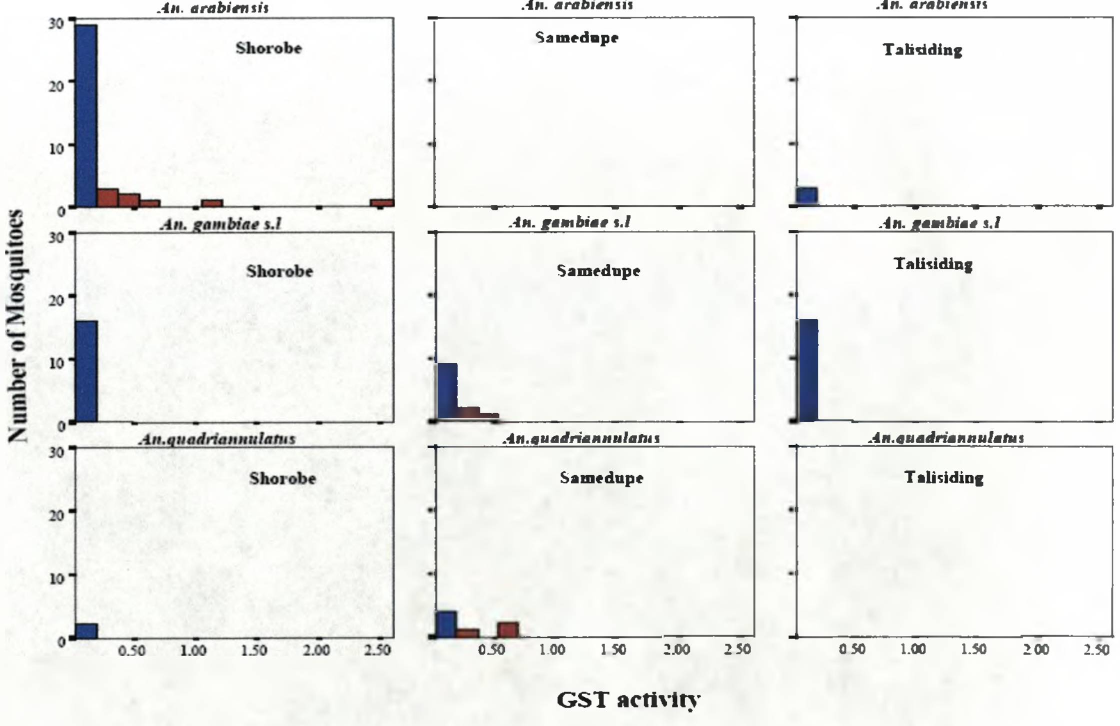


Figure 66. The range of GST activity (mmol CDNB conjugated/min/mg protein) in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in five study sites from Swaziland.

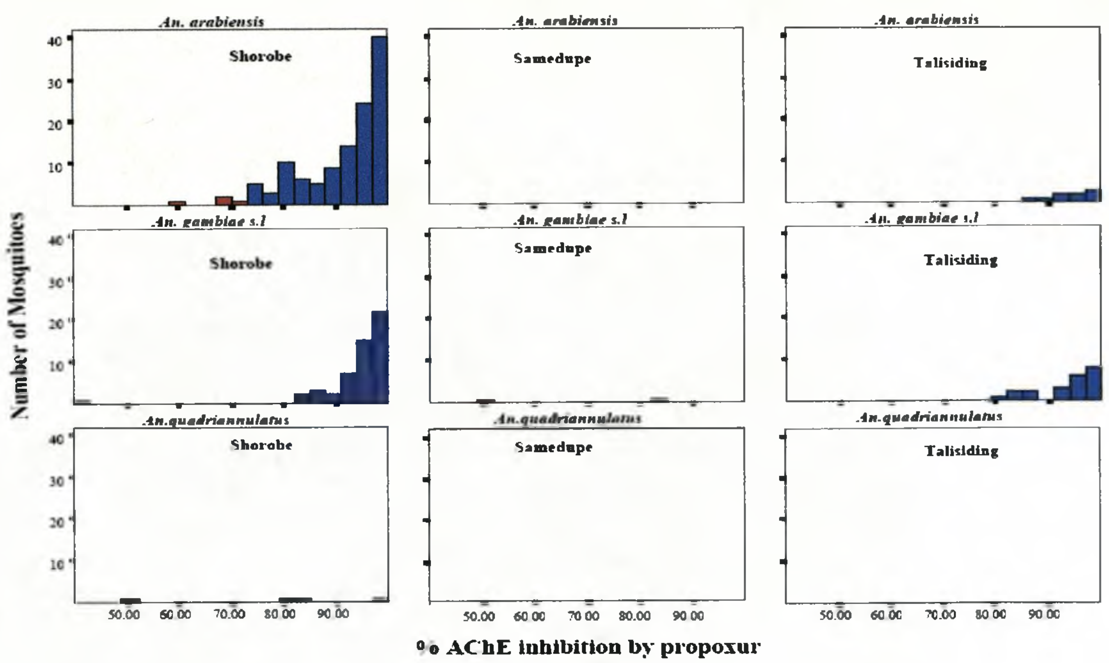


Figure 67. The range of acetylcholinesterase inhibition by propoxur in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in three study sites from Botswana.

Altered AChE resistance mechanism was detected at low levels in *An. arabiensis* from Shorobe ranging from 40% to 100% showing that the altered AChE genes are segregating in this population (Figure 67). Low levels of elevated esterase activity with the substrate ρ-nitrophenyl acetate (ρNPA) were observed from all the three sites (Figure 68).

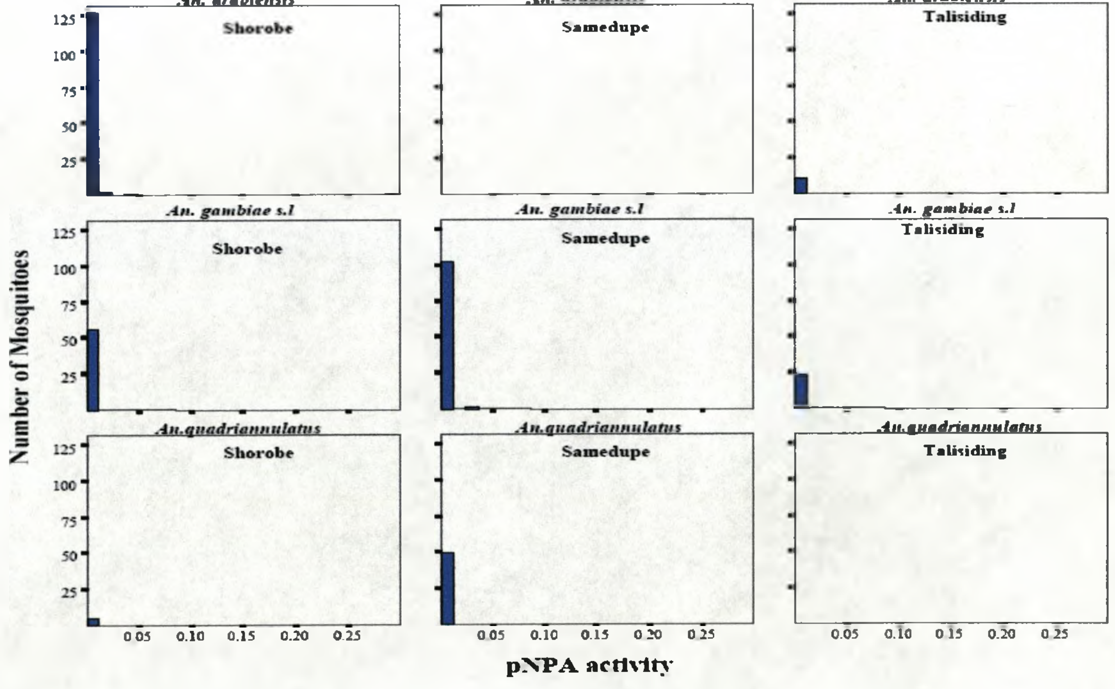


Figure 68. The ranges of ρ-nitrophenyl acetate (ρNPA) in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in three study sites from Botswana.

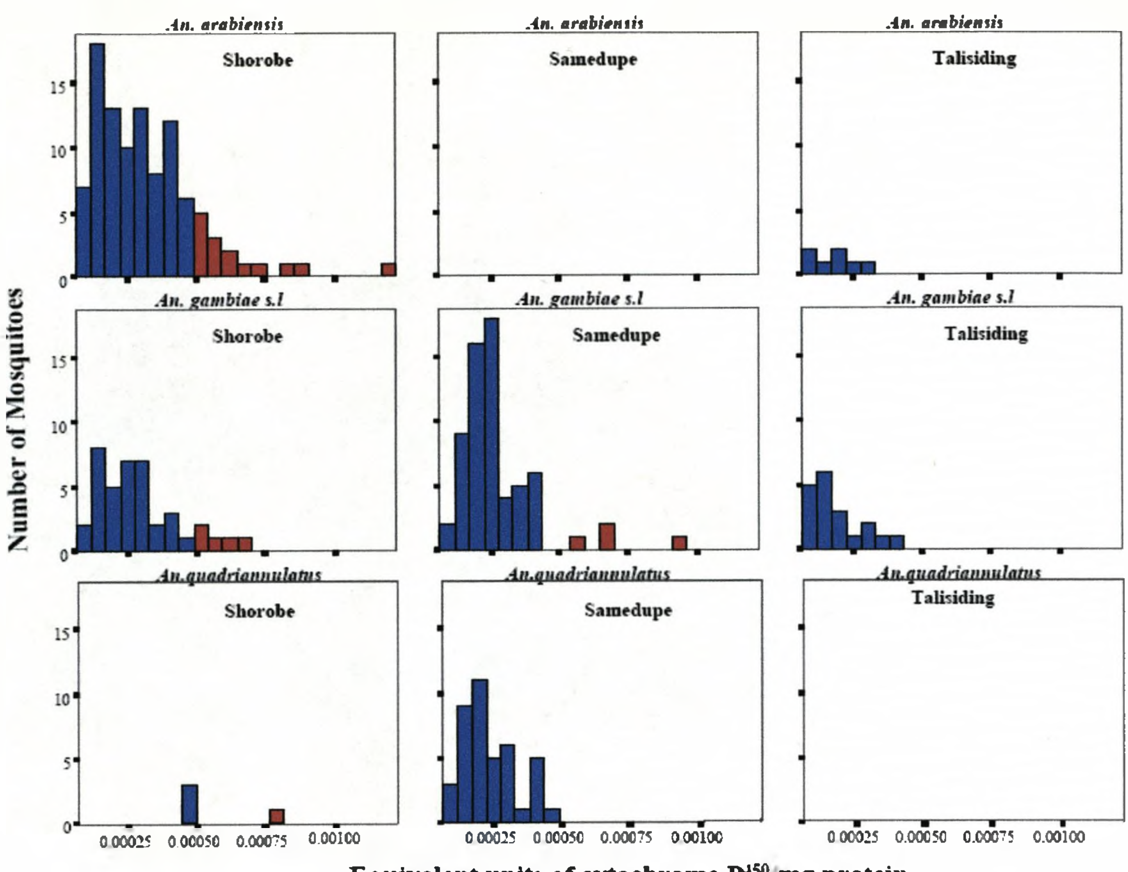


Figure 69. The range of equivalent units of cytochrome P^{450}/mg protein in *An. gambiae* s.l., *An. arabiensis* and *An. quadriannulatus* in three study sites in Botswana.

An. arabiensis from Shorobe and *An. gambiae* s.l from Samedupe showed high levels of monooxygenases as compared to *An. arabiensis* (Durban) susceptible strain indicating the existence of this resistance mechanism in Botswana (Figure 69).

These study areas are along the fringes of the Okavango delta where there is extensive cattle rearing and agricultural activities. OPs are used for animal dipping and therefore be may selecting for altered AChE and esterases in Shorobe. Aerial spraying with endosulfan was used by the tsetse control programmeme in these areas.

2.11 Discussion

The WHO defines insecticide resistance as 'the inherited ability of a strain of some organism to survive doses of a toxicant that would kill the majority of individuals in a normal population of the same species' (WHO 1957). In order to establish resistance

levels in field collected mosquitoes, a susceptible baseline was first set with *An. arabiensis* (Durban, South Africa strain) and *An. albimanus* (Panama strain) for both biochemical and molecular assays. These two strains gave 100% mortality at the WHO discriminating dosages with all the four groups of insecticides used in public health. Brent (1986) reports that on monitoring, the absolute use of resistance (as in 'the population was resistant') causes more problems of misinterpretation than relative use ('population A was more resistant than B') and a quatitative definition of how resistance was categorized and measured should always be given. In this study all field collected samples were compared to both the two susceptible laboratory strains. Brent further cautions that the choice of sensitive reference strains (sometimes merely a single one is used, which was not the case during this study) and any shift in their response with time can affect greatly the value of the index and inferences made and that this has been observed with regard to fungicide resistance. If a reference strain has been kept away from all chemical treatments for years in a laboratory culture, it may be abnormally sensitive (Brent, 1986).

The altered AChE from both the susceptible strains indicated a normal susceptible baseline for OPs and carbamates. The most interesting results from the *An. arabiensis* susceptible strain was with the relatively high levels of glutathione S-transferase, a phenomenon also observed with *An. albimanus* (Mexico) even after 15 years without DDT exposure. No DDT resistance was detected in field caught mosquitoes from KZN during initial surveys, but resistance was subsequently detected after the resumption of DDT usage in this province. A slight elevation of esterase activity in *An. arabiensis* was evident with the substrate ρ-nitrophenyl acetate. The ρ-nitrophenyl acetate is used as an indicator of OP resistance but does not give a precise indication of resistance levels.

In the two South African provinces with malaria (Limpopo and Mpumalanga), co-operation with the business sector was high, allowing documentation of pesticide usage and mapping of breeding sites. However, a detailed calendar and concentration usage of pesticides could still not be accurately developed, which further suggested a need for the ecosystem approach explained in detail in Chapter 4.

WHO susceptibility tests performed on 1-3 day old adult field collected *An. arabiensis* showed cross-resistance to DDT/permethrin. No kdr-type based resistance mechanism has been detected in this species. Metcalf et al., (1951) (in Roberts and Hshieh, 2003) reported

that DDT was a slow-acting poison that strongly influenced insect behaviour. This behaviour was indicated in KZN by the result that approximately 70.8% (N=92 individuals) caught in three houses within three days were not blood-fed, probably the result of repellency of DDT.

The metabolic studies done on 1-3 day old F1 and F15 progeny of females from KZN indicated that elevation of GSTs was the resistance mechanism conferring cross-resistance to DDT/permethrin. The role of GSTs conferring resistance to pyrethroids was explained in detail in section 1.7.1.2. Synergist work (see section 1.6.5.2) is being undertaken at the Medical Research Council, Durban, South Africa, to further elucidate the resistance mechanisms operating in this sample. More work is also being undertaken at the Liverpool School of Tropical Medicine to sequence the kdr gene for possible new mutations of the sodium channel target site in *An. arabiensis*.

The presence of an altered AChE resistance mechanism detected at different frequencies in all the four study sites in Mamfene, KZN indicate the role of OPs and carbamates used in the agricultural sector in the selection of this type of resistance mechanism. The variation of resistance levels due to this mechanism with OPs and carbamates is described in Chapter 5. Makhathini Agricultural Research station had the highest percentage of individuals with the altered AChE resistance mechanism at 21-30% (N=164). This may have been due to the high agricultural pesticide usage in this study area. Studies to look at the linkages between agricultural pesticide usage, insecticide resistance and malaria transmission are underway in this area (see Chapter 4).

Levels of equivalent units of cytochrome P^{450}/mg protein in *An. arabiensis* in all the four sites were higher than that of both the standard susceptible laboratory strains used in this study. This, together with the GSTs detected in this population could be conferring the cross-resistance to DDT/permethrin as observed with the WHO susceptibility tests. More research is being conducted to improve the present moonoxygenase assay, to produce in a more direct determination of cytochrome P^{450}s. Raised levels of GST activity were detected at all sites, but were highest in the Mjindi study area at 0 to 5.2 mmol CDNB conjugated/min/mg protein. The role of this resistance mechanism in conferring cross-resistance between DDT/permethrin was further supported by the metabolic studies. Levels of esterase activity with both α- and β- naphthyl acetate were similar for all the

study areas, the slight differences being probably due to different volumes of the homogenate when pipetting during the experiments.

In Mozambique, WHO insecticide susceptibility tests in all the four study areas (Boane, Bela-Vista, Catuana and Beluloane) showed resistance to pyrethroids especially to lambda cyhalothrin and low resistance to deltamethrin in Boane and Catuana. OP's and carbamate resistance with WHO susceptible tests was not detected except at low levels with bendiocarb at Bela-Vista. A possible explanation for the low level of resistance to these insecticides in bioassays is described in detail in Chapter 5 section 5.2.1. The WHO insecticide susceptibility tests correlated well with biochemical assays, accept for the altered AChE resistance mechanism. Raised levels of altered AChE were detected in all the areas at high levels. All the three genotypes (SS-susceptible, RS-heterozygote, RR-resistant) were registered and the overall frequency of this resistance mechanism was obtained at approximately 10-20% in all the areas. Evolutionary plasticity in resistance has been described in detail in Chapter 5, section 5.21. This has serious implications in malaria vector control where WHO susceptibility tests are the only means of insecticide resistance detection especially in Mozambique where bendiocarb is the insecticide used by the malaria control programme. Work to understand the plasticity response in WHO susceptibility tests with OPs and carbamates in *An. arabiensis* and *An. funestus* is planned at the Medical Research Council, Durban, South Africa in collaboration with the Liverpool School of Tropical Medicine, United Kingdom. This study also looked at the possible origin of insecticide selection pressure in Mozambique described in detail in section 5.2.5. The ecosystem approach to studying the linkages between pesticide usage, insecticide resistance and malaria transmission (see Chapter 4) is also planned in Mozambique to address the question of the origin of insecticide selection pressure. High levels of cytochrome P^{450} monooxygenases were registered in all areas which correlates well with the WHO susceptibility tests. There was a strong correlation of elevated esterases with α- and β- naphthyl acetates and ρ-nitrophenyl acetate. All areas showed high levels of elevated GST's but this were not well correlated with the WHO susceptibility test results.

In Swaziland, cooperation by the agricultural sector permitted the documentation of pesticides used in farms, although calendar and concentration of usage were absent. Difficulties were experienced in collecting adult blood-fed female mosquitoes and

therefore only larval collections were undertaken, which produced low numbers of the malaria vector *An. arabiensis* and therefore no WHO susceptibility tests were performed. Species identification of the larval samples collected from all the areas showed a mixture of *An. arabiensis* and *An. quadriannulatus*. The unidentified samples were recorded as *An. gambiae* s.l. An altered AChE resistance mechanism was detected in Mahlabathini, which lies below the Tambuti citrus farm, itself a possible source of OP and carbamate insecticides that could select this resistance mechanism. Elevated esterases were only observed in two areas of Mahlabathini and Nasaline; the differences observed, α-naphthyl acetate but not with β-naphthyl acetate, could be attributed to pipetting discrepancies during the running of the assay. High levels of elevated esterases with the substrate p-nitrophenyl acetate were observed in all the four areas, highlighting the accuracy of this assay as compared with the other two assays for general esterases. Swaziland has been using DDT for residual indoor insecticide spraying and this could be selecting the high levels of GST's detected in three study areas (Mahlathini, Stilo and Tamboti). More work is required in Swaziland to study the frequency and distribution of the resistance mechanisms.

Collections in Zambia were almost as successful as those performed in Mozambique in terms of numbers. This was mainly due to the anthropophilic nature of the two major malaria vectors, *An. gambiae* s.s (Zambia) and *An. funestus* s.s (Mozambique). However some samples from the Zambian field collections were lost due to the long distance covered during collections and transportation of samples to the Medical Research Council, Durban, South Africa which resulted in high percentage mortality. The cross-resistance with DDT/deltamethrin observed with WHO insecticide susceptibility tests on samples collected from Fiwale, Ndola province are indicative of a kdr-type based resistance mechanism. The kdr PCR diagnostic tests did show positive results with samples from this area. Further collections were made to document the frequency of this resistance mechanism, but so far the results have yielded negative results, suggesting which indicate that the mechanism exists at a very low level in this population. Small scale farmers were co-operative in this area with the documentation of pesticides they use in their small gardens. All mosquito samples from the Fiwale study area were collected indoors in one of the villages in proximity to the vegetable gardens. The insecticide selection pressure is definitely from these gardens as there is no residual indoor insecticide spraying in this village. All insecticides are used all year round and all at the

same time. Agricultural practices in the use of insecticides that could have an impact on the selection of resistance on vectors as well as other insects of agricultural importance are to be studied through the ecosystem approach explained in Chapter 4. Elevated levels of esterases measured with the substrates α- and β-naphthyl acetate were detected only at Fiwale. Elevated GST's were only detected in Chililabombwe province, an area under indoor residual insecticide spraying with DDT and at Fiwale. The altered AChE was found at very low levels only at Fiwale.

Botswana had the same pattern of resistance as shown by the other two countries for *An. arabiensis*. There is intensive animal farming in this country which involves a high frequency of animal dipping and therefore greater insecticide usage than in Zambia and Swaziland. Resistance was detected high in Shorobe which is along the fringes of the Okavango delta. Shorobe is two days drive from Francistown where the malaria control is based, which made it difficult to transport field collected mosquitoes for further analysis. All countries had a broad spectrum of insecticide resistance. More research is now required to determine the spread and frequency of the resistance mechanism detected to date. Implementation of methods for mosquito collections, processing and analysis have now been well implemented in the study countries, which are now in a position to establish and accurately monitor resistance management programmes as part of their national malaria control strategies.

CHAPTER 3

STUDIES ON ENTOMOLOGICAL ASPECTS OF MALARIA TRANSMISSION IN AN EXPATRIATE COMMUNITY IN COASTAL GABON

STUDIES ON ENTMOLOGICAL ASPECTS OF MALARIA TRANSMISSION IN AN EXPATRIATE COMMUNITY IN COASTAL GABON

3.1 Introduction

Gabon is situated in West Africa. The country shares borders with Cameroon to the north, Equatorial Guinea to the east and the Republic of Congo to the west. (www.cia.gov/cia/publications/factbook/geos/gb.html) (Figure 70). Gabon has a population of 1.5 million. Its oil and mineral reserves have helped Gabon to become one of Africa's wealthier countries but malaria is still a major issue. Gabon's ethnic groups consist of Bantu tribes, including four major tribal groupings (Fang, Bapounou, Nzebi, Obamba). The country is divided into nine provinces: Estuaire, Haut-Ogoue, Moyen-Ogooue, Ngounie, Nyanga, Oggoue-Ivindo, Ogooue-Lolo, Oggoue-Maritime and Woleu-Ntem. Cocoa, coffee, sugar, palm oil, rubber, cattle, okoume (a tropical softwood) and fish form the main agricultural products in Gabon.

The study site at Gamba Rabi Shell Gabon is situated in the Oggoue-Maritime province (location on Figure 70) within the Gamba Protected Areas Complex (lat 1°50-3°10S; long 9°15-10°5°E), which comprises of eight areas covering 11, 320km^2 (Thibauli and Blaney 2001). The Gamba complex has a long, sandy littoral zone that borders the Atlantic Ocean. The complex is located in the Congolian coastal forests ecoregion, which is considered one of Africa's richest moist forests (Olson and Dinerstein 1998). It is an area of tropical rainforests, inundated forests, swamps, savannas, lagoons and mangroves. The complex is home to a wide range of species, including the forest elephant (*Loxodonta Africana*), forest buffalo (*Syncerus caffer*), hippopotamus (*Hippopotamus amphibus*), gorilla (*Gorilla gorilla*), chimpanzee (*Pan troglodytes*) and manatee (*Trichechus senegalensis*). It is also inhabited by three species of African crocodile, at least four species of marine turtle, and numerous species of endemic flora (Thibault and Blaney 2001). Gamba village and the Yenzi club represent a tropical African rural setting in Gabon, where there is the potential for high malaria transmission, morbidity and mortality. Malaria control is currently attempted by truck-mounted fogging of malathion on a weekly basis.

Figure 70. Map of Gabon. The red arrow shows the study site, Gamba Village in the southwestern province of Ogooue-Maritime. (Source: www.un.gor/Depts/Cartographic/map/profile/gabon.pdf).

In order to design an effective malaria control strategy for these areas, a detailed entomological survey was undertaken. This allowed the determination of the vector species, their density, distribution and breeding sites. The survey formed the basis of an assessment of the present control strategy. This was used to help develop an integrated approach to malaria control that relies on case management (identification and treatment of clinical malaria cases), combined with selective, sustainable vector control measures, as recommended by the WHO (WHO, 2004 in

www.emro.who.int/rbm/PDF/Global/Strat.FrameIVM.pdf). In Gabon, potential malaria vector control strategies could include environmental management, chemical control, biological control or personal protection. However, to develop an effective vector control strategy, we required local information about vector distribution and behaviour to identify techniques that were effective, affordable and acceptable to the local communities. Identification of the major vector species was needed, and their relative abundance at the study sites. Sixty species of the genus *Anopheles* can transmit malaria, many of these are indigenous to Africa, and could be in Gabon.

This pilot analysis included:

- Time of biting (evening, night, dawn);
- Feeding preferences of adult female mosquitoes (humans or animals);
- Adult behaviour, preference for biting and resting indoors (endophagic, endophilic) or outdoors (exophagic, exophillic);
- Larval habitat preferences;

Feeding prefenrences of adult female mosquitoes (humans or animals) was done by indoor and outdoor collections in houses and cattle enclosures. The third analysis above was undertaken by setting window traps and human bait catches. Identification of breeding sites established larval habitat preferences in Gamba. The pilot study did not only concentrate on entomological indicators of control effectiveness, as preliminary monitoring of the impact of the existing intervention on malaria and community awareness of the disease was also undertaken.

3.2 Aims

The aim of this pilot study was to assess the feasibility of the resistance monitoring approach to be taken in the PhD programmeme and while doing so, investigate the mosquito control operations at a limited site, the Yenzi club. The results enabled us to make adjustments for optimal performance or effectiveness of the control measures implemented there and to advise on the expansion of the control programme to the neighbouring village of Gamba. The pilot study was undertaken for five days in March, 2001, when a small collection was made, but problems were encountered with mosquito abundance due to the dry season. A second visit to the study area was undertaken for

fourteen days from 21st February to the 7th March, 2002, during the main malaria transmission season. An understanding of the mosquito species composition, behaviour, ecology and the epidemiology of malaria in this area was developed to help in the design of an optimal vector control strategy. Visits to the hospital in Gamba and the clinic at the Yenzi club were undertaken to assess malaria case management to supplement information from the analysis of the vectors. This was done by discussions with the General Practitioners in charge of the hospital and the clinic on how best could the two institutions establish a malaria case management system, that is, number of malaria cases, tools used to assess malaria, treatment and recording of this data (see Appendix 1).

3.3 Materials and Methods

Mosquitoes collected in Gabon were all transported to the University of Cardiff, and subsequently transferred to the Liverpool School of Tropical Medicine during the relocation of this PhD. Mosquitoes were collected and processed as described in detail in Chapter 2, section 2.3.1 and 2.3.2. In order to identify the major vector species, and their relative abundance in the Gabon study area, analysis included:

- Time of biting (evening, night, dawn)
- Location of the adult vector relative to the breeding sites.
- Feeding preferences of adult female mosquitoes (humans or animals).
- Adult behaviour, preference for biting and resting indoors (endophagic, endophilic) or outdoors (exophagic, exophillic).
- Larval habitats

The time of biting was done by doing landing catches, carried out by the author and Mr. John Morgan (Liverpool School of Tropical Medicine). The location of the adult vector relative to the breeding sites was done by first identifying breeding sites, followed by landing catches on houses in proximity to the breeding sites. In Gamba village, landing catches were carried out at houses with livestock, primarily goats in this area, in order to determine the feeding preferences for biting and resting indoors (endophagic, endophilic) as well as outdoors (exophagic, exophilic). Larval habitat preferences were measured by identification of breeding sites around and inside the village of Gamba and the Yenzi

club. The study in this area did not only extend to entomological indicators of control effectiveness but preliminary monitoring of the impact of the existing malaria control intervention on disease was also undertaken and community awareness of the disease established.

Evaluation of the mosquito control strategy at the Yenzi club was done by:

a. Identification of the vectors;

b. Insecticide resistance status of the vector;

Window exit traps were placed at four different houses in Gamba, Gabon and monitored daily throughout the study, to establish mosquito species and densities resting indoors.

To measure peak biting times in Gamba, adult female blood fed mosquitoes were collected using the "landing catches" method. Collections occurred between 9.00 pm and 2.00 am Blood fed adult female *anopheles* mosquitoes were placed in oviposition tubes (breeding tubes) and F1 progeny obtained for subsequent insecticide resistance monitoring by bioassay, biochemical and molecular tests and species identification at the Liverpool School of Tropical Medicine. Dead mosquitoes were stored in silica gel for species identification and the molecular detection of pyrethroid/DDT kdr-based resistance using Polymerase Chain Reaction.

A link with the laboratory of the Smithsonian Institute (SI) in Gamba, was established in order to use the laboratory's equipment, microscopes and fridges to be able to identify and store collected mosquitoes, as the clinic did not have the necessary insect processing equipment. Four sentinel sites were established at plaine 3, plaine 3/4, plaine 2 and plaine 5 in Gamba (plaine is equivalent to a zone). Sentinel sites were chosen based on their proximity to potential breeding sites. Window exit traps were placed at these four houses in Gamba and catches monitored daily throughout the visit, to establish the mosquito species and the densities resting indoors.

Five collections points were identified at the Yenzi club. Clean standing water in the area in and around the Yenzi club, which is a potential *Anopheles* breeding sites, were identified. Mosquito larvae present in these sites were collected and reared to adults.

3.3.1 Vector Species

To measure peak biting times, live adult female mosquitoes were collected using the "landing catches" method on myself. As using human beings for mosquito live catches can result in parasite transmission, I was put on prophylaxis with doxycycline. Each session took place between 9.0pm and 2.0am. The temperature range during these sessions was 25 – 30°C. Blood-fed adult female anopheles mosquitoes from these collections were placed in oviposition tubes and F1 progeny obtained for subsequent analysis (see section 2.3.1 and 2.3.2).

One to three day old F1 progeny were to be used for insecticide resistance detection by biochemical and molecular assays. Only biochemical and molecular assays were performed. WHO susceptibility tests were not carried out due to the low number of samples. Dead mosquitoes were stored in silica gel for species identification and the molecular detection of pyrethroid/DDT kdr-based resistance identification by PCR (see section 2.3.5.2).

3.3.2 Biochemical Assays

Biochemical assays were performed on F1 progeny raised from blood-fed adult female mosquitoes and on adults from larval field collections. Assays for the two most important types of resistance mechanism to the four main insecticide groups, decreased target site sensitivity and increased metabolism to non-toxic products were performed as described earlier in chapter 2 (section 2.3.4). These assays measured esterase, monooxygenease and glutathione S-transferases activity, as these are the major enzyme families involved in insecticide metabolism. The fourth biochemical assay measured acetylcholinesterase target site insensitivity, which is responsible for resistance to organophosphorus and carbamate insecticides.

3.3.3 Molecular Assays

Anopheles mosquitoes were morphologically sorted into *An. gambiae* s.l. and non-*An. gambiae* group (Gillies and Coetzee 1987). Species identification of the *Anopheles gambiae* group collection was achieved using the ribosomal DNA-PCR method

developed by Scott et al. (1993) (section 2.3.5.1). Pyrethroid and DDT resistance, resulting from a mutation in the target site of these insecticides, is common in *An. gambiae* from West Africa (Martinez-Torres et al., 1998). The presence of this resistance, was investigated using a PCR based diagnostic test to detect a leucine to phenylalanine mutation of the sodium channel gene (Martinez-Torres et al., 1998) (section 2.3.5.2).

3.4 Results

At all the sites, a mixture of *Anopheles sp.* (30.1%), *Culex sp.* (57.5%) and *Aedes sp.* (12.3%) mosquitoes were collected. *Culex sp.* and *Aedes sp.* were not identified to species level; it was assumed for practical purposes that catches of these genera comprised *Culex quinquefasciatus* and *Aedes aegypti.* Between 9 pm and 10 pm only *Culex sp.* and *Aedes sp.* were biting, with peak *Anopheles sp.* biting at 11.30 pm, when they formed the bulk of the collection (75%) (Figure 71).

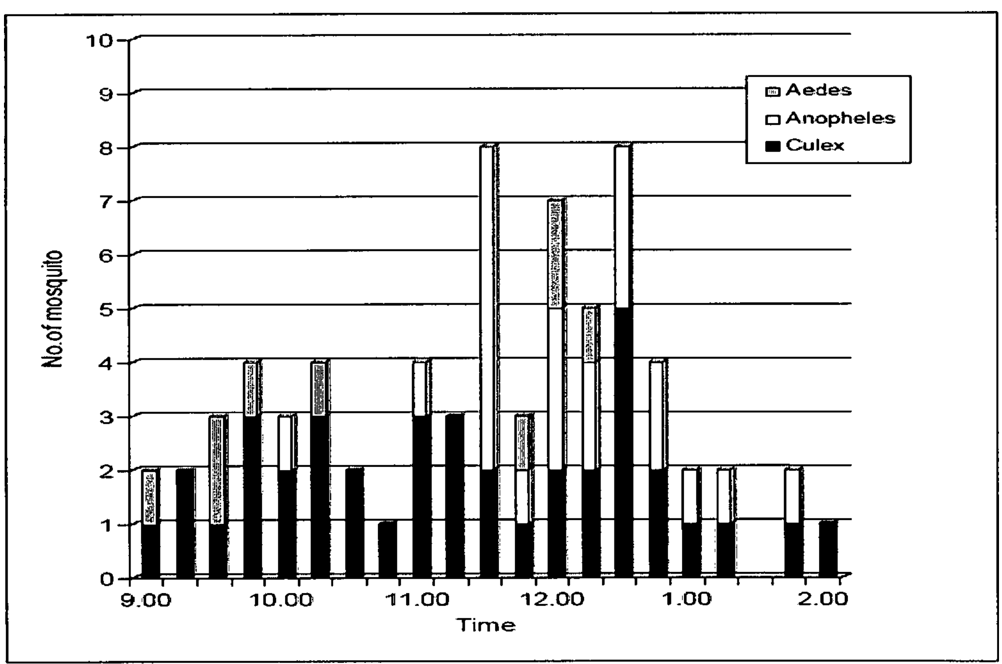


Figure 71. Man-biting mosquito landing catches from 9 pm to 2 am from one person on one night on the 25th February, 2002.

Figure 72 shows the results of a sample of mosquitoes analysed by PCR for species within the *An. gambiae* complex. Specific PCR primers within the reaction mixture produce diagnostic products of different sizes for each species within the *An. gambiae* complex.

In this region of Africa, *An. gambiae* s.l, *An. arabiensis* and *An. melas* are the three most likely vectors. *An. merus* and *An. bwambae*, the other members of the *An. gambiae* species complex, have a more limited distribution and we did not expect to find them in Gabon. Control samples of each of the three potential vectors were run on each gel as reference standards, along with molecular weight markers. The breakdown of the *Anopheles* species within the collections from Gabon is given in Table 30.

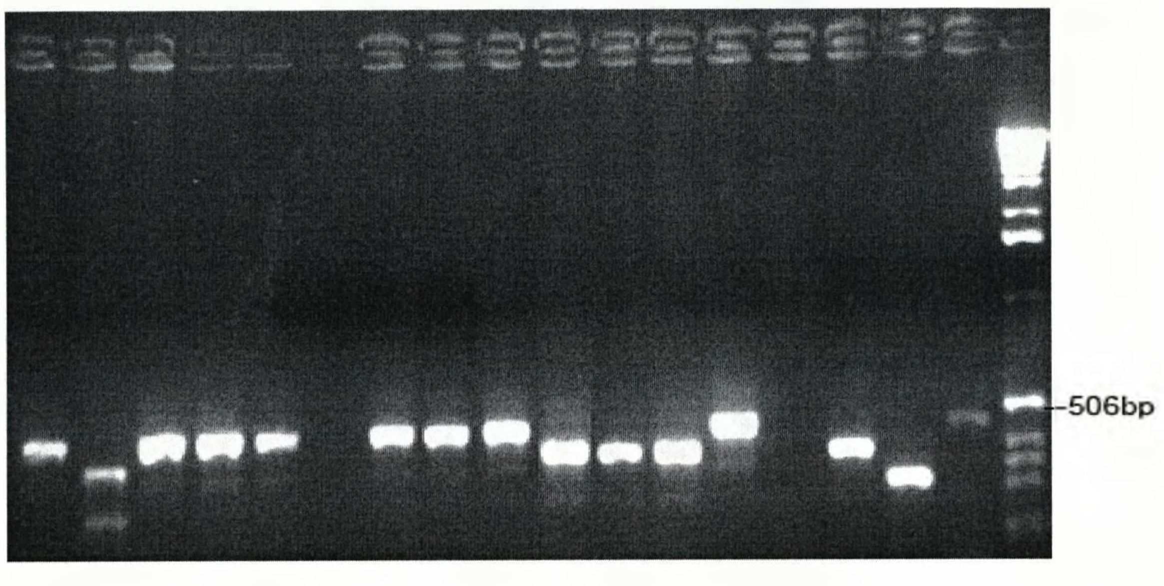


Figure 72. PCR identification of species from the *Anopheles gambiae* complex. PCR products were separated on 1.5% agarose gels. Lanes 1-13: are PCR products using the legs of individual insects from *Anopheles* collected at the Yenzi Camp as the DNA template; C: no DNA control; G: *Anopheles gambiae* standard A: *An. arabiensis* standard; M: *An. melas* standard.

Site	Total *An. gambiae* s.l caught	*An. gambiae s.s*	*An. melas*
Yenzi House 116	29	12	17
Yenzi Lodge	72	56	16
Yenzi Pool	6	6	0
Yenzi Clinic	2	2	0
Total	**109**	**76**	**33**

Table 30. The species distribution of *Anopheles* from different mosquito catches undertaken at the Yenzi Club.

The two species found were *An. gambiae* s.s. and *An. melas*, both members of the *Anopheles gambiae* complex. While both can act as malaria vectors, *An. gambiae* s.s is the major problem and comprised the bulk of the sample (79% of all *Anopheles*) in all apart from Yenzi house 116. No adult female blood fed mosquitoes were collected at the Gamba village.

Biochemical assays performed on mosquito samples from the Yenzi club show the existence of elevated levels of esterase in approximately 30% (42 individuals) of the *Anopheles gambiae* with the substrates alpha naphthyl acetate and beta naphthyl acetate compared to other insecticide susceptible *Anopheles* strains (Figures 73-77).

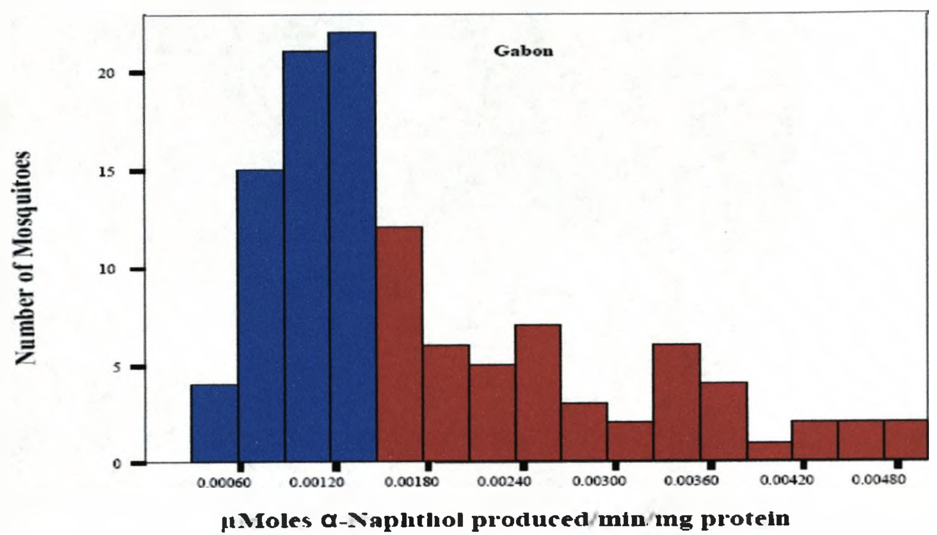


Figure 73. Esterase activity with the substrate alpha-naphthyl acetate in *An. gambiae* s.s Gamba/Yenzi (Gabon). Blue = susceptible and Red = resistant.

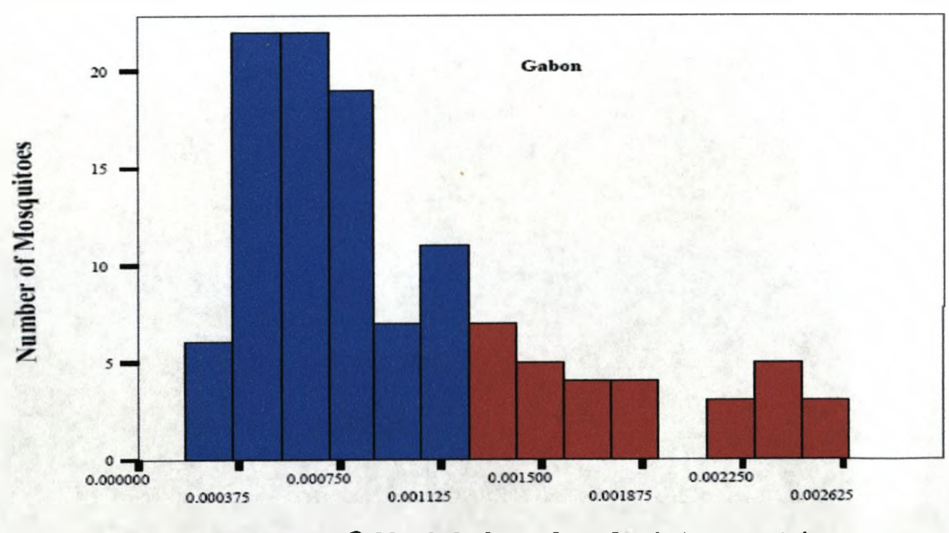


Figure 74. Esterase activity with the substrate beta-naphthyl acetate in *An. gambiae* s.s Gamba/Yenzi (Gabon). Blue = susceptible and Red = resistant.

Figure 75 shows the range of monooxygenase units (equivalent units of cyrochrome P^{450}/min/mg protein) in Gabon *Anopheles* compared to the ranges in susceptible *An. stephensi* and *An. albimanus*. There is no indication of monooxygenase-based pyrethroid or OP resistance in the Gabon mosquitoes.

Figure 76 shows the range of glutathione S-transferase activity in the Gabon *Anopheles*. This is within the range that would be expected of DDT and pyrethroid susceptible insects

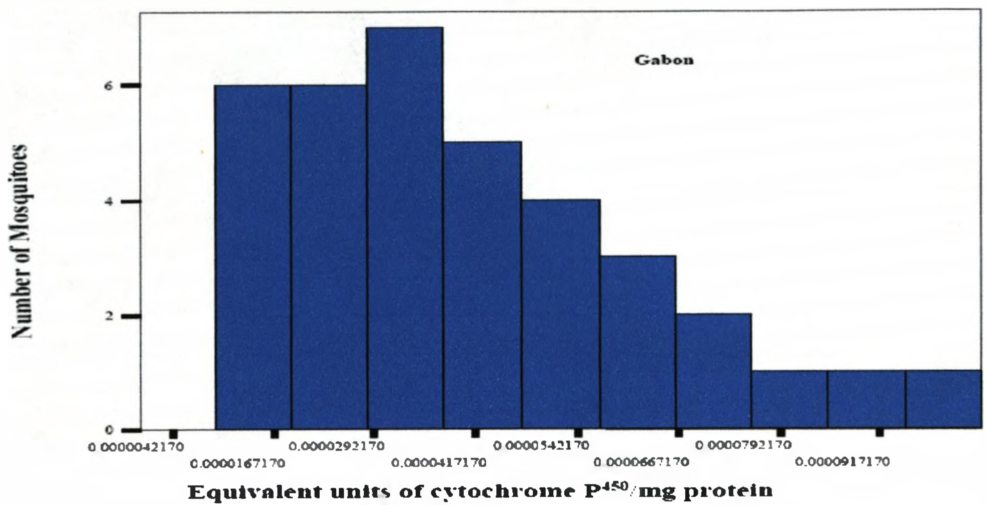


Figure 75. Equivalent units of cytochrome P^{450} in *An. gambiae* s.s. Blue = susceptible.

Acetylcholinesterase is the target site for OPs and carbamates. Mutations in this enzyme reduce its sensitivity to insecticide inhibition producing broad spectrum inhibition. Figure 34 shows that the propoxur inhibition spectrum of acetylcholinesterase in mosquitoes from Gabon is identical to that for other susceptible mosquitoes. Hence, there is no evidence that this broad-spectrum mechanism has been selected by the malathion fogging or other agricultural insecticide use in the region.

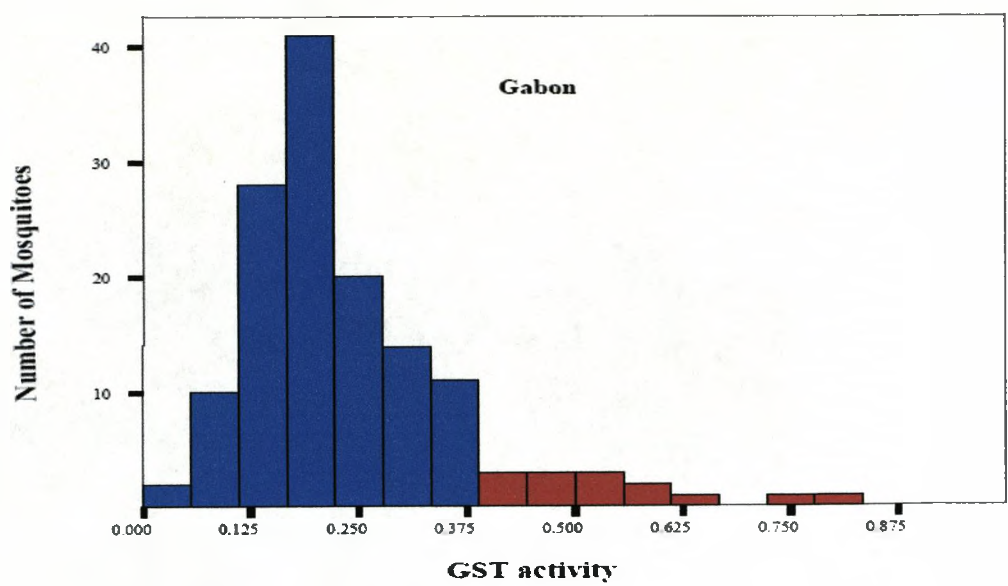


Figure 76. Glutathione S-transferase (GST) activity in *An. gambiae* s.s. Blue = susceptible and Red = resistant.

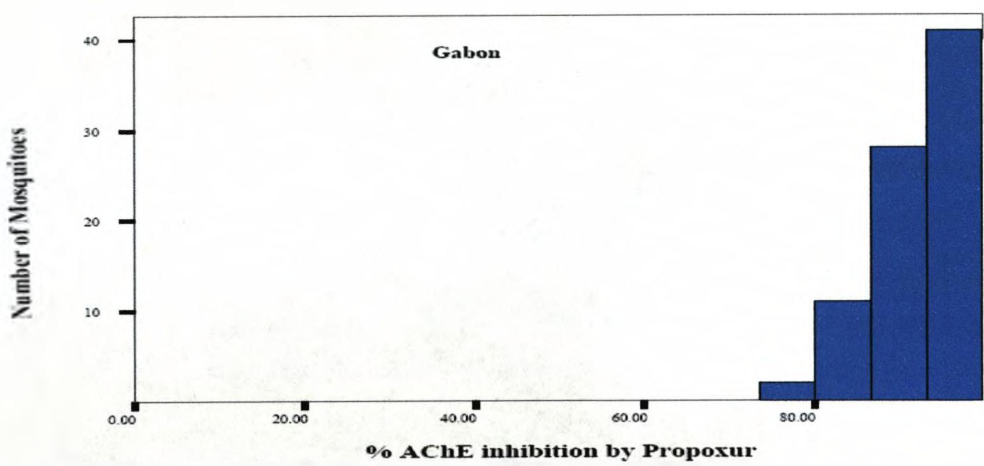


Figure 77. Acetycholinesterase (AChE) inhibition ranges in *An. gambiae* s.s. Blue = susceptible.

The target site for DDT and pyrethroids is the sodium channels on the nerve membrane. Resistance due to a mutation (leucine to phenylalanine) is common in *An. gambiae* from West Africa, while a different substitution (leucine to serine) occurs with a limited distribution in East Africa. These mutations can be detected in individual insects using a simple diagnostic PCR assay. Results of this assay on the Gabon *Anopheles* are illustrated in Figure 78.

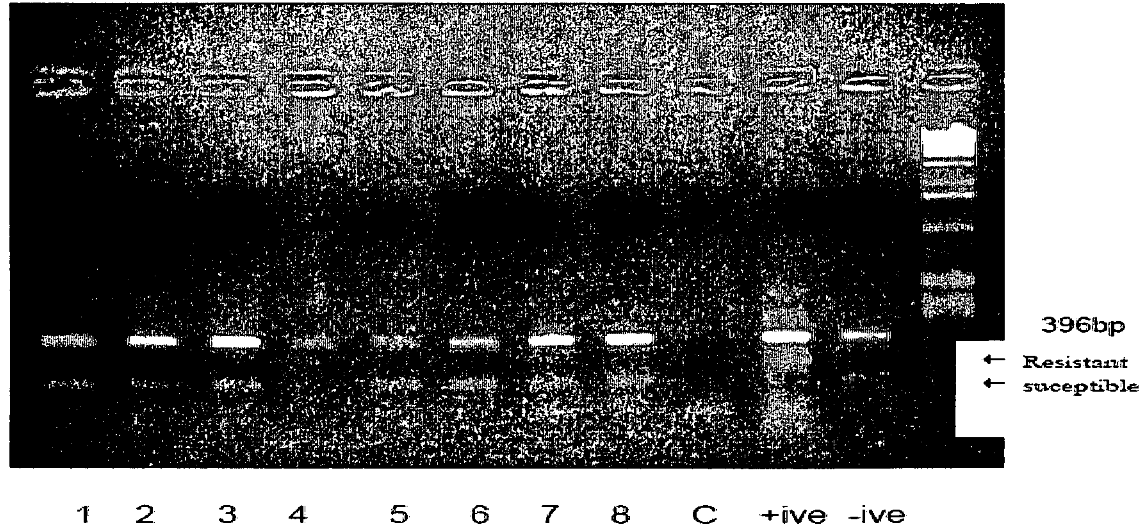


Figure 78. Identification of *kdr* in *Anopheles gambiae* by PCR – based diagnostic test. PCR products obtained using the test on samples after separation on a 1.5% agerose gel. Lanes 1-4: contain 4 samples from Yenzi Camp; 5-8: 4 samples from Gamba: C: no DNA control; +ive: resistant strain VK-per; -ive: susceptible strain Kisumu.

Positive and negative controls (resistant and susceptible insects) demonstrate the different banding patterns expected. The strong upper band indicates that the PCR reaction is working and the correct gene is amplified. The fainter lower band gives the resistance phenotype. All mosquitoes from Gabon had the homozygous susceptible genotype for kdr.

3.5 Discussion

The decision to implement mosquito control at the Yenzi club appeared to be due to complaints about the mosquito populations reaching nuisance threshold levels. Much of the biting nuisance reported by the residents is likely to be due to the large numbers of *Culex* and *Aedes* in the collections. The latter have a particularly unpleasant bite, are more obvious during the day and often produce a stronger inflammatory reaction to their bite than either *Culex* or *Anopheles* mosquitoes. However, the insecticide fogging method employed by Shell in this area has only been demonstrated as effective in controlling mosquito nuisance biting when the adult mosquito population is localized (which is not the case in Yenzi) or when fogging is carried out uniformly over a very large area to prevent re-infestation. This is almost impossible to achieve in this setting in Gabon and apart from that, it is also likely to have little effect on malaria transmission as *An. gambiae* is an opportunistic breeder, which will use any small, clean water body, many breeding sites of *An. gambiae* s.s were found in and around the Carrier village in Gamba

and a nearby quarry in an area about two to three kilometres across the lagoon from Yenzi.

The normal flight range of *An. gambiae* has been found to be usually below one km from breeding sites (Constantini et al., 1996) but *An. gambiae* with the assistance of wind have been shown to fly up to 7 km (Gillies and de Meillon 1968). The spatial distribution of *An. gambiae* was found to be related to the predominant wind direction at night, which suggests that their dispersal from breeding sites is assisted by wind (Lindsay et al., 1995).

Kaufmann and Briegle (2004) found that the flight range of *An. gambiae* can be 9 km when sugar-fed and 10 km when blood-fed, hence mosquitoes from these neighbouring areas could act as reservoirs for re-infestation even if fogging was effective. It was also likely that there is extensive localised breeding in temporary water bodies around the Yenzi club. Effective control by fogging also requires the insecticide fog to drift predominantly through areas where mosquitoes are flying, to be able to provide the contact activity necessary to kill them.

From the observations on the fogging process, it is clear that the fog was concentrated well above the optimum flight height of *An. gambiae* s.s and would therefore, have minimal contact with the mosquitoes. Low wind velocities, stable thermal conditions and 15°C are considered ideal conditions for thermal fogging to allow the fog to travel at ground level (Brattsten and Sutherland 2003). Maximum biting rates for *An. gambiae* s.s, the main vector at Yenzi club, occurs between 16 – 33°C being well above the ideal fogging temperature, which mitigates against using this method for vector control in Yenzi. Fogging for malaria control would also only be effective in the narrow peak biting window for *Anopheles*, which is from 11.30pm to 1.00am. Fogging earlier than this, as regularly occurs in the Yenzi club, may reduce the biting nuisance of *Culex* and *Aedes* mosquitoes but will have zero effect on malaria transmission. Malathion fogging may have a negative impact on other flying insects around the Yenzi club. Although the fogging is localised to Yenzi, such indiscriminate (non-target) use of insecticide is difficult to equate with the present Smithsonian comprehensive study on biodiversity in Gabon to advance management decisions that promote natural resource conservation, particularly when a low level of malathion resistance has already started to develop in the major malaria vectors.

Elevated esterases have been implicated in organophosphate (OP) resistance in a range of mosquitoes. These primarily act as a sink for the OPs, sequestering and slowly metabolizing them, hence the level of resistance produced is proportional to the degree of elevation (Hemingway, 1982). The OP resistance resulting from esterase elevation is broad spectrum, affecting all members of the OP insecticide class (Hemingway, 1982). Resistance levels of 2-10 fold would be anticipated from the levels of esterase elevation present in the *Anopheles* from Gabon. The presence of this resistance mechanism poses a problem for the control method used at the Yenzi club, as malathion, an OP, is the insecticide in use (this PhD).

3.5 Summary and Recommendations

1. A variety of mosquitoes are present in the vicinity of the Yenzi club and Gamba village. *Culex* were the most numerous mosquitoes and these, along with day-biting *Aedes*, form a significant biting nuisance.
2. Two members of the *An. gambiae* complex were identified in the collections. *An. gambiae* s.s, a major malaria vector, constituted 79% of the *Anopheline* catch and anti-malaria activities must be targeted against this species.
3. The *Anopheles gambiae* s.l. had some broad-spectrum organophosphate resistance due to an elevated esterase-based resistance mechanism. This resistance will extend to malathion, the insecticide currently used for fogging.
4. Mosquitoes were fully susceptible to all other insecticides.
5. Fogging, particularly with an insecticide to which there is some resistance, is unlikely to have any positive impact on reducing malaria transmission.

A much better malaria control operation in Gabon could be instigated with a similar budget to that used for fogging, using either indoor residual spraying or providing pyrethroid impregnated bednets.

6. An example of development of malaria information (as used successfully in South Africa) is attached in Appendix 1.

By combining these methods with a robust monitoring system, operations could be targeted to those households or facilities where the highest levels of malaria transmission are occurring. Since this pilot study was undertaken, fogging in Gabon has been

withdrawn and control efforts are now focused on treatment of breeding sites and indoor residual spraying.

CHAPTER 4

DEVELOPMENT OF A MULTI-SECTORAL FORUM FOR PESTICIDE MONITORING, USAGE AND POLICY DEVELOPMENT

DEVELOPMENT OF A MULTI-SECTORAL FORUM FOR PESTICIDE MONITORING, USAGE AND POLICY DEVELOPMENT

4.1 Introduction

Once the pilot study in Gabon was complete the broader study in southern Africa was established using the same principals. However, unlike Gabon where a single company control all the anti-malarial and insecticide–based activities in the study area, in southern Africa numerous stakeholders were involved in insecticide-based activities. The development of insecticide resistance in malaria vector mosquitoes has been associated with pesticide usage in agriculture (Martinez-Torres et al., 1998). This has serious implications for sustainability of vector control, while high levels of disease affect agricultural development (Abamu et al., 2003). Agriculture contributes to the viability of rural areas by generating employment and helps to maintain the rural infrastructure, a point emphasised in World Summit on Sustainable Development, the Johannesburg Declaration, 2003 (www.rdfs.net/ linked-docs/SARDmESP_1XII103.pdf).

Malaria occurs in areas of semi to high agricultural development where agricultural activities are the major means of poverty alleviation. Development is a human problem aimed at changing the human, economic, social and ecological environment leading to improved health (WHO 2005). Forget and Lebel (2001) state how public health thinking has evolved over the last quarter century towards a more global, ecological approach, which basically recognizes that the health problems of the world, including malaria, have had serious impact on human development. It is clear that these problems cannot be solved by a single scientific discipline working alone. This approach in turn has led to natural resource management thinking, including consideration of environmental and social factors together with economic parameters (Forget and Lebel 2001). This has stimulated an integrated approach to management of health and environment (Forget and Lebel 2001).

Due to the complexity of the factors involved in agricultural activities, an ecosystem approach was deemed necessary in South Africa to evaluate the impact of human activities on linkages between total agricultural and public health pesticide usage, vector

resistance and malaria transmission. This approach requires local know-how assisting researchers to guide communities towards solutions to problems that are perceived by them as priorities (Forget and Lebel 2001).

4.2 An Ecosystem Approach in Health

McMicheal (1993) states that 'We are, frankly, not used to thinking of health and disease within an ecosystem-based framework; we prefer to attribute disease causation to events or processes that arise and act at the level of the individual. This, for half a century, has been the dominant expectation of epidemiologists, and their methods are well-honed to this expectation'.

An adoption of an 'ecological' approach to public health action was suggested to public health practitioners over a decade ago (Kickbush 1989). Within an ecosystem, ecology deals with interrelationships between organisms and their environment. Allison (1991) describes an ecosystem approach as a belief in the practical and ethical importance of a holistic understanding of the interactions of living things with each other and the environment. Like ecology, the term ecosystem has multiple meanings, it has been used to describe the entire world, for example, aquatic environments (Rapport 1995) as well as other explanations. Allen et al. (1993) describe the ecosystem as an ecological system occupying a particular place and time with emphasis given to a system description of interacting biota and the environment, including explicitly human activities.

4.3 Ecosystem Health as a Metaphor

The WHO defines health as "a state of complete physical, mental and social well-being, and does not consist only of the absence of disease or infirmity". Human health is seen as the absence of disease, rather than the presence of conditions that constitute wellness. Metaphors are linguistic phenomena where words normally associated with one object are applied to another. Ecosystem health is such a metaphor, which has fundamental psychological importance, being linked to self (through health) and holism (through ecosystem) (Find and Sandstrom 1993).

Find and Sandstron (1993) suggested that people actually see and understand their world through simple slogans and metaphors like 'ecosystem health' rather than being

influenced by complicated theories. The ecosystem metaphor provides a commanding image tapping both health and environmental concerns (Worthington 1983). Find and Sandstron (1993) state that, for both scientists, and the lay public alike, the ecosystem health metaphor provides a method of common engagement, a 'metaphorical resource' that can be indirectly pursued through human actions directed at human kind. Eyles and Donovan (1986) describes a well society as one in which people can meet their basic needs, poverty has been reduced, people are socially and economically mobile and respectful of the dignity of others and they have access to good services in a stable, democratic and participatory environment. Eyles and Donovan (1986) report how the production of harmful crops like tobacco and the use of potentially toxic products such as pesticides which facilitate monocultures, can displace more varied agriculture in large regions of the world. This has considerable impact on the diversity and sustainability of ecosystems, as well as on human health. The 'ecosystem' consequences of such factors can be described as well as the human health and well-being consequences.

The Ecosystem Approach, (an approach taken through the multi-sectoral forum within this PhD) is outlined by Forget and Lebel (2001) as followed 'for purpose of planning and information gathering, the user, according to the task at hand and the scope of the process, may define the limits of a given ecosystem. While in general the limits selected will circumscribe an ecological space such as a watershed or a region, we can also designate a farm, an urban subdivision or a rural community as an ecosystem'.

It is, however, clear that the precise definition of an ecosystem in this context may be controversial. For the purpose of this PhD, the ecosystem was described as a system which incorporates a set of different living organisms that interact with their physical environment. This is based on the working definition proposed by the Canadian Council of Ministers of the Environment in 1996.

4.4 Sustainability

Sustainability has a wide range of definitions. However, the economic and development literature shows that most definitions are centred around economic, environmental and social welfare objectives (Cernea 1993). Three associations have been put forward by Cernea (1993):

1. sustainability of constant consumption,

2. sustainability of constant stock of natural resources and
3. sustainability of intergenerational equity.

The third association was utilised in this PhD, which was suggested by the World Commission on the Environment and Development (1987). This third association stipulates that sustainability is a process of change in which the exploitation of resources, the direction of investment, the orientation of technological development and institutional change are made consistent with future as well as present needs' (World Commission on Environment and Development 1987). In this PhD, sustainability was considered with regard to effective malaria and agricultural pest control, as a result of stimulating the judicious use of insecticides, resulting in continued effectiveness of vector control, increased agricultural productivity, improved health and well-being and poverty alleviation within the communities.

This approach therefore incorporates the concept of environmental epidemiology, defined as the study of the spatial and temporal distribution of a disease in relation to possible environmental factors (Committee on Environmental Epidemiology 1997). The approach provides a forum for understanding the dynamics of agricultural pesticide usage, development of vector insecticide resistance and the resultant effect this would have on malaria incidence which would lead to implementation of more effective control strategies.

4.5 Aims

The aim of the multi-sectoral forum was to provide a discussion opportunity in which all relevant stakeholders could participate aiming to develop a protocol for integrated pest and pesticide management and usage. A second aim was to make recommendations for policy decision making in both the agricultural and health sector through which appropriate interventions could be developed and maintained. This experience could then form the basis for recommending similar initiatives in the remaining malarious provinces of South Africa and the entire SADC region, where pesticides are used to promote agricultural production and to control malaria vector mosquitoes. This approach should

enable the different stakeholders to advocate and agree on strategies for improving agricultural production, including measures to ensure appropriate pesticide use.

4.6 Background

The ecosystem approach for the development of this multi-sectoral forum for pesticide monitoring, usage and policy development was adopted as a result of two workshops held in Kenya for the Systemwide Initiative on Malaria and Agriculture (SIMA). The first workshop was in May 2001 and the second in May 2002. Forty one participants representing the following institutes and countries attended the May 2001 workshop.

CGIAR Centres (IWMI, IPGRI, WARDA AND ICRAF):

- Other international centres and institutions of advanced research (ICIPE, DBL, NRI, University of Copenhagen);
- Donor representatives (IDRC, DFID, USAID, UNEP);
- Six countries from the East and southern African region (Mozambique, South Africa (represented by myself), Zimbabwe, Tanzania, Uganda, Kenya);

Above abbreviations stand for the following institutes:

ICIPE - International Centre of Insect Physiology and Ecology
USAID - the U.S. Agency for International Development
DBL - Danish Bilharziasis Laboratory
UNEP - United Nations Environmental Programme
IPGRI - International Plant Genetic Resources Institute
IWMI - International Water Management Institute
IDRC - International Development Research Centre
CGIAR - Consultative Group on International Agricultural Research
WARDA - Africa Rice Centre
ICRAF - the International Council for Research in Agroforestry
DFID - Department for International Development

The second workshop was an IDRC-supported capacity building and proposal development training workshop with 20 participants from Kenya, Mozambique, South

Africa, Uganda, and Zimbabwe. It consisted of lectures and a field visit to demonstrate the ecosystem approach to human health, with specialized training in GIS applications as well as group sessions on proposal writing. Each country was represented by a scientific leader (myself for South Africa), a social scientist and an environmental scientist. All country teams were requested to submit a pre-proposal to SIMA after which, four teams (from South Africa, Zimbabwe, Uganda and Mozambique) were awarded US$12,500 each, to develop a full proposal. Uganda and Zimbabwe were first awarded funds for two-years for proposals on ecosystem approaches to malaria control in April 2003. Later, funds were made available for a third country (South Africa), but this award was delayed after negotiations were made to put this on hold as this PhD study was near to being completed.

When documenting pesticide usage in agricultural and health sectors during this PhD, it became clear that a more inclusive approach was needed in order to obtain data and influence the behaviour of the different sectors. An ecosystem approach was introduced in KZN during this PhD to provide a forum for communities to develop an integrated pest and pesticide management and usage programme with the objective of sustainable and efficient vector control. To achieve this objective a vector resistance monitoring, agricultural pesticide usage and pesticide residue monitoring programme was developed. This process was initiated in order to promote interactions between communities and the environmental, health and agricultural sectors, which would lead to increased awareness, exchange of information and joint action in anti-malaria and agricultural activities. Recommendations for policy decision making in both the agricultural and health sectors would be made and through this forum appropriate interventions, which should result in effective malaria control, as well as allowing technological improvement (pesticide choice) to be introduced that would benefit the health of the local communities.

Research was undertaken by the Agricultural Research Commission, Plant Protection Research Institute (ARC-PPRI) based in Pretoria in South Africa. The study areas included in this PhD to evaluate insecticide loads in aquatic environments with special reference to water sources and sediments were, Ndumo, KwaJobe and the Tembe Elephant Park in the Ubombo district, northeastern KZN. This study was also designed to establish whether the insecticide residues originated from use in agricultural or malaria control activities. Without this study, no data would be available, as the South African

water quality monitoring programme does not include pesticide monitoring in these rural areas. Furthermore, the project was aimed at predicting the possible risk of insecticide resistance development in malaria vectors. This study showed insecticide contamination of the water environment in the above areas and their data indicated residues representative of pyrethroid, organophosphorus, organochlorine and carbamate insecticide groups (Water Research Commission (WRC), report 2003).

The ecosystem approach (see section 4.2) was used to develop a framework and initiate a process that should improve the socio-economic well-being of the rural population dependent on the Pongola River system in KZN within this PhD study area. There is an on-going project, funded by the South African Water Research Commission (SAWRC) through the Centre for Environment and Development, aiming to create a shared vision of the use of the river that will reflect a diverse and sustainable economy, by promoting co-operative governance over the resource. Issues of concern include the release of water from the Pongola Dam to mimic the natural flooding, as well as current and future land use of the floodplain. In an effort to alleviate poverty, the government has been promoting irrigated agricultural developments in an area adjacent to the floodplain, the Makhatini Flats, which form part of this PhD study area (Water Research Commission (WRC), report, 2003).

The ecosystem approach was also employed by the SAWRC to improve the livelihoods of the local population through more ecologically sensitive cultivation and increased conservation of resources. A key issue was the need to balance water use between the subsistence farmers on the floodplain on one side with the water requirements of the larger scale irrigated agricultural developments on the other side (SAWRC pers. Comm.). Irrigation in the region also has the potential to increase malaria incidence due to an increase in suitable breeding sites, and therefore needs to be carefully planned and managed.

Studies done by ARC-PPRI on water samples in the area clearly show the presence of pesticide accumulation in several study sites. This was also followed by an investigation into the magnitude of pesticide exposure and uptake by women and children in small-scale farming (ARC-PPRI report). A strong correlation between agricultural pesticide usage and vector resistance was also established in sentinel sites in the study area during

this PhD. With the intended increase in commercial farming area, the use of pesticides will increase, calling for urgent attention if this is to occur in a format that will not negatively impact malaria control (Makhathini Farmers Association pers. Comm.). A Cotton Ginny is in the final stages of construction in anticipation of receiving large quantities of raw cotton from local farmers. Water for all purposes is obtained from naturally occurring water bodies, mainly rivers, none of which are tested by the Department of Water Affairs for the presence of chemicals (ARC-PPRI report and Gumede, pers. Comm.). No effort is therefore being made to address the potential health risks, experienced by the locals due to exposure to unsafe water.

It is envisaged that the Ecosystem Approach to address problems associated with pesticide usage and effects on vector control will incorporate the many systems upon which these factors impact. The participatory process will ensure that the opinions of all stakeholders are expressed, and taken into consideration, in the formulation of policy recommendations. This will increase the chance for a long-term sustainability of agriculture and environmental systems in the region. This project will also provide new knowledge and tools to address issues of gender, poverty, health and food security, thus helping to ensure long-term environmental integrity.

4.7 Examples of Selected Ecosystem Projects

The goal of the International Forum on Ecosystem Approaches to Human Health held on May 18-23, 2003 in Montreal, Canada, provided a platform for a discussion of ecosystem approaches to human health, the evidence from the field and the relevance of these approaches to improving health and well-being. The examples below are projects using such approaches.

4.7.1 Brazil

Title: An Ecosystem approach controlling communicable and emerging disease.

To study the strong links between stressed ecosystems and communicable and emerging diseases, the workshop participants used an ecosystem approach to human health to develop a conceptual framework to identify, adapt and disseminate methods for

preventing communicable and emerging diseases through better management of the ecosystem. Policy recommendations arose from the workshop, tackling the need for implementation of integrated community-based and sustainable strategies to prevent and control communicable and emerging diseases.

4.7.2 Cuba

Title: Revitalizing central Havana

Purpose of the project was to rehabilitate Cayo Hueso, one of Havana's oldest and most run down districts, where 170,000 people crowd into three square kilometres of tenements. The community helped to develop and apply the necessary interventions. This was by the National Institute of Hygiene, Epidemiology and Microbiology (INHEM) and the University of Manitoba.

4.7.3 Mexico

Title: Fighting malaria without DDT

The southern Mexican state of Oaxaca is home to 80% of all the malaria cases in the country. With the phasing out of DDT, there is urgency to search for safe and effective controls. The project brings public health researchers, anthropologists, malaria and environmental specialists from the National Institute of Public Health of Mexico, the National Programme of Vector-borne Disease, the Ministry of Health of the State of Oaxaca, and the Centre for integral training of voluntary community health workers together with community members to find solutions.

4.7.4 Ethiopia

Title: Balancing food self-sufficiency, soil degradation and income.

Health and agricultural researchers from the International Livestock Research Institute used an ecosystem approach to find solutions to the problems of poor human health and nutrition, soil degradation and lack of cash income. Their study discovered that the use of

chemical fertilizers resulted in only small increases in food production, but caused significant soil and nutrient loss.

4.7.5 Cote d'Ivoire

Title: Exploring the impacts of hydroelectric dams.

The hydroelectric dam built during the 1980s in the Buyo area has transformed the local landscape and communities. The most significant socio-economic impacts have been changes to agricultural practices and the creation of a local fishery. A research team from the University d'Abobo-Adjame, Abidjan, is working with communities in Buyo to assess the impact of the dam on the local ecosystem and on people's health. The ultimate aim is to develop cost-effective interventions for improving the health of both.

4.8 Objectives

The Ecosystem Approach within this PhD programmeme was designed to address the following three objectives:

1. To monitor the development and spread of insecticide resistance in southern African mosquito vectors.
2. To determine the relative importance of anti-malarial and agricultural pesticide usage in the selection of insecticide resistance.
3. To help establish a rational policy for insecticide choice and use for southern Africa, on the basis of the above.

4.9 Materials and Methods

As stated earlier (see section 1.11), two of the main objectives of this PhD were to determine the relative importance of anti-malarial and agricultural pesticide usage in the selection of insecticide resistance and to help establish a rational policy for insecticide choice and use for southern Africa. To achieve these objectives, a participatory research method was employed. This involved participatory problem definition which resulted in building of a shared agenda for action. Two workshops were held in October 2003 at the Department of Health, Jozini, KZN. The first workshop was a result of visits to the

institutes, groups and organizations (Table 31) involved to discuss the ecosystem approach aimed at solving the problems of insecticide resistance and the perceived association of vector insecticide resistance with agricultural pesticide usage and malaria transmission. During the identification of stakeholders, the author ensured that both men and women were included in the participatory process. Women formed the bulk of the stakeholders due to the migration of men to the cities for better jobs. The second workshop was held in November 2003 at the Makhathini Agricultural Research Station, Mamfene, Jozini. A Participatory Rural Appraisal (PRA) tool was employed during this second workshop to assess the ecosystem structures, in order to develop community-based solutions to the malaria problem in the southern African region. This also provided an opportunity to determine and understand the gender-specific impacts of ecosystem health risks. All the workshops held were chaired by the author, also including communications with all the different stakeholders and the choice of the steering committee which will foresee the implementation of the project and its management.

The PRA is an action research tool that involves community members in defining and working to solve local concerns (www.camp.umich.edu/acadpgm/urp/utepsymposium/publication/McDade.pdf), for example, problems associated with pesticide usage in both health and agricultural sectors in this PhD. The PRA method stresses local knowledge, empowerment, and sustainability in addressing issues like natural resources, agriculture and health. The tool has been used among international NGOs, operating in developing countries over the past decade and certain governments (www.camp.umich.edu/acadpgm/urp/utepsymposium/publication/McDade.pdf).The PRA tool applies a different number of methods mostly decided upon by the researcher. The method applied in this study was a mapping exercise, that is, modelling of the problem (Figure 80). This exercise, conducted in Zulu, the language spoken in KZN, was a starting point for recognizing and discussing differences in agricultural practices in the form of pesticide usage in the community. The PRA tool was also used to explore local perceptions about wealth and therefore helping to design and develop the sequence of interventions to address the socio-economic issues.

The multi-sectoral approach was considered to be the most suitable strategy used to investigate the many factors associated with insecticide resistance selection in this project, i.e. implementation of insecticide resistance management strategies in southern Africa. A

variety of potential stakeholders in South Africa were contacted to establish their support and willingness to participate. To assess the extent to which this issue is perceived to be of concern to the community, workshops were held at which their support was canvassed and local representatives elected (Table 31). The participatory process was successfully used to establish a stakeholders' forum with representatives from local farmers as well as those detailed in table 31.

The stakeholders' forum identified from the different institutional and community sectors was divided into two groups, the collaborating partners and partners based on the different capacities of the sectors. The roles of the principal collaborating partner are listed in table 32 as well as the roles of other collaborating partners. The partners were also allocated roles as listed in table 31. The steering committee was chaired, and chosen by the author to oversee the implementation of the project from all the collaborating partners and partners listed in table 32. Efforts were made to reduce barriers or obstacles that could preclude or debilitate equitable participation within the stakeholder forum.

New linkages within SIMA and the IDRC Ecosystem Approach initiative were investigated to ensure that projects with similar strategies are combined in southern Africa. Through this interdisciplinary team approach opportunities were provided to learn from each other, thereby building regional capacity in the Ecosystems Approach in health research.

Due to the limitation of a PhD programme, the study was restricted to investigating the links between agricultural practices and pesticide usage, the resistance status of *An. arabiensis* and malaria transmission in one malarious province only, the northeast of KZN. To undertake this linkage analysis, four villages, they were Kwa-Jobe, Makhathini, Ndumo and Mzinyeni ecologically characteristic of the target communities in the region, were identified based on the initial insecticide resistance analysis of this study. These sites had the highest levels of insecticide resistance encountered during the study period.

The Agricultural Research Council (ARC) also carried out research in these four locations to evaluate insecticide loads in aquatic environments with special reference to water sources and sediment (ARC-PPRI). The ARC-PPRI study was aimed at determining whether the insecticide residues originated from use in agricultural programmes or from

the malaria control initiatives. This study provided analysis of insecticide contamination of the aquatic environment in the villages. The combined data analysis from the two studies, this PhD as well as the ARC-PPRI study, helped to establish a stakeholders' forum to look at the linkages between the recent selection of insecticide resistance in *An. arabiensis*, selection pressure due to pesticide usage in agriculture as well as malaria incidence.

To achieve this objective, a preliminary workshop was held at the Department of Health, in Jozini, north-eastern KZN, to identify institutional and community sectors that are directly or indirectly associated with the problem and to determine their potential status and roles in the stakeholders' forum (Table 31).

4.9.1 Selection of the Study Area

The study area was located in KZN, South Africa and the pilot study was conducted in Ingwavuma and Ubombo districts of the province (Figure 18, section 2.2.1). The villages identified were Kwa-Jobe, Makhathini, Mzinyeni (in Ubombo district) and Ndumo and and the Tembe Elephant park (an area with no malaria control and agricultural activities) (in Ingwavuma district).

The study sites were situated in the low-lying, semi-tropical malarious area of north-eastern KZN. Within the area, small and large-scale agriculture was practiced. The farming practices included cattle farming and crop production, including cotton, sugarcane, wheat and various vegetables. Historically, this rural area has not experienced high levels of economic and infrastructural development, but is now being specifically targeted by the government for developments such as agriculture and tourism.

Subsistence farming by the local communities is being supplemented by the introduction of cash crops e.g. sugar, cotton and vegetables, all of which use high levels of pesticides to enhance their productivity. Farming activities are predominantly carried out by women due to the migration of men for economic reasons. The traditionally patriarchal communities live in scattered households close to their agricultural activities, each consisting of an extended family unit. So the majority of forum's participants were women.

4.10 Results and Discussion

4.10.1 Participatory Workshop

A participatory workshop (Figure 79a, b and c) was held in October 2003 at the Makhathini Research Station in Jozini. A problem mapping exercise was undertaken through this participatory workshop involving stakeholders listed in table 31. This involved a multi-disciplinary and participatory process which provided a forum in which all relevant stakeholders could participate to develop a protocol for integrated pest and pesticide management and pesticide usage. The group made recommendations for policy decision making for both the agricultural and health sectors and established a community vehicle through which appropriate interventions could be developed and maintained. All participants in this workshop were given the opportunity to voice their knowledge about malaria, insecticide resistance and agricultural practices in the form of pesticide usage.

4.10.2 Problem Mapping

Problems were encountered with the documentation of pesticide usage in the agricultural sector. This led to the introduction of a cross-disciplinary approach, which involved all the different institutes, described in table 31 and figure 79a, b and c. This group started to jointly address this issue in an intra-disciplinary and participatory approach.

Figure 79a. Explanation of research tools for agricultural-economics research and analysis by a researcher from the Department of agricultural-economics, University of KZN using the PRA tool conducted in Zulu.

Figure 79b. Report back from participatory discussions on pesticide usage, gender and socio-economic issues by one of the group leaders using the PRA tool.

Figure 79c. Problem mapping exercise involving pesticide usage, vector resistance, malaria transmission and socio-economic issues involving women farmers and farm labourers by the author.

Figure 79 (a,b & c). Participatory workshop. Farming community participatory workshop at the Makhathini Agricultural Research Station, Makhathini Flats, Jozini, north-eastern KZN undertaken to introduce farm workers (primarily women) and farmers, to skills aimed at promoting interactions between communities and environmental, health and agricultural sectors, which should increase awareness, exchange of information and joint action in insecticide – based anti-malaria and agriculture activities.

This process advocated the judicious use of pesticides in both the agricultural and health sectors. Women were invited to participate in the forum as they formed the bulk of farmers and farm labourers in almost all the rural areas of South Africa. Figure 80 illustrates how improved understanding and monitoring of pesticide usage should help to reduce pesticide accumulation in breeding sites and therefore decrease the insecticide selection pressure on the malaria vectors, resulting in more efficient malaria vector control programmes. Similarly, well coordinated judicious use of pesticides in agriculture should result in increased agricultural productivity, which in turn will improve the women's economic status and therefore enable them to make decisions regarding the health status of their offspring.

Institute	Status	Role
Medical Research Council South Africa	Principal collaborating partner	Project and budget management, vector resistance, GIS, capacity building
Agricultural Research Council South Africa	Collaborating partner	Pesticide residual studies, Environmental risk, capacity building
Department of Agricultural-Economics, University of Natal	Collaborating partner	Agricultural-economics research and analysis, capacity building
Department of Health including Bathesda Hospital, Jozine, KZN	Collaborating partner	Malaria control expertise and case data, pesticides in breast milk, capacity building
Non Governmental Oganisations (NGOs) – Multi Initiative Development Trust, Jozini, KZN	Collaborating partner	Facilitation, information dissemination, community liaison, capacity building
Farmers association, Jozini, KZN	Collaborating partner	Access to farmers, pesticide suppliers and usage
Department of Agriculture, Jozini, KZN	Partner	Information about agricultural practices, land use, capacity building
Department of Water Affairs, Jozini, KZN	Partner	Expertise on water systems, use and management
Municipality, Jozini, KZN	Partner	Political support
Pesticide suppliers, South Africa	Partner	Information about pesticide policies and use
Faith Based Organizations, Jozini, KZN	Partner	Spiritual influences
Traditional healers, Jozini, KZN	Partner	Treatment for malaria and pesticide exposure
Farm labourers, Jozini, KZN	Partner	Study participants
Women's groups, Jozini, KZN	Partner	Information about gender issues
Traditional authorities, Jozini, KZN	Partner	Political support
KwaZulu-Natal Conservation Services	Partner	Information about environmental use and management

Table 31. Institutional and community sectors identified in the establishment of the stakeholders' forum, with their status and roles.

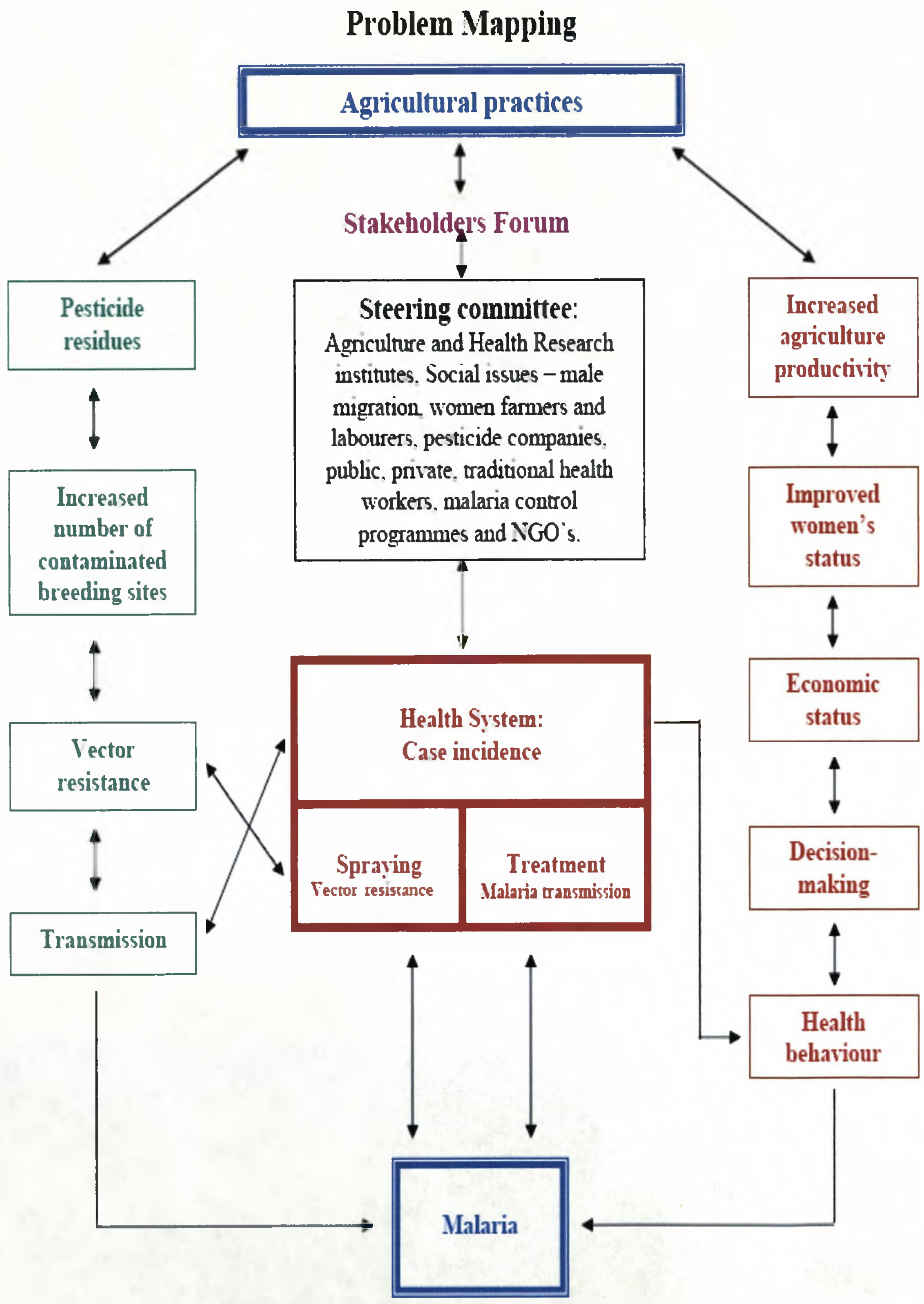


Figure 80. Problem mapping exercise. Overview of the problem mapping exercise undertaken to study the links between agricultural practices in the form of pesticide usage, the resistance status of the Anopheles and malaria transmission in north-eastern KwuZulu/Natal and how the different stakeholders perceive these links.

4.10.3 Development of Vector Resistance Monitoring, Agricultural Pesticide Usage and Pesticide Residue Monitoring Programmeme Agreed by the Forum

To be able to implement sustainable pesticide usage programmes, vector resistance monitoring, agricultural pesticide usage and pesticide residue monitoring programmes were developed. The activities established by this study through the forum related to achieving resistance monitoring and management were as follows:

1. Develop a database on insecticide use patterns in the study area, based on updated information collected through community participation mechanisms.
2. Collect data on malaria cases in the study area using the existing information systems on malaria statistics.
3. Monitoring for insecticide resistant mosquitoes and insecticide residues in the aquatic environment in the study area.
4. Determine possible correlation of insecticide residue levels in the aquatic environment with resistant mosquito population and malaria transmission.
5. Determine the proximity of the women's homes to the agricultural sites and breeding sites.
6. Determine demography, social aspects and economic status of the farmers and labourers to establish any association with malaria incidence.
7. Determine the hydrological aspects in the area.
8. Undertake a baseline Knowledge, Attitude and Practices (KAP) study.
9. Develop and pilot test research protocols for determining the relationship between demography, socio-economic status, agricultural practices and malaria incidence amongst farmers, with an input from stakeholders through workshops.
10. Conduct a literature review of economic research on malaria in southern Africa.
11. Conduct a desk-top study of the local and regional economies.
12. Conduct a base-line survey of the socio-economic status of participants in the study area.
13. Develop measurements for assessing future changes in the economic well-being of households in the study area.

14. Determine the factors affecting farmers' management practices.
15. Undertake a financial analysis of best management practices for farmers.
16. Training of communities and stakeholders in the participatory process.

4.10.4 Development of methods to be employed

This study looked at possible methods that should be used in this process, which will form a collaborative effort to be conducted by all the collaborating partners from research organisations, government departments and the community. Methods proposed to be used in this process were as follows:

1. Investigate the hydrological aspects of the Pongola River catchment and tributaries selected for inclusion in the study.
2. Decide upon the location of the sentinel sites through stakeholder participation prior to the implementation of the study.
3. Develop research protocols to determine the relationship between demography, socio-economic status, agricultural practices and malaria incidence amongst farmers, with input from stakeholders through workshops.
4. Identify study participants, both near and far from the selected river system through sound statistical methods.
5. Implement community mapping exercises to be able to illustrate and discuss malaria risk with stakeholders.
6. Identify issues to be addressed in the survey questionnaire with the stakeholders and researchers during a workshop. Select community members to undertake the survey.
7. Design survey questionnaires in collaboration with the statistician, database manager, GIS specialist and researchers. Discussions with the community representatives will be held prior to the development of the questionnaires to ensure that all the relevant issues are addressed. The data collection process will also be agreed upon in consultation with the community representatives. Questionnaires will be presented to the MRC's ethics committee before the commencement of the pilot survey. Thoroughly train local inhabitants to undertake pilot and final surveys in local language towards investigating the following:

a) Methods and patterns of pesticide and land use by local farmers. This will be done by participant observations and interviews.

b) Health issues regarding malaria incidence and treatment, general well-being, access to and use of health facilities, economics of health and health seeking behaviour, symptoms relating to the use of pesticides, and breast feeding activities.

c) Agricultural and economic factors relating to land use and ownership. This includes agricultural practices as well as identifying variables that can assist in monitoring changes in these factors over time.

d) Socio-economic, cultural and gender issues, including demography of households and the social relationships that effect agricultural practices, health status and behaviour both within the home and the local community.

e) Ecological issues relating to the Pongolo River flood plain as influenced by the flooding of the river and associated agricultural practices.

8. A literature review of relevant information on the region will be undertaken and an inventory of plans and projects in the region compiled.

9. Monitor insecticide resistance in malaria vectors. Both adult blood-fed females and mosquito larvae will be collected from houses, breeding sites and cattle enclosures and tested for insecticide resistance as described in Chapter 2.

10. Pesticide residue monitoring will be undertaken from water and sediment samples from selected study sites during four sampling intervals. The sampling intervals will be selected, coinciding with seasonal fluctuations in pesticide use, hydrological flow and mosquito sampling for resistance measurements. The number of samples will be established in agreement with a statistician, and the positions decided upon in collaboration with hydrologists. The samples for water analysis and vector resistance will be collected during four sampling intervals twice during the transmission season; In order to cover the seasonal cycle, one will be taken before and the other one after the peak transmission period. Collected samples will be stored and transported under appropriate conditions.

11. Community representatives will be issued with a simple camera and film in order to record the activities that are relevant to this study. These will be

compiled into A0 laminated posters at the end of the project and returned to the communities.

12. Analysis from the community mapping exercise will include:
    - Methods and patterns of pesticide and land use by local farmers, detected participant observations and interviews.
    - Health issues regarding malaria incidence and treatment, general well-being, access to and use of health facilities, economics of health and health seeking behaviour, symptoms relating to the use of pesticides and breast feeding activities.
    - Agricultural and economic factors relating to land use and ownership. This includes agricultural practices as well as identifying variables that can assist in monitoring changes in these factors over time.
    - Socio-economic, cultural and gender issues including demography of households and the social relationships that effect agricultural practices, health status and behaviour, both within the home and the local community.
    - Ecological issues relating to the Pongola River flood plain as influenced by the flooding of the river and associated agricultural practices.
13. Spatial data for all components of the project will be collected and added to the existing data repository of the Malaria Research Programme's (MRP) GIS laboratory, some of which are already available. Data not currently available will be captured through the use of Global Positioning System (GPS) receivers or obtained from the relevant source. Questionnaire and survey results will be double-punched into a 4th generation relational database and managed by the database managers in the MRP.
14. A descriptive analysis of the survey results will, where possible, be accompanied by statistical analysis. The analysis will comprise the results of investigations described above, under point 4 as well as the following:
    a) A multivariate statistical technique (e.g. Principal Components Analysis and Cluster Analysis) to provide a description of heterogeneity in socio-economic well-being amongst surveyed households.

b) Regression analysis and discriminate analysis to identify the socio-economic characteristics associated with the use of best management practices with respect to malaria control objectives in the region.

c) Correlation between insecticide residue levels in the water environment, resistant mosquitoes and malaria transmission.

d) Partial budgeting and capital budgeting techniques to analyze the specified best management practices.

e) Quantitatively analysis of water samples using Gas Chromatographic methods. The presence of pesticide residues will be confirmed using either Gas Chromatography with Mass Spectrophotometry or other appropriate techniques. Sampling, extractions and analysis will be done according to internationally accepted and vetted methods. Active ingredients per insecticide chemical group (pyrethroids, organochlorines, organophosphates and carbamates) will be analyzed.

15. Attribute data collected through questionnaires or laboratory tests will be attached to the spatial data and displayed to enable a spatial analysis. GIS will also be used to simultaneously display different components of the data to allow an integrated spatial analysis of how they affect and are affected by each other. It will also enable an investigation into the small-scale variations of and between social, economic and agricultural factors within the region. The vector-based GIS software package MapInfo, will be used to carry out the analysis, copies of which are currently in use in the MRP and for which sufficient experience is available to undertake the research. To determine the possible correlation of insecticide residue levels in the water environment with resistant mosquito population and malaria transmission, GIS will be used to map temporal and special distributions of data collected on both pesticide residues and resistant mosquito occurrences.

16. A final workshop will be organised on completion of all investigations to formulate policy recommendations regarding the appropriate agricultural pesticide usage.

4.11 Implementation and Management

Tables 32 and 33 below show collaborating institutions with their comparative advantages and roles as well as the logical framework

Collaborating Institution	Comparative advantage	Role
Medical Research Council, Durban, South Africa.	Administrative support Vector analysis Social Studies GIS	Manage and coordinate Undertake vector analysis Organize meetings and workshops Undertake spatial analysis Manage budget Coordinate final report production Data capture, maintain database Spatial analysis using GIS
Water Research Commission, Unit for Pesticide Science, Tswane, South Africa.	Residual pesticide analysis Environmental studies Risk assessments	Undertake pesticide residue analysis and risk assessment.
Agricultural economist, School of Agricultural Sciences and Agribusiness, University of KZN	Agricultural-economic research and analysis	Undertake agricultural-economic research and analysis
Maputaland Infrastructure Development Initiative (MIDI Trust), Jozini.	Community based NGO Social studies	Facilitation, progress monitoring and information dissemination Community liaison
Department of Health, and Bethesda Hospital, Jozini.	Environmental and malaria control Malaria case data Pesticide exposure	Malaria metric information Pesticide exposure information
Department of Agriculture and Farmers Association, Jozini.	Agricultural research and extension Access to farmers, pesticide suppliers and usage	Information of agricultural practices Develop practices according to policy recommendations Agricultural community interface

Table 32. Shows organisation of the steering committee and their respective roles.

Narrative Summary	Measurable Indicators	Means of Verification
Goal Sustainable malaria and agricultural pest control due to judicious use of insecticides	1. Malaria vector resistance 2. Pesticide residues 3. Effective malaria control	1. Susceptibility, molecular & biochemical tests 2. Residue analysis 3. Malaria information system
Purpose Appropriate insecticide policy	Agricultural and malaria insecticide policies	Project steering committee presentations to National Malaria Advisory group and department of agriculture and health
Outputs Digital maps of pesticide usage Digital maps of pesticide residues Digital maps of malaria vector, resistance & breeding sites Digital maps of economic status of communities Published papers and reports		
Activities 1. Develop appropriate instruments to capture household economic and KAP data and enumerator 2. Malaria vector collections, processing and analysing 3. Water sample collections, processing and analysing 4. Data collation, double punching database and analysing 5. GPS study sites 6. Literature surveys 7. Community liaison and workshop 8. Training	1. Questionnaires developed and administered 2. Samples collected and analysed 3. Samples collected and analysed 4. Project databases 5. Study sites completed 6. Manuscripts collated 7. Workshops held 8. Number trained	1. Questionnaires 2. Field and laboratory techniques 3. Field and laboratory 4. Data punchers and managers 5. GPS 6. Library 7. Training programmes

Table 34. Logical framework to lead the steering committee through the cause-effect relationships between goal, purpose, outputs and activities.

4.11 Outputs

The knowledge base that we have started to develop with this approach will be used to produce a comprehensive database of digital maps. These will reflect the spatial and temporal distribution of agricultural and malaria control pesticide usage patterns, pesticide distribution of agricultural and malaria control pesticide usage and breeding sites, demography, economic status of the community and the hydrology of the study area. Qualitative data on social aspects will be incorporated into the database. This will also lead to continuous development of new knowledge and tools to reduce malaria risks in different agricultural and socio-economic settings. This work will use situation analysis reports, peer review publications and workshops to disseminate information obtained on the links between health and agriculture for reducing malaria risks and therefore enhancement of human health through improved ecosystem and natural resource management.

The forum will promote interactions between communities and the environmental, health and agricultural sectors, which will lead to increased awareness, exchange of information and joint action in anti-malaria and agriculture activities. It will also lead to stakeholder capacity development for interdisciplinary participatory research and therefore promotion of holistic approaches to malaria reduction, based on improved management and utilization of natural resources. It will subsequently lead to implementation of ecosystem-based and environmentally-sound best practices for reductions in malaria risk at study sites with known malaria and social-ecological characteristics. This work will help to assess the impact of interventions aimed to control malaria and agricultural productivity and document their impact on the sustainability of insecticide usage in both programmes.

4.12 Discussion.

The stakeholders' forum was only fully implemented in north-eastern KZN, but the high levels of acceptance and enthusiasm for the scheme suggest that it could now form the basis for recommendations to commence similar initiatives in the remaining malarious provinces of South Africa, as well as the entire southern African region where pesticides are used in agricultural production. The forum process advocates for the judicious use of pesticides in both the agricultural and health sectors. Women were invited to participate in

the forum, as they form the majority among farmers and farm labourers in almost all the rural areas of South Africa. This is due to male migration to the cities to seek employment. Figure 80 illustrates that improved understanding and monitoring of pesticide usage should now help the women to reduce this usage in farming and hence avoid pesticide accumulation in breeding sites. This will consecutively reduce the insecticide selection pressure on the malaria vectors, resulting in more efficient malaria vector control programmes. Similarly, well coordinated judicious use of pesticides in agriculture should result in increased agricultural productivity, which as a result will improve the women's economic status and therefore enable them to make decisions regarding the health status of their offspring. The beneficial effects of this system will now need to be monitored and fostered over time.

During the course of two workshops, a project team and management structure, including a steering committee, was established (Table 31). Based on the discussions during the workshops, it became clear that the development of insecticide resistance in insects of both health and agricultural importance had serious implications for all the stakeholders for sustainability of vector control as well as agricultural developments. Since agriculture contributes to the viability of rural areas by generating employment and helps to maintain the rural infrastructure, the issue was perceived as a serious concern by the communities.

Development is a human problem aimed at changing the human, economic, social and ecological environment leading to improved health. The established stakeholders' forum became aware how health problems, especially malaria in this case, cannot be solved by a single scientific discipline standing on its own and therefore a holistic approach to address health issues was urgently needed in their community. In order to address these issues, this study looked at activities that introduced a cross-disciplinary and participatory approach to provide a forum for participation to develop an integrated pest control programme, pesticide management and insecticide usage programme with the objective of sustainable and efficient vector control, by insecticides.

The major accomplishment of this pilot study has been both conceptual and methodological so far. That is, the concepts and methods to be employed have been successfully debated and understood by the different stakeholders during the two participatory workshops. No empirical data exists at the moment to supply a

comprehensive numerical estimate of the linkages between pesticide usage, vector resistance and malaria transmission and therefore the health status of the given ecosystem but the forum should allow these linkages to be fully investigated. The stakeholders were successfully included in both the definition of the system and its description (see problem mapping, Figure 80). Stakeholders have now formed a part of the research and are therefore involved in developing the solutions to insecticide resistance selection, which will lead to a higher chance of sustainability of the system. Their involvement at this stage was in problem identification, planning and implementation of the pesticide usage and resistance management and monitoring and they will consequently therefore take part in the analysis of the data obtained through the system. They now own the problems, the solutions and their means to solve them.

4.14 Summary and Conclusions

The multidisciplinary forum used participatory rural appraisal (PRA) research to identify how communities perceive linkages between pesticide usage, vector resistance and malaria. The above agreements for future research have now established the framework in which the stakeholders' forum will operate in the future. This along with the resistance baselines established during this PhD will lay the groundwork for the next decade of insecticide based malaria control activities in South Africa.

The stakeholders' forum, although established as part of this PhD, is intended as a long term on-going process, which will continue as part of my standard activities upon my return to the MRC in Durban after completion of the thesis. The ecosystem approach process envisaged, will analyze the complex situation in the developing agricultural sector of the malaria endemic areas of KZN in regard to insecticide resistance, pesticide use, environmental, demographic, social and health data in respect to the study community. It will create awareness amongst farmers concerning inappropriate agricultural usage practises and enforce the need for development best management practices.

Long term benefits from the planned work will be that community participation in the project will enable community inputs in policy making, thereby making it oriented towards the needs of the community.

Sustainable malaria control through insecticide use, as well as technological improvement (pesticide choice), will benefit the local communities' health as well as their economic status. The improved pesticide use practises in agriculture and malaria control should result in increased agricultural productivity and better health, leading to enhanced economic status within the community. This will lead to an improvement of the environment through integrated pest control and pesticide management thus increasing the longevity of the natural ecosystem, providing sustainable tourism opportunities and economic growth. Based on a better understanding of the situation, policy decisions will be formulated with regards to pesticide use in the area by all sectors.

Experience from this work in KZN will from the basis for recommendations to inform similar initiatives in the remaining malarious provinces of South Africa and the SADC region, where pesticides are used to increase agricultural production. This will enable the different stakeholders to advocate and agree on strategies for improving agricultural production, including measures to ensure appropriate pesticide use. The information dissemination strategy will be targeted at local, provincial, national and international communities and institutions through a variety of media on appropriate pesticide use, agricultural practices and health related issues. This will not only be targeted at malaria and agricultural communities, but also at the variety of disciplines affected by this process. For example:

- Communities in malaria prone areas – workshops, community radio stations, community information booklet, posters and drama (all in the local language);
- Policy makers – meetings, workshops, reports on policy recommendations;
- Researchers – peer-review publications, local and international conferences on research finding, recommendations, new methodologies and tools;
- Developers – internet access, newspaper articles on research findings and implications of development on malaria incidence;
- Agricultural extension officers – workshops, reading material and reports;
- Media – press releases and interviews.

Monitoring will be carried out by providing financial evaluation of suitable technologies and farming practices in the form of pesticides. Follow-up risk perception and exposure

investigation, as well as environmental contamination measures and farmer knowledge regarding pesticide use. The participatory process will continually be evaluated Monitoring will also include levels of insecticide resistance in vector populations, levels of pesticide accumulation in water bodies and breeding sites, risk perceptions associated with malaria, the environmental effects of flooding on the Pongola floodplain and levels of pesticide residues in breeding sites and water bodies.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

5.1 Discussion

The current PhD was established as part of a much broader integrated programmeme to build capacity in insecticide resistance detection and management in southern Africa. As the southern African programmeme was exclusively developed at the national control programmeme level, it was essential in the first place to undertake a smaller scale pilot programmeme to establish basic study parameters. The site in Gabon was chosen, being closely aligned to a large malaria naïve expatriate community where intensive insecticide-based vector control operations had been undertaken for more than two decades.

The conclusions (see Section 3.5) were reported to the Shell management team, who subsequently, in consultation with their expatriate community in Gabon, agreed to stop fogging activities and concentrate on improving house screening by insecticidal treatment of screens and increasing information flow on malaria prophylaxis and treatment.

In addition to having little beneficial impact on disease control, the malathion fogging may have a negative impact on other flying insects around the Yenzi club. Although the fogging was localised to Yenzi, such indiscriminate (non-targeted) use of insecticide was difficult to reconcile with the Smithsonian comprehensive biodiversity study currently being undertaken in the Gabon area to advance management decisions that promote natural resource conservation.

The findings from this pilot mosquito control evaluation and baseline survey in Gabon were summarised in Section 3.5. An example of a malaria information system (as used successfully in South Africa) was given to the control programmeme managers (Appendix 1). This could be used to help instigate a better malaria control operation in Gabon, with a similar budget to that used for fogging, by either indoor residual spraying or by providing pyrethroid treated bed nets. Through combining these methods with a robust monitoring system, operations could be targeted to those households or facilities where the highest levels of malaria transmission are occurring.

Shell is currently considering how to improve their malaria control operations at all their sites and have used the Gabon study as a template for further studies in Nigeria. The successful completion of this pilot study allowed me to develop systems that could be more broadly applied to the national malaria control programmemes in southern Africa.

5.2 Southern African Region

After the successful Gabon study larger scale analysis was undertaken in southern Africa. Broad spectrum resistance to all the four major insecticide groups was detected in mosquitoes from the countries sampled, using WHO susceptibility tests and molecular and biochemical assays.

All the tools needed to detect and monitor insecticide resistance in malaria vectors in the southern African region were successfully implemented in the larger scale study and a range of collaborators were trained in a variety of entomological techniques. This cohort of researchers will now form the basis of the entomological surveillance capability for malaria in southern Africa for the foreseeable future.

5.2.1 AChE Based Resistance

An altered AChE resistance mechanism was detected at different frequencies in all three main regional malaria vectors. This mechanism produces resistance to both OPs and carbamates, although the resistance levels conferred can vary, as previously indicated by Hemingway et al. (1986). Earlier studies showed that when log normal activity is plotted against log inhibited AChE value, the inhibition levels of AChE from homozygous (RR), heterozygous resistant (RS) and homozygous susceptible (SS) did not overlap (Hemingway et al., 1986; ffrench-Constant and Bonning 1989). The heterozygotes plotted as % inhibition alone overlapped with both the homozygous populations at either end of their distribution ranges. The AChE inhibition percentages with 0.1M propoxur for homozygous susceptible should be >80% as was the case with both *An. arabiensis* and *An. gambiae* s.s. However, inhibition levels can be lower than this for fully susceptible insects. For example, inhibition with 0.1M propoxur was >60% for fully susceptible *An. albimanus* (Penilla 2001).Occasionally in the inhibited well activity values > 100% were observed. This apparent increase is an artefact in samples with low baseline AChE

activity, as propoxur in the inhibited wells has some absorbance at 405 nm (Hemingway et al., 1986). This absorbance is usually masked by the AChE activity, but where this is low the absorbance from the propoxur becomes significant. The problem of unambiguous identification of the three genotypes (RR, RS and SS) using the biochemical assay for altered AChE performed in this study, was solved by (ffrench-Constant and Bonning 1989) using scatter plots of uninhibited and propoxur inhibited AChE activity of *An. albimanus*, *An. nigerimus* and *C. pipiens* on a log scale.

The altered AChE mechanism often produces a low level of resistance that is not detected in the heterozygous form by standard WHO bioassays. In this study, complete susceptibility to propoxur and bendiocarb with WHO bioassays, was recorded except for 0.41% survival on bendiocarb in Bela-Vista, Mozambique, an area which also registered some homozygous resistant genotypes with the altered AChE assay.

The results from Bela-Vista, Mozambique are in agreement with findings in southern Mexico. Studies with *An. albimanus* by Arredondo-Jimenez et al. (1993a) which showed complete susceptibility to bendiocarb with WHO susceptibility tests, despite evidence of an altered AChE mechanism. This suggests that the WHO discriminating dosages for carbamates are set at such a high level that they may only detect homozygous resistant mosquitoes with this mechanism. The different levels of resistance conferred by this mechanism to carbamates was indicated by (ffrench-Constant and Bonning 1989) and with OPs by (Hemingway et al., 1993). Large variation in susceptibility in bioassays was not observed in this study in contrast to Mexican *An. albimanus* where multiple resistance mechanisms were segregating. Boane registered 100% mortality for both carbamates and OPs, Bela-Vista had 100% mortality to both malathion and fenitrothion but some resistance to bendiocarb.

Evolutionary plasticity in resistance has been studied for the AChE-based resistance mechanism (Raymond et al., 1996) as well as for monooxygenase-mediated resistance (Raymond et al., 1998). Raymond et al. (1996) investigated environmental parameters responsible for resistance variation in order to understand the plasticity of response. Their conclusion was that the dominance of insecticide resistance could not be directly inferred from the expression of the acetylcholinesterase (*Ace*) gene, as the dominance level for insecticide resistance was not a fixed value, but depended on environmental parameters, a

phenomenon that has serious implications for resistance management. (Curtis et al., 1978) investigated the influence of different dominance levels on the evolution of insecticide resistance, but did not consider dominance as a variable parameter. Based on bioassay data from this study as well as those from Mexico (Penilla 2001), it may be necessary to further understand the plasticity response of resistance, the actual levels of resistance that are conferred due to different AChE mutations in the three malaria vectors in southern Africa, and the impact if any tht this will have on bendiocarb efficacy when used for indoor residual spraying, as this insecticide has now been introduced for malaria control by the programmes in South Africa and Mozambique.

The AChE-based resistance mechanism is also a potential marker for studying natural population sub-structure. Potential population substructure was observed in the Mexican study by (Penilla 2001) who detected differences in the frequency of the AChE-based resistance mechanism in *An. albimanus* specimens obtained by different collection methods. Detailed comparison of this sort were not carried out in this PhD study due to the limited time available for mosquito collections from the different countries, which did not allow for routine collections by multiple methods The Mexican study included detailed identification of the insecticide selection pressure, a vital parameter for setting up a resistance management strategy. A similar exercise was undertaken in this study by mapping the distribution of resistance mechanisms in southern Africa and by implementation of a stakeholders' forum to document pesticide usage in both the health and agricultural sectors. Gaining data from these stakeholders' fora is ongoing.

In South Africa, the presence of an altered AChE-based resistance mechanism was associated with the heavy agricultural use of insecticides. A similar situation was observed in Zambia, linked to small-scale vegetable farming and in Botswana where it occurred in areas with aerial spraying of endosulfan for tsetse control and extensive cattle farming in the Okavango delta region. *An. gambiae* s.s was easily collected in Zambia, but it was difficult to transport the live specimens back to the laboratory in South Africa without a high adult mosquito mortality, hence data for this country is reliant on a relatively small number of specimens.

5.2.2 Esterases

Biochemical assays can be used to detect general esterase activity, with most esterase enzymes having some activity with the naphthyl acetates. Esterases belong to a large family of enzymes that are able to catalyse the hydrolysis of carboxylic and phosphoric acid esters, termed carboxyesterases and phosphoric triester hydrolases, respectively (Reiner 1993; Walker 1993). In the biochemical assays, an increase in naphthyl acetate metabolism gives an indication of a quantitative change in one or more esterases in the mosquito. In this study an increase in esterase activity was detected in all three southern African vector species.

The WHO susceptibility tests with pyrethroids showed high levels of resistance to lambda cyhalothrin (in Boane and Bela-Vista, Mozambique) and high levels of resistance to permethrin in South Africa. Deltamethrin and permethrin resistance were also detected in Mozambique, but at lower levels than that for lambda cyhalothrin. Cross-resistance between DDT and deltamethrin was detected from Fiwale, Zambia. No resistance to any of the OPs used was detected in South Africa, Mozambique or Zambia. Esterases primarily confer OP resistance, as by definition they will bind OPs and they may also give pyrethroid resistance. The elevation of esterases in many OP-resistant *Culex* populations is due to gene amplification (Karunaratne et al., 1993; Jayawardena et al., 1994; Vaughan et al., 1997; Karunaratne & Hemingway 1998; Small et al., 2000). The molecular basis of esterase elevation in the southern African vectors is still unknown.

Pyrethroid insecticide resistance due to elevated esterase was detected in the late 1980s in *An. albimanus* from Guatemala (Beach et al., 1989; Brogdon and Barber 1990). (Georghiou et al., 1972) described resistance to both fenitrothion and deltamethrin in populations that were heavily selected by years of organophosphorus insecticide use on cotton crops. Some permethrin hydrolysing esterases have been characterized in the agricultural pests *H. armigera*, *H. punctigera* and *B. tabaci* (Gunning et al., 1996; 1999). Several species, including *M. domestica* and *Culex* mosquitoes have shown elevated levels of esterases which confer cross-resistance between organophosphates and pyrethroids (Soderlung et al., 1990).

The existence of mosquito populations with low frequencies of elevated esterases and altered AChE mechanism, as observed in Mozambique, South Africa, Zambia and Botswana, could eventually result in the selection of high levels of OP or pyrethroid resistance if pressure with these insecticide classes is intensified. Currently the selection pressure with both insecticide classes is relatively low. Resistant mosquitoes carrying both mechanisms were rare, also being reflected in the Mozambique data where all the OPs used gave 100% mortality in WHO bioassays.

5.2.3 Cytochrome P450 Monooxygenases

Cytochrome P^{450} monooxygenases are important in the metabolism of a wide range of xenobiotic and endogenous compounds through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, nitrogen and thioether oxidation. A large number of substrates can be metabolized by this enzymatic system due to the multiple isoforms of P^{450} that exist in each organism and the broad substrate specificity of some of these isoforms (Scott et al., 2000). Insecticide resistance often involves increases in one or more of these enzymes. Diagnosis of resistance due to increased monooxygenase activity has often been based upon the ability of the cytochrome P^{450} synergist piperonyl butoxide (PBO) to inhibit the resistance phenotype. However, this synergist interacts with both esterases and monooxygenases. Biochemical assays can now be used to indirectly estimate cytochrome P^{450} content in individual mosquitoes (Brogdon et al., 1997), giving two routes of implicating P^{450}s in resistance, although both are imperfect.

Exposure to the synergist PBO, before a WHO pyrethroid bioassay tests with mosquitoes from Boane in Mozambique indicated that resistance to pyrethroids was primarily due to monooxygenases. Synergist studies are often performed on colonised resistant strains that have been established from field-collected samples. However, *An. funestus* is a very difficult species to establish in the insectary and therefore F1 survivors from field-collected samples, which had been exposed to lambda cyhalothrin were used instead. The results of the synergist study do not give conclusive evidence for the role of the monooxygenase-base resistance, but this coupled with the high resistance observed with WHO susceptibility tests on lambda cyhalothrin and low resistance to both permethrin and deltamethrin, associated with elevated estimates of cytochrome P^{450} in the

biochemical assays, suggest the presence of the monooxygenase-based resistance mechanism in *An. funestus* in Mozambique. This was subsequently confirmed by our collaborators on the MIM programme in South Africa, who were able to show in colonised *An. funestus* that pyrethroid selection increased P^{450} levels (Brooke et al., 2002).

Monooxygenase-mediated resistance can be due to increased expression of one or more P^{450}s involved in detoxification of the insecticide. Alternatively, P^{450} elevation may result from gene amplification, or resistance may result from a change in the structural gene itself (Scott et al., 1999; Liu and Scott 1998; Seifert et al., 2002). Efforts are now on-going in *An. funestus* to isolate the specific P^{450}s involved in resistance.

Resistance in different populations may utilize the same P^{450}s or different ones. Scott et al. (2004) investigated the genetic basis of resistance in a strain of housefly (NG98) from Georgia, USA that had developed 3700-fold resistance to permethrin and compared this to other permethrin resistant housefly strains from the USA and Japan. Their conclusion was that monooxygenase-mediated resistance appears to have evolved using different P^{450}s, and possibly different regulatory signals controlling P^{450} expression, even in strains selected with the same insecticide. Work is now underway in both Liverpool and South Africa to determine exactly which of the 100 plus P^{450} genes in *An. funestus* from Mozambique are elevated. Pin-pointing these genes may allow us to monitor specifically for P^{450} genes rather than relying on the indirect assessment of monooxygenase based resistance with the biochemical assay.

5.2.4 Glutathione S- Transferases (GSTs)

GSTs are a diverse family of dimeric proteins found in almost all living organisms. They were originally studied for their role in detoxification of endogenous and xenobiotic compounds. GSTs in insects are involved in the detoxification of a number of insecticides being largely organophosphates, organochlorines and cyclodienes (Reidy et al., 1990; Clark et al., 1986; Grant and Matsumura 1989; Fournier et al., 1992). They are involved in the 0-dealkylation or O-dearylation of organophosphorus insecticides (Hayes et al., 1988; Haung et al.,1998), as a secondary mechanism in the detoxification of the active oxon-analogues of organophosphorus insecticides (Hemingway et al., 1991) and in the dehydroclorination of organochlorines (Clarke and Shamaan 1984.). GSTs confer

resistance to pyrethroids by detoxifying lipid peroxidation products induced by the pyrethroids, thereby protecting tissues from oxidative damage and/or by binding pyrethroid molecules in a sequestration mechanism, thus offering passive protection (Vontas et al., 2001).

The GST assay used in this study detects gross-changes in the pool of all GST enzymes that are able to use CDNB as a substrate. Like monooxygenase-based resistance, GST-based resistance can be difficult to interpret as different patterns of upregulated GSTs give different insecticide resistance spectra, but show similar activity profiles with general substrates like CDNB. GSTs can have a role in pyrethroid resistance along with esterases and monooxygenases. They may be producing some of the observed pyrethroid resistance in Mozambiquan *An. funestus* by reducing the oxidative damage caused by the insecticides.

5.2.5 Sodium Channel Target Site "Kdr"-Based Resistance

The kdr-type based resistance mechanism is recessive in most species. It gives resistance to pyrethroids and DDT. Lund and Narahashi (1983) showed that in insecticide intoxicated susceptible insects, the inhibited sodium channel target was left permanently open and that the opening of only a few percent of the sodium channel was sufficient to cause death of the insect. As heterozygous (RS) insects possess 50% susceptible channels, they are phenotypically similar to susceptible insects in the presence of insecticide (Lund and Narahashi 1983). If resistance was produced by the insecticide closing instead of opening the channel, the gene would be codominant to dominant, as is the case with altered AChE-based resistance mechanism. GABA-gated chloride channels are affected differently by insecticides. Clark et al. (1994) showed how avermectins leave channels permanently open, while cyclodienes leave them in a closed position. Resistance to avermectins was recessive in *M. domestica* and *L. deamlineata* (Argentine & Clark 1990; Konno & Scott 1991) but resistance to cyclodienes was codominant to dominant in *M. domestica* (Georghiou 1969) and *T. casteneum* (Beuman & Stuart 1990).

The major pyrethroid resistance detected in *An. funestus* in this study was not kdr-based. However, there was evidence of kdr in *An. gambiae* s.s from Zambia. The mutation found in the Zambian material was identical to that commonly found in West Africa, suggesting

a common origin and subsequent spread south of this resistant genotype already reported for Burkina Faso and the Ivory Coast. The detection of kdr in Zambian *Anopheles* represents the southern most extreme of this resistance mechanism detected to date and supports the hypothesis that the resistance was selected several decades ago by DDT treatment and was subsequently reselected when pyrethroids were introduced.

The origin of pesticide selection pressure has been emphasized in this study. It is of vital importance to determine the origin of the pyrethroid resistance problem in Mozambique, where agricultural activities until recently have been low due to the civil war, as compared to other countries in this region. However pyrethroid resistant *An. funestus* were still common (in this PhD, Casimiro (MSc) and Brooke et al., 2002). However, it has been noted that cotton production in Mozambique has increased substantially over the last 5 years. Cotton is one of the most insecticide intense agricultural crops and this may have contributed significantly to the pyrethroid selection pressure on *An. funestus* in Mozambique. Alternatively, resistance may have been selected in South Africa and has spread to Mozambique. Insecticide usage in South Africa is variable and primarily dependent on the type of crop production. It is not clear whether the insecticide pressure responsible for resistance in *An. funestus* in Mozambique was initially from there or from the other countries that share its borders. It is possible that selection occurred through run-off from insecticides applied for agricultural purposes in neighbouring countries, but this is unlikely as most insecticides do not survive long in water and therefore would not travel large distances. Insecticide persistence rates in different water bodies will allow calculation of the distance they are likely to be transported from the site of their original application. For example, insecticide residues have been detected in one of the national parks in the study area of KZN in South Africa an area far from any agricultural activities, where no insecticides are used regularly (in this PhD and ARC report 2003).

In sites like Shorobe, Botswana, an area far from agricultural activities, insecticide residues are present as a result of drifting of aerial sparying and cattle dipping. Pesticide contamination in national parks is likely due to animal dipping from surrounding areas, however, concentrations are likely to be lower than those needed to select for resistance, being, in the 1 – 0.01 ppm range for most insecticide treatment at the larval stage. Detailed analysis of insecticide concentrations in various water bodies in the study sites in Mozambique is needed in order to provide information on the origin of selection pressure,

a similar exercise is also needed in other southern African countries. In South Africa, this already takes place with the implementation of a cross-disciplinary process to address this issue. This Ecosystem Approach will provide information necessary to design a joint policy on agricultural pesticide usage in Mozambique, a country targeted for increased agricultural activities, with the already implemented malaria control.

Future work will involve the full implementation of the multidisciplinary approach to establishing sustainable pesticide implementation strategies through studies of the linkages between pesticide usage, vector resistance and malaria. This would first be implemented in KZN, South Africa. This approach will then provide information to design a joint policy on agricultural pesticide usage in other SADC countries, for example, Mozambique, a country targeted for increased agricultural and malarial control activities.

An altered AChE resistance mechanism was detected at different frequencies in all three main regional malaria vectors. This has serious implications for malaria control as OPs and carbamates have been targeted as replacements for DDT and pyrethroids. More work is planned to study the changes in the AChE gene in *An. arabiensis* from South Africa. In addition a detailed study of resistance mechanisms involved in observed cross-resistance between DDT/permethrin in *An. arabiensis* in KZN will be undertaken. More work is also planned to further study the frequency and distribution of the kdr-type resistance mechanism detected in Fiwale, Zambia.

Appendix 1

Notes on case data collection form

1. Case numbers: These are assigned to malaria positive patients and should be unique for purposes of uniquely identifying each individual malaria case in the database. It is used among other things to detect duplicate entries or search for a particular case in the database. Its format is also important for database purposes and a possible method is the following: Y02/10/001

Case 001 of October 2002.

Yenzi Clinic would use Y to start its case numbers; Gamba Village Hospital G. The second 2 digits are the last 2 digits of the current year, digits 4 & 5 the current month, and the last three, the number assigned to the nth case for Yenzi or Gamba, for that month in that year, each new case getting the next number with leading zeroes. The case number is also useful for cross-checking other data such as blood smear date.

1. Blood smear date: the date on which the positive blood smear was taken from the patient.
2. Referred to hospital: If a patient is referred to hospital after diagnosis at the clinic, this column should be ticked so that if the patient's information has been recorded at the clinic, it will not be re-recorded at the hospital, thus creating duplication. (The malaria information system will however allow more data to be added to that already entered at the clinic if necessary, for example, if the patient dies in hospital).
3. Age: Entered in whole years of life completed, thus any baby up to the age of 1 year exactly, is entered as 0. If age is unknown, 999 is entered.
4. Village name: Place of residence of patient: Yenzi or Gamba. This is the first part of the 2 or 3 part address that uniquely identifies all dwellings/homesteads/ houses in the 2 settlements.
5. If the former is Gamba, the Plain No. (1 – 5) must be entered here, and forms the 2nd part of the address for Gamba homes.
6. House no.: Forms the last part of the address e.g. Village: Gamba, Plain: 1, House no.: 123, uniquely identifies a home.

7. Plasmodium: Positive blood smear results are entered here, and code given at the bottom of the form.
8. Origin of infection: It is usually necessary to know whether the malaria has been contracted within one or other of the villages, or whether it has been brought in from outside e.g. Libreville. Codes may be established for recording this information.
9. Treatment: Drug used for treatment may be entered here.

Geolocation of the homesteads:
Each home in Yenzi Club and Gamba Village will have its geographic position recorded by establishing its latitude and longitude using GPS, and recording these and the address (village name, plaine number and house number) on a data collection sheet provided for the programme (see Table 34).

Village Name:		Plain No. :		Date:		Collected by :	

House No.	Household Head Name	No. of Structures	Latitude			Longitude			Population
			Deg	Min	Sec	Deg	Min	Sec	

Table 19. GPS data collection form for the geolocation of the homesteads at Yenzi club and Gamba Village. N.B. New form for a new plain or village must be used. All data sheets for one plain must be kept together.

The malaria information system will be developed in Microsoft Access, and will consist of three basic components, which interact in a manner which is transparent to the user. The system is designed to be user-friendly and does not require the user to have knowledge of the software used to its development.

1. Data entry will be performed through front-end data imput sreeens, which will be installed at each of the health facilities. These are designed to make data entry easier for the user through use of built-in lists of permitted values where possible, reducing typing and error. They are also designed to reduce as many data enty errors as possible at the point of entry, by the setting permitted ranges, the automatic entry data where possible based on other data entered, and the above lists, among others. Data entered will go into the second component, a relational database (see Figure 81).
2. The master relational database should be housed on the computer at Gamba Village Hospital, and a working database at the clinic, from where data will be transferred to the hospital and added to the master database at set intervals. This database will store both malaria case data and geographic data on all dwellings, and by its relational nature, will be able to map the former to its relavant dwelling.
3. Pre-defined required output from the system will be in the form of automated table, report, graph or map production.

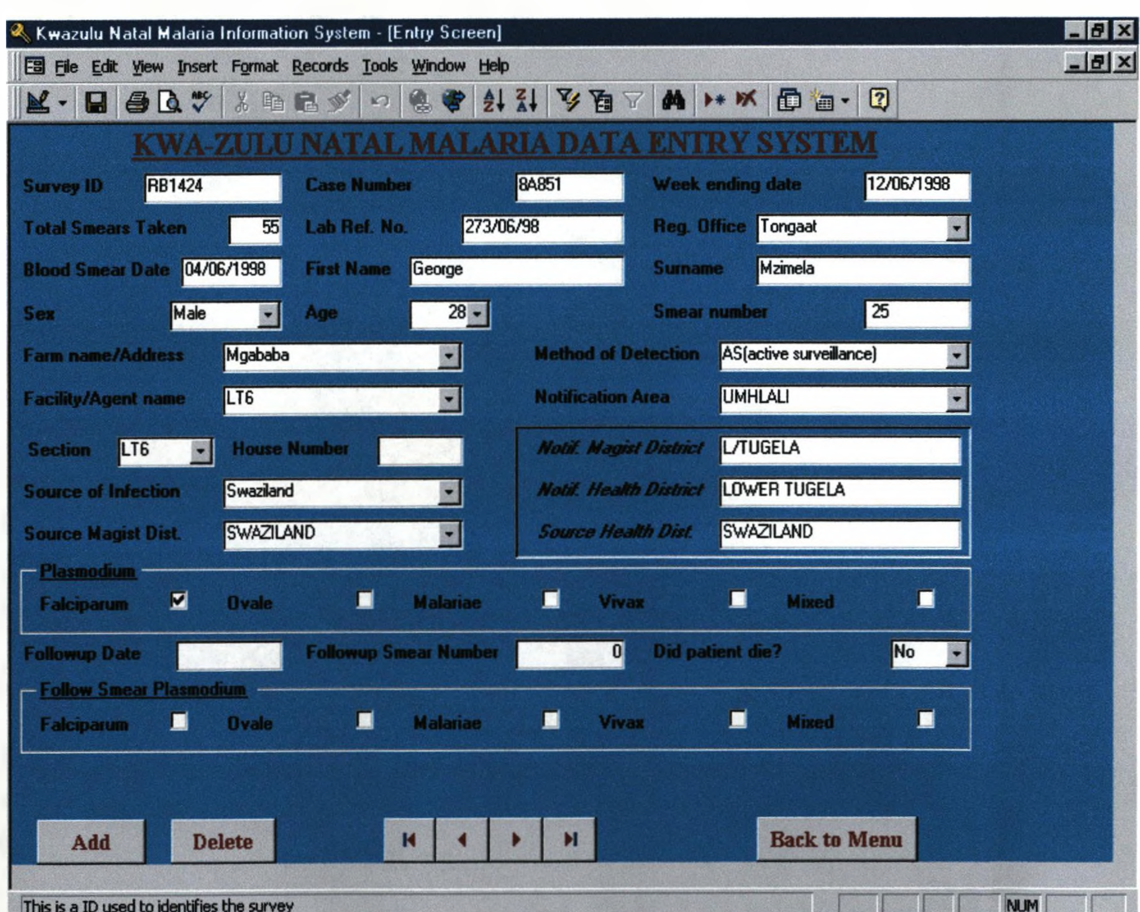


Figure 81. Malaria Sample data entry screen. This is the main form for data entry into the programme. This form is used to collect all data on one malaria case. It may be used to add new cases, update or correct existing cases and delete cases.

The above work can be done with two copies of Microsoft Office Professional. One copy of Geographic Information System (GIS) software such as HealthMapper or MapInfo for mapping the location of malaria cases. This data should always be backed up between the clinic and the hospital, an external modem for the transfer of data between the two places will be necessary and will be facilitated through existing telephone links. Below are number of other issues that need to be considered while implementing the above system.

1. Data verification: Checking and error correction on data entered must be performed. The input system traps as many errors as possible, but cannot trap all.
2. Duplication of case records must be checked for, as patient data captured at the clinic should not be recaptured at the hospital if the patient is referred there. Patient data may be updated, corrected or added to at any time using the daa entry screens.
3. If there is an e-mail connection between the clinic and the hospital, the transfer of patient data may be automated using programme code.
4. Training of users needs to take place. This may consist only of use of the imput and output components, or also include training of the doctor based in Gamba if he requires to be able to perform any ad hoc quiries on the data, for analysis that has not been requested in the system requirements specification.
5. A written requirements specification should be made available to the developer of the system. This should include required input, output (and output format) and copies of any existing data collection forms, reports, etc.
6. A user manual should be written by the developer, and copies made available to all users.
7. If relevant, GPS trainining is important to obtain the spatial distribution of malaria cases and vectors.

The report was well received by the programme managers in Gabon and as a result of this initial work fogging was stopped in Yenzi. The expatriate community were taught how to identify *Anopheles* from *Culex* and *Aedes* mosquitoes and indoor residual treatments were increased. The reduction in malaria cases and major reduction in vector control costs in Gabon subsequently prompted Shell to take on similar recommendations at their large operational bases in Nigeria. An initial visit to sample the mosquito populations in Warri, Port Harcourt and Lagos was undertaken in late 2004.

Appendix 2

MIM/TDR Insecticide Resistance Workshop

7th to 12th February 2000

The workshop was held in the library of the National Malaria Research Programme, Medical Research Council, Durban. The proceedings commenced at 8.00 am each day.

Day 1, Monday 7th

Welcome: *Dr. Brian Sharp - 5 minutes*

1. Status/objectives of the MIM/TDR insecticide resistance project:

General overview, time line and progress: *Dr. Brian Sharp - 15 minutes*

Workshop objectives: *Dr. Brian Sharp - 15 minutes*

2. Review of insecticide resistance, implications and research needs:

Prof. Janet Hemingway - 30 minutes

3. Distribution of Insecticide Resistance and implication of resistance in *An. funestus*:

Prof. Maureen Coetzee - 15 minutes

4. Training to date: *Prof. Janet Hemingway -10 minutes*

5. Discussion - *30 minutes*

10.00-10.30: Tea Break

6. Data collection and analysis to date by the seven southern African partners –

5 minutes each (40 minutes).

a. Larval collections

Blood fed adult females

b. F1's from b.

Southern African Collaborators:

Mr. Ron Masendu	Blair Research Institute, Zimbabwe
Ms. Sonia Casimiro/Mr. Nelson Cuamba	Ministry of Health, Mozambique
Mrs. Violet Siachinji	TDR Research Institute, Zambia
Mr. Mokgweetsinyana Setshwano	Malaria Control, Botswana
Mr. Richard Kamwi	Malaria Control, Namibia
Mr. Quinton Dlamini	Malaria Control, Swaziland

13.00 – 14.00 Lunch

7. Data processing techniques and susceptibility testing, theory and practical:

By Dr. Graham Small, Mr. Peter Mohloai and Mr. Basil Brooke – 2 hours

a. Bioassays

b. Data recording

Sample storage

15.00 – 15.30 Tea

8. Discussions on practical problems – *1 hour*

19.00: Dinner

Day 2, Tuesday 8th

Practicals – ***all day***

10.00- 10.30 Tea

13.00 – 14.00 Lunch

15.00 – 15.30 Tea

Day 3, Wednesday 9th

1. Report on forthcoming Burkino Faso meeting

Dr. Brian Sharp - 15 minutes

2. Development of a collaborative proposal for year 3 on surveillance and research in respect to insecticide resistance in southern Africa. *All day*

10.00- 10.30 Tea

13.00 – 14.00 Lunch

15.00 – 15.30 Tea

Day 4, Thursday 10th

1. Finalize collaborative proposal

2. Specific country work-plan

3. Practical

10.00 - 10.30 Tea

13.00 – 14.00 Lunch

15.00 – 15.30 Tea

Day 5, Friday 11th

1. Outline of training programme for 3 partner institutions and finalization of training agenda.

2. Practical and data analysis.

10.00 - 10.30 Tea

13.00 – 14.00 Lunch

15.00 – 15.30 Tea

19.00 Dinner

Institutes Training Programme 13th to 28th 2000

Biochemical and molecular techniques:

Dr. Graham Small, Mr. Peter Mohloai and Mr. Basil Brooke

Optimization of PCR for species identification

Optimization of PCR for kdr detection

Biochemical assays

Oxygenases (mfo)

Acetylcholinesterases (AChE)

Esterases

Glutathione -s- transferases (GST)

Proteins

P-Nitrophinyl acetate (p-NPA)

Data analysis and recording.

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