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SUSCEPTIBILITY AND RESISTANCE TO INSECTICIDES AMONG MALARIA VECTOR MOSQUITOES IN MOZAMBIQUE

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SUSCEPTIBILITY AND RESISTANCE TO INSECTICIDES AMONG MALARIA VECTOR MOSQUITOES IN MOZAMBIQUE

By

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ABSTRACT

Insecticide resistance in malaria vector mosquitoes reduces the efficacy of insecticide in killing and can therefore cause a major problem for malaria vector control by insecticides. In Mozambique, pyrethroid resistance in *Anopheles funestus* was first detected in December 1999 in the southern corner of Maputo Province. Since then, various collections have been made at selected sites through out the country and WHO standard susceptibility tests and biochemical assays were conducted to determine the susceptibility status and the major resistance mechanisms, in the F1 generation of field collected mosquitoes. Three malaria vector species: *Anopheles funestus* s.s., *Anopheles gambiae* s.s. and *Anopheles arabiensis* were identified in this study by Polymerase Chain Reaction (PCR) and their distributions plotted. The susceptibility data indicate that the *Anopheles funestus* s.s population in southern Mozambique is widely resistant to pyrethroid and with low levels of carbamate resistance evident at six localities. No resistance to organophosphate and DDT was observed at any study sites. Biochemical tests indicate the presence of an altered acetylcholinesterase in all collection localities with the exception of Massinga district. Elevated esterase activity with substrate α-naphthyl acetate were detect in Boane with a probable role in organophosphate resistance. Elevated GST were detected in Boane, Moamba and Catembe. Very low levels monooxygenase titres were registered in all the localities in Mozambique, which suggest that this resistance mechanism is not operating in these areas. Pyrethroid resistance in the *Anopheles gambiae* complex was detected only in *Anopheles arabiensis* from one locality. No resistant to other groups of insecticide were observed. Altered acetylcholinesterases were registered in all collection localities and in both species: *Anopheles gambiae* s.s. and *Anopheles arabiensis*. Elevated esterase with substrate α-naphthyl acetate were detected in *Anopheles arabiensis* at only one locality. Elevated GSTs were detected at all localities and in both species.

The implications of the findings for malaria vector control in Mozambique are discussed.

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I dedicate this work to memory of my father Feliciano Casimiro.

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ABREVIATIONS

WHO	World Health Organization
DDT	Dichloro-diphenyl-trichloethane
HCH	Hexachlorocyclohexane
BHC	Benzene hexachloride
An.gambiae s.s.	*Anopheles gambiae sensu strictu*
An.gambiae s.l.	*Anopheles gambiae sensu lato*
An.arabiensis	*Anopheles arabiensis*
An. merus	*Anopheles merus*
An. melas	*Anopheles melas*
An. quadriannulatus	*Anopheles quadriannulatus*
An. albimanus	*Anopheles albimanus*
An. funestus	*Anopheles funestus*
An. rivulorum	*Aopheles rivulorum*
ITNs	Insecticide treated nets
NMCP	National Malaria Control Programme
IRS	Indoor residual spraying
PCR	Polymerase Chain Reaction
MRC	Medical Research Council
SW	Salt water
FW	Fresh water
DNA	Deoxyribonucleic acid
GABA	Gamma amino butyric acid
OP	Organophosphate
GST	Glutathion S-Transferase
AChE	Acetylcholinesterase
MFO	Mixed Function Oxidase
DNTB	Dithiobis 2-nitrobenzoic acid
ASCHI	Acetylthiocholine iodide
*P*NPA	Para nitrophenyl acetate
SSCP	Single Strand Conformation Polymorphism

PREFACE

The work described in this thesis was carried out in the National Institute of Health, Mozambique and in the Medical Research Council, Durban from March 2000 to September 2002, under supervision of Professor C. Appleton. It was co-supervised by Dr. B. Sharp, Medical Research Council, Durban.

This study represents original work by the author and has not been submitted in any form to another University. Where use has been made of the work of others it is duly acknowledged in the text

SLR Casimiro

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CHAPTER 1

INSECTICIDE RESISTANCE

1.1 Introduction

Insecticide resistance in malaria vector mosquitoes reduces the efficacy of insecticides in killing and is therefore of major concern in regard to effective malaria vector control by insecticides. According to the Seventh report of the WHO (World Health Organization) Expert Committee on Insecticides "Resistance to insecticides is the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to the majority of individuals in normal population of the same species" (WHO 1957).

Chemical insecticides have been used against *Anopheles* mosquitoes in malaria control programmes for over 60 years, with varying success [Park Ross 1936 cited by (Hargreaves 2000)]. Between the two World Wars, anti-larval operations were the traditional, and almost the only malaria control measures applied on a relatively large scale in Africa. During this period, pyrethrum was used for indoor residual spraying (Kouznetsov 1977). Dichloro-diphenyl-trichloroethane (DDT) and dieldrin with their residual effects replaced pyrethrum as an indoor vector control strategy shortly after the second world war (Sharp & Le Sueur 1990). The common and widespread use of these insecticides in Africa resulted in the emergence of resistant strains of malaria vectors mosquitoes and this can lead to authorities limiting or banning their use (Coetzee *et al.* 2000a). The development of synthetic pyrethroids in the past decade has seen this insecticide class replacing DDT for house spraying in malaria control programmes. Pyrethroids are currently the only practical insecticides for impregnating bednets due to their low mammalian toxicity, a widely promoted alternative vector control strategy to house spraying (Curtis 1990).

Vector control remains a central element of most antimalarial campaigns but, in only a few countries of Africa, namely, Ethiopia, South Africa, Swaziland, Botswana, Namibia and Zimbabwe, has sustained vector control been achieved through the indoor residual spraying (IRS) (WHO 1985). In Mozambique, vector control through annual IRS with synthetic pyrethroids, is the major strategy used for malaria control.

The efficacy of chemical control measures depends on a number of factors: the vector species; the pesticide in use and particularly the degree to which the local vector species have acquired resistance to it; the method of application of the insecticide; the type of formulation; the nature of the surface to be treated; the management of the control programme; the thoroughness of application of the control measure and the climatic conditions (WHO 1997).

One of the major problem that has resulted from residual spraying in some countries has seen the evolution of vector resistant to DDT and other chemicals (WHO 1986). Resistance to organochlorine was first observed in *Aedes* mosquitoes in the late 1940s (WHO 1997). In 1946, WHO reports indicated only two species of *Anopheles* resistant to DDT (WHO 1992) but by 1986, 106 species of mosquitoes worldwide were known to have developed resistance to organochlorine insecticides, 38 species had developed resistance to organophosphorus insecticides and 17 species were resistance to carbamates (WHO 1997)

In Africa, the first case of insecticide resistance, involving *Anopheles gambiae s.s.* was observed in 1967 in Bobo Dioulasso (Burkina Faso) and attributed to the use of DDT against cotton pests (Chandre *et al.* 1999). Pyrethroid resistance in *An.gambiae* s.s. has been documented from both West and East Africa (Elissa *et al.* 1993; Vulule *et al.* 1994, 1996; Darriet *et al.* 1997; Martinez-Torres *et al.* 1998). The primary resistance mechanism in these populations is of the *Kdr*-type, which gives broad-spectrum resistance to all pyrethroids and DDT.

A multiple resistance pattern covering organochlorines, organophosphates and carbamates is rare but has been described in some populations of *Anopheles albimanus*, *Anopheles antroparvus* and *Anopheles sacharovi*. This is generally due to the widespread use of various insecticides for agricultural pests in neighbouring areas and severe contamination of anopheline breeding places (Onori *et al.* 1993).

In Southern Africa, the first case of pyrethroid resistant in *Anopheles funestus*, was reported in 1999 from the Kwazulu-Natal province of South Africa (Hargreaves *el al.* 2000).

Recently, Brooke *et al.* (2001) found evidence of resistance to pyrethroids and the carbamate propoxur, in *An. funestus* s.s. populations from the Beluluane region of southern Mozambique.

More recently, altered acetycholinesterases and elevated monooxygenase attributed to organophosphates and pyrethroids respectively have been detected in *Anopheles arabiensis* at Mamfene, an agricultural area in South Africa (P Mohloai, 2000, per. Comm.[1]).

1.2 Insecticides classification and modes of action

Insecticides are chemicals, which are used to control insect pests. There are several classifications of insecticides, however, for public health insect control the insecticides can be divided into four major groups: Organochlorines, organophosphates, carbamates and pyrethroids (WHO 1997).

1. 2.1 Organochlorines

There are four groups of organochlorines: Diphenyl aliphatics, HCH (Hexachlorocyclohexane), cyclodienes and polychloroterpenes.

1.2.1.1 Diphenyl aliphatics

This is the oldest group of the organochlorines. In this group the insecticide used in public health is DDT which was originally synthesized in 1854. The first field trials of DDT took place during the Second World War (Onori *et al.* 1993). DDT is probably the best known and most notorious chemical of the 20th century. The mode of action for DDT has never been clearly established (Ware 2002). The target site of DDT are the Na^+ channel proteins (Sawicki 1987).

1.2.1.2 Cyclodienes

This group of organochlorines appeared after World War II: In this group dieldrin was the insecticide employed in public health. Most of the cyclodienes are persistent insecticides and are stable in soil and relatively stable to the ultraviolet of sunlight. Unlike DDT and HCH, the cyclodienes have a positive temperature correlation-their toxicity increases with increases in the surrounding temperature. The modes of action are also not clearly understood (Ware 2002). The target sit of cyclodienes is altered gamma amino butyric (GABA) (Ffrench-Constant *et al.* 1991).

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[1] Mr P Mohloai, Medical Research Council, P.O. Box 70380, Overport, Durban.

1.2.1.3. Polychloroterpenes

Only two polychloroterpenes were developed: toxaphene in 1947, and strobane in 1951. Toxaphene had by far the greatest use of any single insecticide in agriculture while strobane was relatively insignificant. Toxaphene is rather easily metabolised by mammals and birds, and is not stored in body fat nearly to the extent of DDT, HCH and cyclodienes. Toxaphene and strobane act on the neurons, causing an imbalance in sodium and potassium ions, similar to that of the cyclodiene insecticides (Ware 2002).

1.2.2. Organophosphates

This group of insecticides, widely used in agriculture is now commonly employed in public health, mainly because several insect vectors of disease have become resistant to chlorinated hydrocarbons. Their general mode of action is due to the inhibition of the enzyme cholinesterase in arthropods as well as in mammals (Onori *et al.* 1993).

The OP (organophosphates) are generally divided into three groups: Aliphatic, phenyl and heterocyclic derivates. The most common OP belonging to the aliphatic group used in public health is Malathion which is a wide-spectrum insecticide that was introduced in 1950 (Cecchine *et al.* 2000). The phenyl OPs are generally more stable than the aliphatics, thus their residues are longer lasting. Fenitrothion is one of the most common phenyl used in public health (Ware 2002).

1.2.3 Carbamates

Carbamates were originally extracted from the Calabar bean, which grows in West Africa. The extracts of this bean contain physostigmine, a methylcarbamate ester (Baron 1991). Carbamates are derivatives of carbamic acid, as organophosphates are derivatives of phosphoric acid. Like the organophosphates, carbamates as a class are not generally persistent in the environment. Their mode of action is that of inhibiting the vital enzyme cholinesterase.

The first successful carbamate insecticide, carbaryl (Sevin ®), was introduced in 1956.
In insects the effects of organophosphates and carbamates are primarily those of poisoning of the central nervous system, since the insect neuromuscular junction is not cholinergic, as mammals.

Two distinct qualities have made carbamate popular: Its very low mammalian oral and dermal toxicity and an exceptionally broad spectrum of insect control (Ware 2002).

Propoxur (Baygon ®) and Bendiocarb are the carbamates most commonly used in public health.

1.2.4. Pyrethroids

The pyrethroid insecticides are typically esters of chrysanthemic acid having a high degree of lipophilicity (fat solubility). The original compounds were the natural pyrethrins, which are isolated from the flowers of *Chrysanthemum* (Blonquist 2002).

The pyrethroids have an interesting evolution, which divided into four generations: The insecticide used in public health belonging to the last generation, include: lambdacyhalothrin (Karate®), cypermethrin (Cymbush®), cyfluthrin (Baythroid®), deltamethrin (K-Othrin®), All of these are photostable (Ware 2002).

Pyrethrins and synthetic pyrethroids insecticides are nerve poisons, which act by contact with larval and adult mosquitoes. Most of the pyrethrins are unstable being quickly oxidized in the presence of air and generally in the presence of water. They are generally of low toxicity to human beings (WHO 1997). The target site of this group of insectice are the Na+ channel proteins (Martinez-Torres *et al.* 1998).

1.3 History of malaria control in Mozambique

1.3.1 Indoor residual spraying

In Mozambique, malaria control was initiated in 1946 with IRS using DDT and partially BHC (Benzene hexachloride) in the southern part of the country in the semi-urban area of Maputo city and in the rural area of Limpopo Valley (Soeiro1956). Prior to the introduction of malaria control in southern Mozambique, between 1937 a nd 1938 an overall parasite and spleen rate of 92.1% and 65.3% was recorded in children one to five years old (Ferreira 1958). The main malaria vectors *An. gambiae s.l.* and *An. funestus* were also widespread and found in high densities indoors throughout the malarious areas.

In Maputo following the introduction of malaria control by house spraying with DDT malaria admissions dropped dramatically. From 733 cases in 1944 to 328 after 1946 to a low of 214 and 94 in 1952 and 1954, respectively, following the extension of residual spraying in 1950 (Figure 1). In the Limpopo Valley after the introduction of malaria control (IRS) parasite and spleen rates in children under a year old declined from 62.7% and 59.4% respectively in 1953 to 23.6% and 21% in 1954 and to 17% and 1% in 1955 (Figure 2).

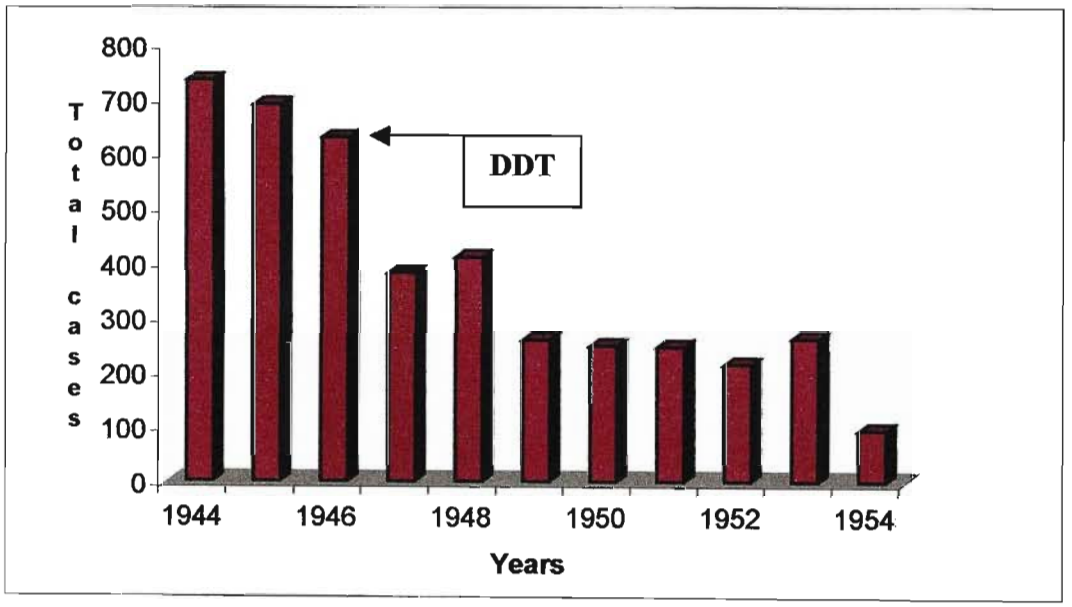

Figure 1. Annual totals of clinical malaria cases admitted to the Maputo Central Hospital, for the period 1944-1954 (Soeiro 1956).

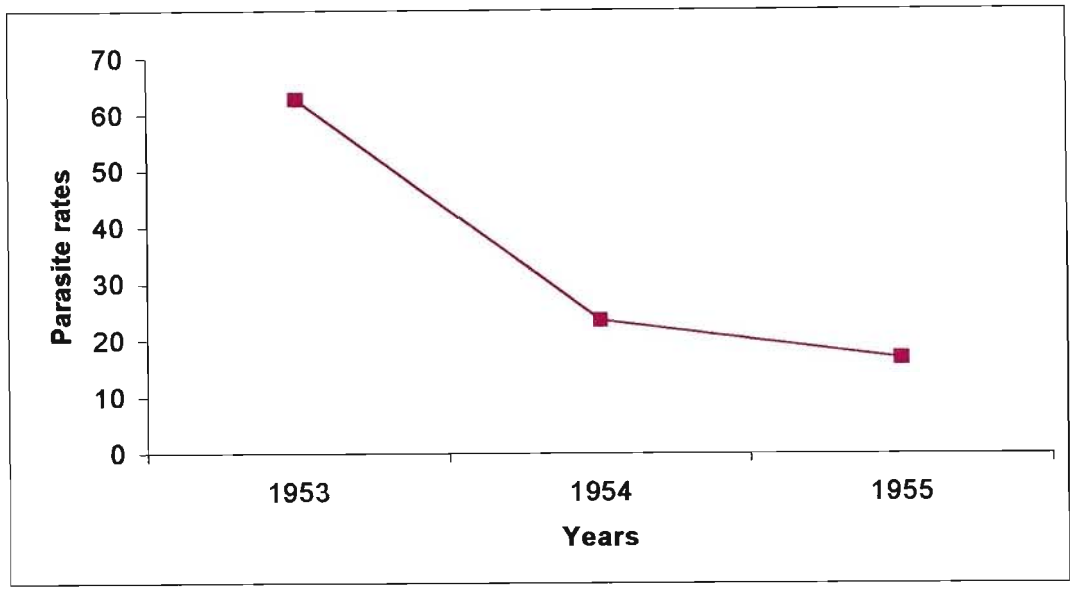

Figure 2. Malaria infection in children 0-1 year old for the period 1953-1955 in the Limpopo Valley, Southern Mozambique (Soeiro 1956).

The introduction of residual insecticides also had a marked impact on the densities of indoor resting mosquitoes of *An. gambiae s.l.* and *An. funestus* in sprayed houses (Soeiro 1956). Vector control efforts were abandoned in 1956, however between 1946-1956 good control was achieved with overall parasite ratios being kept consistently below 10% for the ten year period (Soeiro 1956).

In 1960 following an agreement between the government and the World Health Organization (WHO), a malaria eradication pilot project was initiated in the southern parts of Mozambique. The objective was to determine whether malaria transmission could be interrupted by applying DDT house spraying combined with surveillance and therapeutic measures. Although malaria transmission was never interrupted, dramatic reductions in malaria prevalence were achieved between 1961 and 1971, but these were mainly in the southern parts of the country where malaria control activities had been carried out since 1946 (Schwalbach & De La Maza 1985; Martinenko 1992), Figure 3. Failure to interrupt transmission was attributed mainly to population movements from other parts of the country, which made it impossible to prevent constant renewal of the reservoir of infection.

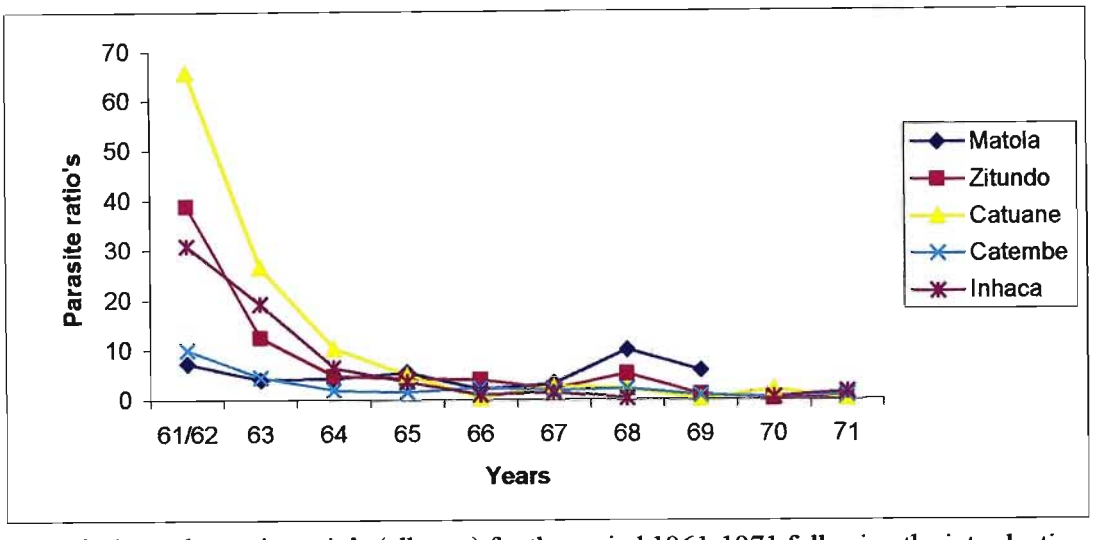

Figure 3. Annual parasite ratio's (all ages) for the period 1961-1971 following the introduction of the malaria eradication experiment in 1960 in southern Mozambique (Schwalbach & De La Maza 1985).

The escalation of civil war in the late 1970's led to a complete breakdown of control measures. As a result, in the mid 1970s, after the eradication campaign, house spraying was confined to regions of the Limpopo Valley, Maputo City, Quelimane and Nampula and by the 1980s control operations were restricted to the Maputo area (Martinenko *et al.* 1989).

During the 1990s some alternative insecticides were tested for IRS and insecticide treated nets (ITNs) in Mozambique, to assess product efficacy and impact, safety and other factors under local circumstances. Large-scale operational IRS field trials of two pyrethroids: lambdacyhalothrin 10% wettable powder, trademark Icon® 10 WP (Cuamba & Dambo 1994; Franco 1994) and cyfluthrin 10% wettable powder, trademark Baythroid® 10 WP (Martinenko *et al* 1995), were evaluated entomologically and epidemiologically for malaria control by the Instituto Nacional de Saúde in conjunction with the Programa Nacional do Controlo da Malária. In 2000 the LSDI (Lubombo Spatial Development Initiative) Malaria Control Programme, a regional initiative between South Africa, Swaziland and Mozambique aimed at protecting communities from malaria and to create a suitable environment for economic development and promotion of eco-tourism in the area. The programme introduced IRS in Maputo province using carbamates (Propoxur and Bendiocarb) due to

the discovery of pyrethroid resistance in *An. funestus* populations in this area (B L Sharp, 2000, pers.comm.[2]).

1.3.2 Insecticides treated nets

ITNs are not extensively used in Mozambique. The first pilot project of ITNs was carried out in Boane district between 1994 and 1998. Recently two community based malaria prevention and control initiatives have been successfully initiated in Zambézia and Gaza provinces.

The Zambézia initiative was officially launched in May 2000 and to date more than 100,000 ITNs have been sold and about 100,000 people have participated in the community capacity development activities (Ministério da Saúde 2001). The Gaza initiative was developed in response to a flooding emergency caused by two devastating cyclones, which affected southern Mozambique during the first quarter of 2000. As part of the rehabilitation efforts for flood-affected families, the Ministry of Health and UNICEF and several non-governmental organization distributed 200,000 ITNs to flood-affected families in conjunction with community capacity development/education. The population protected is estimated to be 575,670 (Ministério da Saúde, 2001a).

A malaria knowledgement attitute practice (KAP) surveys carried out before the implementation of ITNs in Gaza, indicates that in rural areas, knowledge of the role of mosquitoes in malaria transmission was less than 30%. Ten months after the distribution, participatory malaria sessions were repeated, nets were retreated and a KAP survey was carried out in the communities that received nets and almost 100% of interviewees stated that they knew of the existence of malaria, 91% of respondents cited mosquitoes as a means of transmission, compared with only 30% in the baseline pre-intervention study in rural Gaza (Ministério da Saúde 2001a). It is envisaged that ITNs will become one the major strategies for malaria control by the NMCP (National Malaria Control Programme) in places where IRS is not applicable.

[2] Dr BL Sharp, Medical Research Council, P.O. Box 70380, Overport, Durban.

1.3.3 Chemoprophylaxis and chemotherapy

Following independence, a campaign of chloroquine chemoprophylaxis was instituted throughout the country with the objective of reducing the morbidity of malaria. Chloroquine was distributed to school children and villages once a week at a dosage of five mg/Kg. The targeted population coverage was not reached because of logistical problems. In 1983, resistance of *Plasmodium falciparum* to chloroquine was detected and the chemoprophylaxis campaign was stopped (Martinenko 1992). By 1999, resistance of *Plasmodium falciparum* to anti-malarial drugs, especially the first-line treatment for non-complicated malaria, chloroquine, varied between 15% and 40% depending on location (Ministério da Saúde 2001).

Drug resistance presents a major obstacle to effective case management, particularly at the periphery where capacity for clinical and laboratory diagnosis is weak. Currently there is ongoing work to monitor the extent of this problem (Ministério da Saúde 2001).

1.3.4 Current malaria control efforts

Initially malaria control was concentrated in Maputo and Limpopo valley and was gradually extended to other parts of southern Mozambique following the initiation of the malaria eradication pilot projects. The NMCP was only established in 1982 but initially only had limited coverage due to the internal strife of the consequent disrupted health services. With the gradual revival of health services in the early 1990s malaria control was directed towards accurate diagnosis and prompt treatment of malaria cases combined with vector control.

Due to a scarcity of resources malaria vector control measures have been limited to spraying in suburban areas of the majority of the provincial capitals mostly with lambda-cyhalothrin and in limited areas with deltamethrin (Barreto 1996). For the first time since the eradication experiments, house spraying has been introduced in rural areas by the malaria control programme of the LSDI covering three districts of Maputo province (Namaacha, Matutuine and Boane) and will expand in 2002 to a further two districts (Marracuene and Moamba) an area of approximately 12500 square kilometres.

1.4 Motivations and aims of this study

In Mozambique, malaria is the primary cause of ill health, accounting for approximately 40% of all outpatient consultations. Up to 60% of in-patients in pediatric wards are admitted as a result of severe malaria illness. Malaria is also the leading cause of death in hospitals in Mozambique, accounting for almost 30% of all deaths recorded. Estimated prevalence in the two to nine years age group ranges from 40% to 80%, with as many as 90% of children under five years of age infected with malaria parasites in some areas. Malaria is also a major problem affecting pregnant woman in the rural areas. Approximately 20% of women are parasitaemic (MISAU 2001a)

Because of the high intensity of transmission and the inadequate coverage of the public health system, the NMCP has relied on IRHS with lambda-cyhalothrin in provincial capitals and some districts as one of the main preventive measures against malaria. DDT was the first insecticide used for house spraying in 1946 and was subsequently used for residual house spraying for many years, especially in the Southern areas of Mozambique. During this period susceptibility tests revealed no resistance to DDT. Since 1993, lambda-cyhalothrin has been used for residual house spraying, but there is a general lack of baseline information on the susceptibility levels of mosquitoes to this insecticide. Further, the use of a range of insecticides for agricultural activities has increased recently in rural farming areas, which will result in increased exposure of mosquito population to these insecticides. This in turn, may select for resistance in mosquitoes, which would comprise vector control.

In countries like Mozambique, where IRHS is the main method used to control malaria vectors, it is essential to do susceptibility tests, to detect any reductions in susceptibility levels and the emergence of resistance sufficient to cause a control failure.

The aims of this study were to evaluate the susceptibility of malaria vectors in Mozambique to the insecticides available to vector control and to determine the possible mechanisms of resistance involved. Standard susceptibility techniques combined with biochemical and molecular based techniques were employed in this study.

1.5 Materials and methods

1.5.1 Description of study area

Mozambique is situated in Southern Africa between latitude 10°27' and 26°53'South and longitude 30°12" and 40°51'East, bordering to the north with Tanzania, to the west with Malawi, Zambia, Zimbabwe and Swaziland and to the south with South Africa. To the east is the Indian Ocean, extending for 2,470 Km. The land area covers 799,380 Km^2.

The country presents three types of relief, from the coast to the interior:

1. The coastal plain that occupies 40% of the country and where the majority of the population is concentrated.
2. The plains with varying altitudes between 200 and sea level.
3. The plateau and mountainous area occupying a relatively small part of the national territory, with altitudes above 1000 metres.

The study was carried out in nine provinces in three regions of Mozambique: The Southern, Central and Northern areas. The mosquitoes collection sites were selected based on high-risk malaria provinces (case notification), agricultural activities and areas with a history of IRHS. Geographic Positioning System points were taken at all collection sites. Where this was not possible geo-referencing of localities were undertake for later cross-referencing.

The study areas are represented in Figure 4. The actual malaria vector control programmes at these localities are outlined in Appendix 1.

Southern region

In this region mosquitoes were collected from four province (18 localities): Inhambane (Homoine and Massinga); Gaza (Chokwe and Manjacaze); Maputo (Manhiça, Marracuene, Moamba, Ressano Garcia, Boane- Bairro 2, Boane – Bairro Manguisa, Boane- Bairro Jimo, Namaacha, Catuane, Bela-Vista and Catembe) and Maputo City (Polana Caniço and Benfica). The climate in this region is characterized by a hot rainy season normally from November to April, and a cool dry season from May to October. The annual rainfall is 400-1000 mm and mean temperature is 22-24 °C.

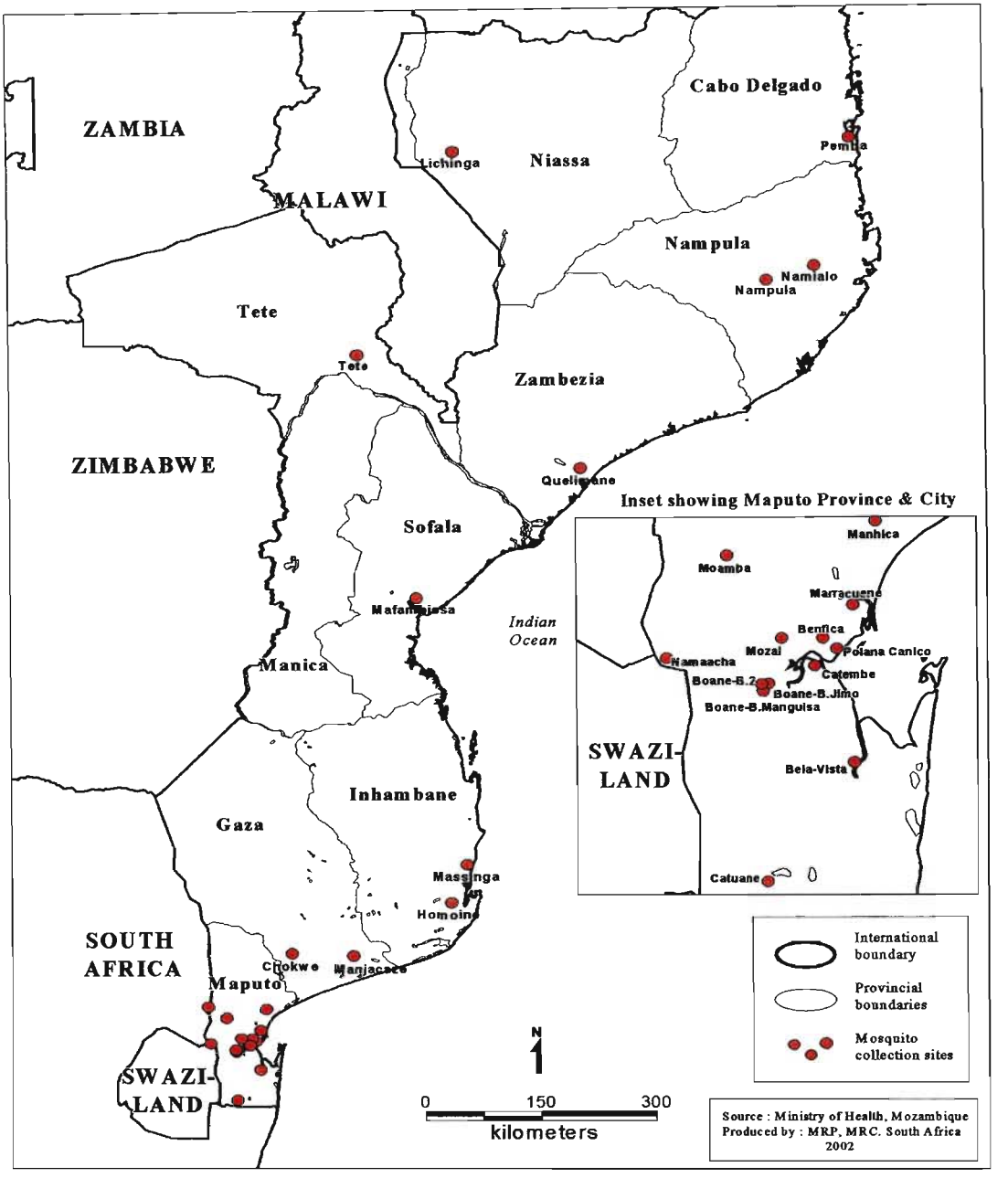

Figure 4. *Anopheles gambiae* s.l. and *Anopheles funestus* s.l. collection sites in Mozambique: 2000-2002.

Vegetation is typically savannah with isolated tropical forest in the southern corner of Maputo province. The altitude is normally less than 100 m with exception of the Lubombo mountains along the border with South Africa and Swaziland, which vary between 500-1000m (Schwalbach & De La Maza 1985).

Central Region

In this region samples were collected from three provinces (three localities): Zambézia (Quelimane), Tete (Tete) and Sofala (Dondo). The mean annual temperature is between 24-26 °C, with the exception of western Manica province and northern Tete Province where the mean annual temperature is 18-20 °C and the annual rainfall is 800-1800 mm.

The climate is wet tropical, modified by altitude. The vegetation is predominally African savannah with tropical forest, alluvial grasslands plus mountain forest on the high plain of Manica (Schwalbach & De La Maza 1985).

North Region

In this region samples were collected from three province (four localities): Nampula (Namialo and Nampula City), Cabo-Delgado (Pemba) and Niassa (Lichinga).
The average annual rainfall is 800-1.800 mm and the mean temperature is 22-26°C, with the exception of Niassa province where the annual mean temperature is between 18-20°C.
The climate of this region is wet tropical with the exception of central Niassa province where the climate is dry tropical. The vegetation is predominally typical forest (Schwalbach &De La Maza 1985).

1.5.2 Mosquito collections

Mosquitoes were collected in houses early in the morning (6.00-10.00H), using an aspirator and torch, during the period March 2000 to July 2002 (Appendix 2). Adult mosquitoes caught in the field (Maputo province and Maputo City) were transported to the laboratory of the National Institute of Health in Maputo in a cooler box covered by a wet towel and were then placed in breeding tubes. An icepack was placed in the cooler box on hot days to prevent death of mosquitoes

from heatstroke during transportation. Adults mosquitoes caught in the other areas, situated far from the laboratory were placed individually in breeding tubes for egg laying and then transported to the laboratory in Maputo. Those that laid eggs were coded, then identified using morphological keys (Gillies & De Meillon 1968; Gillies & Coetzee 1987) and stored in the freezer in Eppendorf tubes and transferred to the MRC (Medical Research Council) Durban, where species identification by PCR (Polymerase Chain Reaction) were undertaken. Those belonging to the *An. funestus* were subjected to PCR-based SSCP (single strand conformation polymorphism) (Koekemoer *et al.* 1999) and those belonging to the *An.gambiae* complex were subject to the polymerase chain reaction of rDNA following the procedures of Scott *et al.* (1993) (Chapter 3).

CHAPTER 2

SPECIES IDENTIFICATION AND DISTRIBUTION OF THE *ANOPHELES GAMBIAE* COMPLEX AND *ANOPHELES FUNESTUS* GROUP IN MOZAMBIQUE

2.1 Introduction

2.1.1 The *Anopheles gambiae* complex

The primary vectors of malaria in Africa and its offshore islands are mosquitoes of the *An. gambiae* Giles complex (Gilles & De Meillon 1968; White 1974).

The sequence of events leading to discovery of the complex *Anopheles gambiae* in the early 1960's have been well described (Paterson 1964 & 1968). A vast amount of research had shown it to be a complex of at least six morphologically indistinguishable species showing pronounced ecological and behaviour diversity (Ribbands 1944; Muirhead Thomson 1948; Service 1993). Three of these sibling species are adapted to fresh-water breeding sites: *An. gambiae* s.s, *Anophleles arabiensis* and *Anopheles quadriannulatus*; two are salt-water breeding: *Anopheles merus* and *Anopheles melas* and one to mineral water: *Anopheles bwambae* (Davidson 1977; White 1974; Gilles & Coetzee 1987; Service 1993).

Anopheles gambiae s.s. and *An. arabiensis* (White 1974), separately or together, constitute the mass of familiar *gambiae* s.l. population in and around continental Africa. *Anopheles gambiae* s.s. is an efficient vector of malaria and filariasis in Africa. Females of this species show a high degree of anthropophily (White 1974). Recently the situation in regards to *An.gambiae* s.s. has become more complicated. In West Africa this species is now split into five different units designated with a non-Linnean nomenclature, as chromosomal forms Bamako, Bissau, Forest, Mopti and Savanna (Touré *et al.* 1983; Coluzzi *et al.* 1985).

Anopheles arabiensis is the primary vector of malaria in many parts of Africa. This species was classified as endophagic with partial or complete endophily (White 1974). However Sharp & Le Sueur (1990) show this species to have mixed feeding and resting behaviour, biting and resting both indoor/outdoor and feeding man/bovine.

In, general *An. gambiae* s.s. predominates in humid situations, whereas *An. arabiensis* is relatively successful in arid zones.

Anopheles quadriannulatus is less widespread in its distribution and has been found in three widely separated areas: Zanzibar (Odetoyinbo & Davidson 1968 [cited by White 1974]), Ethiopia (Turner 1972 [cited by White 1974]), and extensively in southern Africa (Paterson 1963). In Zanzibar and Southern Africa, *An. quadriannulatus* is markedly exophilic while it tends to be endophilic at high altitudes in Ethiopia (White 1974; Coluzzi *et a.* 1979). This species feeds principally on animals other than man (Mahon *et al.* 1976). Recently Hunt *et al.* (1998) found that the Ethiopian population of *An. qudriannulatus* is a different species and designated it as *An. qudriannulatus* B.

Anopheles merus is confined to the East coast of Africa, adjacent inland areas, coastal islands and at inland localities in association with salt pans (Muirhead-Thomson 1951; Gilles & Meillon 1968; White 1974; Gilles & Coetzee 1987; Mosha & Petrarca 1983; Sharp 1983; Le Seur & Sharp 1988; Sharp 1990; Service 1993). This species is regularly zoophagic (White 1974) and has been shown to be involved in low rate malaria transmission (Muirhead-Thomson 1951) and efficient filariasis transmission in Tanzania (Bushrod 1981) and Kenya (Mosha & Petrarca 1983). *Anopheles merus* plays an unexpectedly important role in malaria transmission in coastal Tanzania (Temu *el al.* 1998).

Anopheles melas is a malaria vector in West Africa (White 1974; Davidson 1977; Gilles & Coetzee 1987; Service 1993); this species is the sole member of the complex known to feed readily on goats and sheep (White 1974). Muirhead-Thomson (1948) considered that they probably do not discriminate between man, cow, pig and goat.

Anopheles bwambae is known only from the Semliki forest area of the Uganda/Zaire border, where breeding is apparently confined to mineral water swamps, vegetated principally with *Cyperus laevigatus* sedge, and formed by geothermal activity in the Rift valley (White 1974). This species is a local vector of malaria and filariasis in the Bwamba County (White 1985).

In southern Africa four members of the *An. gambiae* complex occur: *An. gambiae* s.s., *An. arabiensis*, *An. quadriannulatus* and *An. merus* (Shelley 1973; Petrarca *et al.* 1984; Sharp *et al.*

1987; Le Sueur & Sharp 1988; Sharp & Le Sueur 1990; Paskewitz *et al.* 1993; Coetzee *et al.* 2000).

2.1.2 The *Anopheles funestus* group

Anopheles funestus Giles has been shown to be an important malaria vector, in some cases playing a more important role than *An. gambiae* Gilles and *An. arabiensis* Patton (Fontenille *et al.* 1997).

The *Anopheles funestus* Giles is comprised of at least nine members: *An. funestus*, *Anopheles vaneedeni* Gillies & Coetzee, *Anopheles parensis* Gillies, *Anopheles aruni* Sobti, *Anopheles confusus* Evans & Lesson, *Anopheles rivulorum* Lesson, *Anopheles fuscivenosus* Leeson, *Anopheles lessoni* Evans, and *Anopheles brucei* Service (Gillies & Coetzee 1987; Koekemoer *et al.* 1999; Hargraves *et al.* 2000; Brook *et al.* 2001; Kamau *et al.* 2002). Only two species within this group are implicated in malaria trasmission: *An. funestus* and *An. rivulorum*. The later has been implicated as a minor vector in Tanzania (Wilkes *et al.* 1996).

Within this group, *An. funestus* is the most abundant and widespread in southern Africa, and is highly endophilic, endophagic and anthropophilic. The other species are typically more limited in density and distribution, and mainly bite animals outdoors (Bruce-Chwatt 1954; Hackett *et al.* 2000). However they avidly bite humans outdoors in the absence of other hosts (Gillies & De Meillon 1968). *Anopheles rivulorum* is the second most abundant and widespread species in the *funestus* group (Hackett *et al.* 2000).

2.2 Distribution of the *Anopheles gambiae* complex and *Anopheles funestus* group of species in Mozambique.

Little is known about the distribution of malaria vectors and their behavioural status as it relates to malaria transmission in Mozambique. However a survey by Petrarca *et al.* (1984) showed that four species of the *An. gambiae* complex occur in Mozambique: *An. gambiae* s.s., *An. arabiensis*, *An. merus* and *An. quadriannulatus*. *An. gambiae* s.s. was shown to occur in the central-northern regions (north of the Save river) from the coast to the western mountains. On the coast its distribution is often sympatric with that of *An. merus*.

Anopheles arabiensis is the most widely distributed species of the *An.gambiae* complex within the country, while *An. merus* is confined to the coastal regions as well as in inner areas where the rivers are tidal and brackish and/or the soil is saline and *An. quadriannulatus* was only found in a southern locality, Bela-Vista-Maputo area (Petrarca *et al.* 1984).

Published distribution records of the *An.gambiae* complex species in Mozambique are presented in Appendix 3.

De Meillon (1941) showed that the *An. funestus* group is widely distributed within the country. Recently Mendis *et al.* (2000) demonstrated that *An. arabiensis* and *An. funestus* are equally important vectors of malaria in Matola, a coastal suburb of Maputo, southern Mozambique.

2.3 Species identification

2.3.1 Species identification of the *Anopheles gambiae* s.l.

Since the recognition of the complex in 1962, precise identification of each species has been carried out using various methods, including morphological characteristics for primary identification of members of the *An.gambiae* complex, but they remain of limited value (Coluzzi 1964; Davidson *et al.* 1967; Gillies & Coetzee 1987).

There are two morphological characters of SW (saltwater) species, which are useful in separating them from the FW (freshwater) species. The eggs of both *An. melas* and *An. merus* are characterized by being longer and the deck opening on the dorsal surface broader than those of FW *Anopheles gambiae* s.l. This characteristic was used with success by Muirhead-Thomson (1945, 1948).

Muirhead-Thomson (1951) introduced a physiological method for identification of *An. merus* and *An. melas*. This method is based on an observed differential response to saline waters, which distinguishes the first stage larvae of the three freshwater-breeding, from those of the saltwater-breeding forms. Sharp (1983) using colonized *An. merus*, *An. arabiensis* and *An. gambiae* s.s. extended this test in the laboratory to include all instars.

Crossbreeding is a technique that has been used with much success in elucidating cryptic species. Here species identification is based on hybrid sterility of interspecific hybrids (Paterson 1964 [cited by Sharp 1990]). This technique is scarcely practical for the routine identification of field samples and in this role it has largely been superceded by genetic (Coluzzi 1968; Coluzzi & Sabattin 1967; Green 1972; Hunt 1973), electrophoretic (Mahon *et al.* 1976) and PCR based techniques (Collins *et al.* 1988; Scott *et al.* 1993).

Carlson & Service (1979) investigated the possibility of identifying adults of both sexes of *An. gambiae* and *An. arabiensis* by extracting and analyzing their cuticular hydrocarbons. The preliminary results of this study merit more detailed appraisal of these non-volatile and chemically inert cuticular hydrocarbons for the separation of *An. gambiae* and *An. arabiensis* and other species within the gambiae complex.

Bushrod (1981) successfully separated *An. merus* from the FW species of the *An. gambiae* complex (*An. gambiae* s.s. and *an. arabiensis*) in Tanzania by plotting the number of coeloconic sensilla against the palpal ratio.

Coetzee *et al.* (1982) and Coetzee (1986) showed that *An. gambiae* and *An. arabiensis* could be distinguished from *An. merus* /*An. quadriannulatus* by the width of the pale band at apex of hind tarsus three and base of hind tarsus four. Sharp *et al.* (1989) evaluated the effectiveness of this method of identification of *An. gambiae* s.l. species in Natal and the pooling of *An. arabiensis* data from this study resulted in only 56% correct identification. This result seriously detracts from the use of leg banding for the separation of the *An. gambiae* complex members in the Kwazulu-Natal region.

More recently, molecular methods have been devised which use differences at the DNA (deoxyribonucleic acid) level to distinguish species by the Polymerase chain reation (PCR) (Paskwitz & Collins 1990; Scott *et al.* 1993; Bredenkamp & Sharp 1993; Flavia *et al.* 1997; Fettene *et al.* 2002; Fanello *et a*l. 2002).

The PCR assay developed by Paskwitz & Collins (1990) and Scott *et al.* (1993) is based on species-specific fixed differences in the ribosomal DNA (rDNA) region, which includes part of the 28S coding region and part of the intergenic spacer (IGS) region. The method uses a universal (UN)

primer that anneals to a sequence shared by all members of the complex, in combination with specific reverse primers AR, GA, QD and MR that bind to unique sequences of each sibling species, respectively, *An. arabiensis*, *An. gambiae* s.s., *An. quadriannulatus* and *An. merus* or *An. melas*.

Flavia *et al.* (1997) developed new primers to identify the Mopti (M) and Savanna (S) forms within *An. gambiae* s.s. Therefore, the identification of each *An. gambiae* s.s. specimen to the level of molecular form with this technique requires two PCR reaction followed by an enzyme digestion, or only two PCR reactions.

Fettene *et al.* (2002) developed a PCR to distinguish between species A and B of *An. quadriannulatus* s.l. as well other member of the *An. gambiae* complex.

Fanello *et al.* (2002) propose a new method for differential identification of sibling species in the *Anopheles gambiae* Giles complex (Diptera: Culicidae), including simultaneous separation of M and S forms within *An. gambiae* s.s.. This method is a combination of the protocols established by Scott *et al.* (1993) and Flavia *et al.* (1997).

2.3.2 Species identification of the *Anopheles funestus* group

Differentiation of species comprising the *funestus* group is difficult using traditional taxonomic measures (Hackett *et al.* 2000). However four members of this group: *An. brucei* Service, *An. confusus* Evans & Lesson, *An. lessoni* Evans, and *An. rivulorum* Leeson, can be identified from characteristics of the egg and larval morphology. Only *An. fuscivenosus* Leeson is known in the adult stage and the others four species are morphologically almost indistinguishable in all life stages (Gillies & Coetzee 1987).

Cytogenetic methods have been used to identify female adults of two species: *An. parensis* and *An. funestus* (Green & Hunt 1980). This method uses half-gravid females and is rapid. However, *An. vannedeni* and *An. funestus* are homo-sequential species, which complicates identification

Recently, Koekemoer *et al.* (1999) developed a PCR -SSCP assay, which discriminates between four members of the *funestus* group: *An. funestus*, *An. vaneedeni*, *An. rivulorum* and *An. lessoni*.

Because the PCR products show no species-specific size differences when electophoresed on agarose gel, Hackett *et al.* (2000) developed a second PCR to identify *An. funestus* and *An. rivulorum* using the second ribosomal DNA internal transcribed spacer.

2.4 METHODS

2.4.1 Species identification of the *Anopheles gambiae* complex by Polymerase Chain Reaction

DNA extraction

Individual males and females were assigned to a species of the *An. gambiae* complex using rDNA probes in an rDNA-PCR diagnostic assay. Mosquitoes were placed in separate Eppendorf tubes and homogenized in 100 µl of distilled water. The homogenate was boiled for 10 min and then spun for 1min. Two micro litre samples of this were used as the template for PCR as described by Scott *et al.* (1993).

PCR reagents were obtained as a Kit from Promega.

The PCR technique was performed according to a protocol slightly modified from the one described by Scott *et al.* (1993). PCR was done in 24 µl total volume containing: 2.5 µl 10 X PCR buffer (Promega); 2 µl of a solution containing 50.0 nmol of each of dATP, dCTP, dGTP and dTTP; 0.8 µl of each of the primers (6.25ng of GA, 12.5 ng each of UN and MR, 18.5 ng of AR, and 25 ng of QD): 0.175 µl (0.625 units) Taq DNA polymerase; 2.5 µl MgCl2 (25mM); 15.2 µl distilled water and 2 µl of DNA sample.

Reaction vessels were placed in the thermal cycler and PCR was carried out with a programme of 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds.

Each PCR reaction was performed with three controls: one negative control with the PCR mix and without any DNA template; one positive control for *An. gambiae* s.s. and one positive control for *An. arabiensis*.

Amplified product (13 µl) was electrophoresed in 2% agarose-Tris-borate-EDTA gel containing ethidium bromide. The amplified fragments were visualized over a UV transilluminator. Fragment

size was estimated by comparison with size markers (pGEM, Promega). Bromophenol blue was used as a dye front indicator.

In the presence of appropriate template DNA, characteristic fragments are produced: *An. arabiensis*, 313 base pairs (bp); *An. quadriannulatus*, 150 bp; *An. gambiae* s.s. 390 bp; *An. merus*, 464bp.

2.4.2 Species identification of the *Anopheles funestus* group by Polymerase Chain Reaction

An. funestus group wild specimens and those from families reared from wild females were identified to species level using the PCR-SSCP (Koekemoer *et al.* 1999). This technique distinguishes *An. funestus*, *An. leesoni*, *An. rivolorum* and *An. parensis/An. vaneedeni.*

The PCR mixture contained the following 2.5 µl of 10x reaction buffer (500 mM KCL) of each primer, 200 µM of each dNTP, 2 U thermos table taq DNA Polymerase. Amplifications conditions were 30 cycles of denaturation at 94 °C for 30s, annealing at 40 °C for 30s, and extension at 72°C for 30s, final extension at 72°C for 10 min. 6 µl of the product was electrophoresed on 2% agarose gel. The remaining PCR product was later used for SSCP analyses. Two controls were included, one negative control with the PCR mix and without any DNA template and one was positive for the SSCP electrophoresis was obtained by amplifying extracted DNA from *An. funestus*.

Single strand conformation polymorpism electrophoresis was done according to Hiss *et al.* (1994) cited by (Koekemoer *et a.l* 1999), with minor alterations. Mutation Detection Enhancement gel solution, two times concentration from FMC Bio-Rad Protein ii xi 2-d Cell and with 1-mm tick gels. Before denaturing three µl formamide was added to 10 µl PCR product. The centrifuge tubes (0.5ml) were put into ice immediately after denaturing and 10 µl PCR product was electrophoresed for ~ 16 h at 200 V. The electrode buffer was 0.6 MTBE and a final concentration of TBE in the gel was 0.05 M. A 1 Kb-size marker was loaded in the first lane on the left-hand sede to show the orientation of the gel.

2.5 Results and discussion

A total of 275 members of the *An. gambiae* complex were identified by PCR. 185 were *An. arabiensis*, 70 *An. gambiae* s.s. and 20 *An. quadriannulatus*. Species identification is essential in resistance studies because the resistance status differs in the various sibling species due to different selection pressures (Curtis 1987).

The distribution of members of the *An. gambiae* complex and their relative frequency are represented in Table I and are shown in Figure 5. The distribution of records from published data are given in Figure 5a.

Anopheles gambiae s.s. was identified from three localities, *An. arabiensis* from 12 localities and *An. quadriannulatus* from six localities. Collections made as part of this study show that *An. arabiensis* is widely distributed in southern Mozambique, although this species was also found to occur in the central region of Mozambique (Sofala and Tete province). In the Northern area (Nampula) only *An. gambiae* s.s. was found.

Sympatric association was found to occur between the fresh-water breeding species: *Anopheles gambiae* s.s. and *An. arabiensis* at two localities (Homoine and Mafambissa) and between *An. arabiensis* and *An. quadriannulatus* at five localities: Tete, Chokwe, Boane, Catuane, Moamba and Marracuene (Table I). Although, Petrarca *et al.* (1984) found a sympatric association between *An. gambiae* s.s. and *An. arabiensis* in Tete Province.

In areas where sympatric association between *An. arabiensis* and *An. quadriannulatus* was found, the relative frequency of *An. arabiensis* was high in comparison to *An. quadriannulatus*, except in Tete and Marracuene where *An. quadriannulatus* showed higher frequencies relative to *An. arabiensis*.

From the samples collected three species of the *An. gambiae* complex were identified namely: *An. gambiae* s.s., *An. arabiensis* and *An. quadriannulatus*.

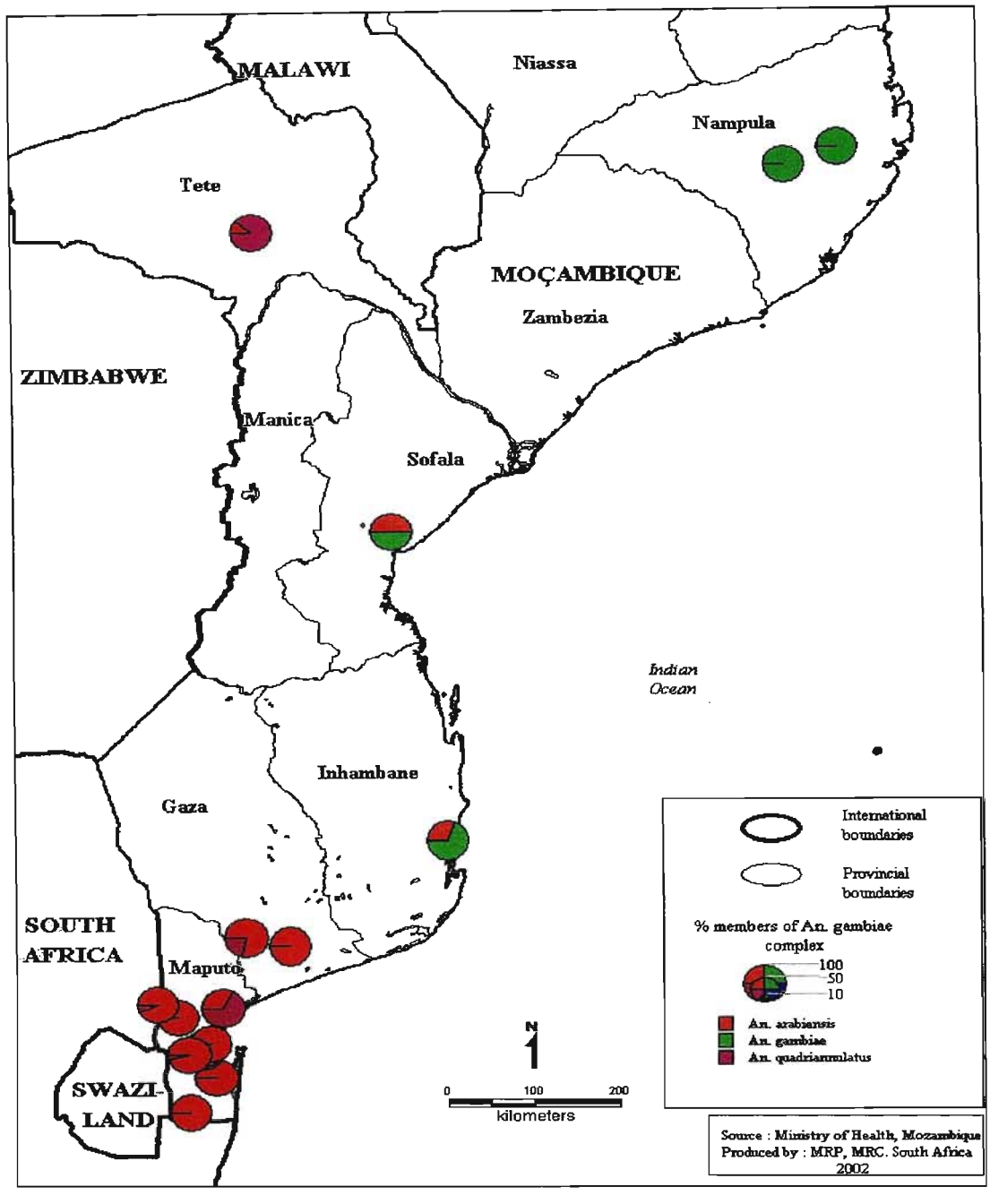

Figure 5. Distribution of members of the *Anopheles gambiae* complex based on collections made from 2000-2002.

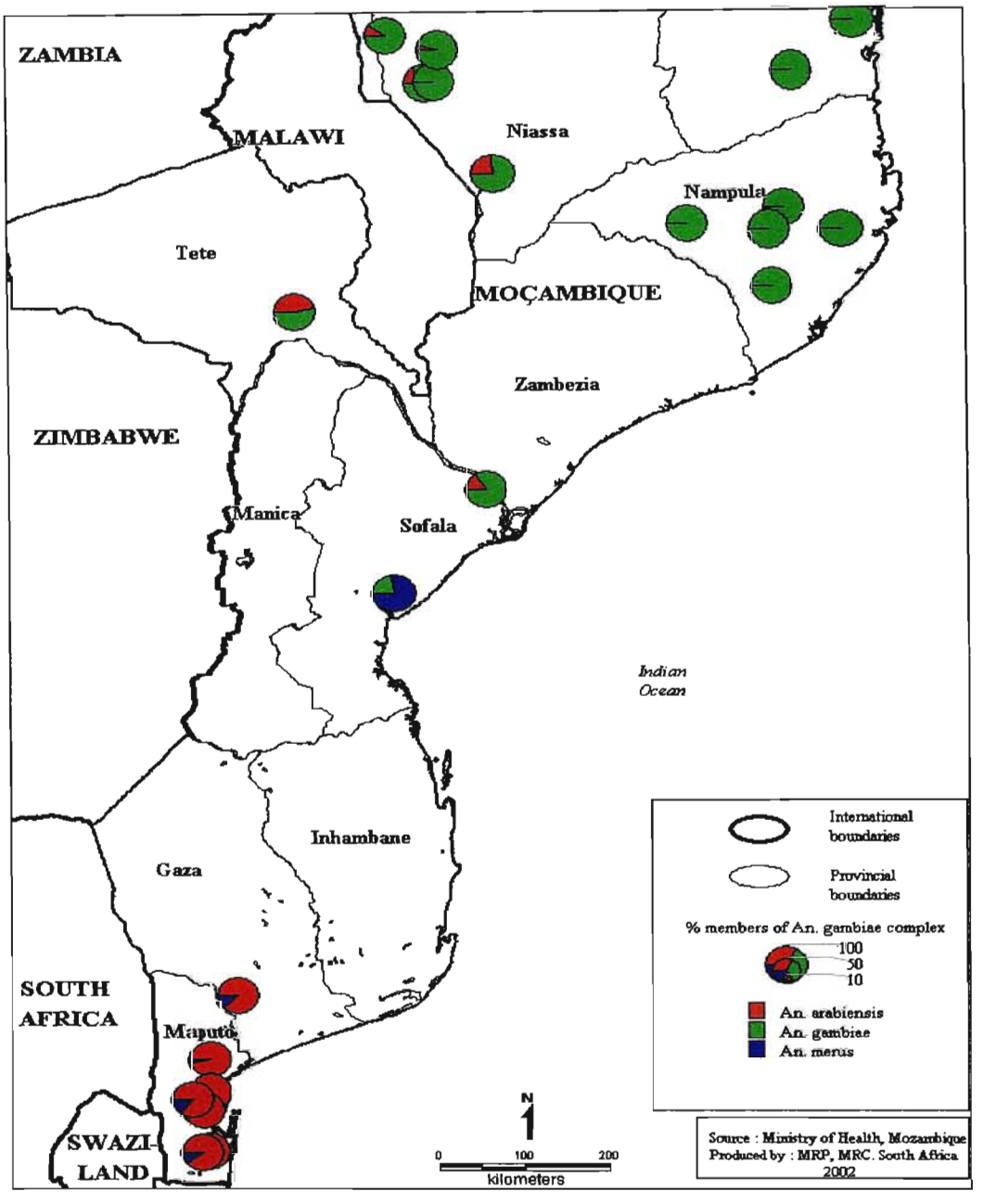

Figure 5a. Published distribution records of member species of the *Anopheles gambiae* complex in Mozambique.

Table I. Distribution of *Anopheles gambiae* s.s., *Anopheles arabiensis* and *Anopheles quadriannulatus* idendified from 16 localities in Mozambique (2000-2002).

Province Locality	Total	*An. arabiensis*		*An. gambiae* s.s.		*An. quadriannulatus*	
		N	%	N	%	N	%
Nampula							
Namialo	10	-	-	10	100.00	-	-
Nampula	6	-	-	6	100.00	-	-
Sofala							
Mafambissa	10	5	50.00	5	50.00	-	-
Tete							
Tete	9	1	11.11	-	-	8	88.89
Inhambane							
Homoine	71	22	30.99	49	69.01	-	-
Gaza							
Chokwe	23	18	78.26	-	-	5	21.74
Manjacaze	2	2	100.00	-	-	-	-
Maputo-Province							
Bela-Vista	2	2	100.00	-	-	-	-
Boane- Bairro 2	21	20	95.24	-	-	1	04.76
Catuane	15	14	93.33	-	-	1	06.67
Moamba	21	20	95.24	-	-	1	04.76
Ressano Garcia	27	27	100.00	-	-	-	-
Marracuene	6	2	33.33	-	-	4	66.67
Manhica	8	8	100.00	-	-	-	-
Maputo-City							
Benfica	2	2	100.00	-	-	-	-
Polana Canico	42	42	100.00	-	-	-	-
Total	275	185	67.27	70	25.45	20	7.27

White (1974) stated that four species: *Anopheles gambiae* s.s., *An. arabiensis*, *An. quadriannulatus* and *An. merus* are found in Southern Mozambique. However in the present study *An. merus* was not detected by PCR. Subsequently, Donnelly *et al.* (1999) identified 15% *An. merus* and 85% *An. arabiensis* but no *An. gambiae* s.s. among 122 specimens of *An. gambiae* s.l. from Matola in the southern region.

Anopheles merus is highly exophilic/exophagic (White 1974). This could be one of the reasons why this species was not found during the sampling, as all collections were indoor resting catches. Other reasons could be due the technique used in this study. The rDNA-PCR that distinguishes the five more common and widespread members of the *An. gambiae* complex failed to consistently identify specimens of *An. merus* in Tanzania (Rensburg *et al.* 1996). Three specimens from Bagamoyo, identified chromosomally as *An. merus* gave only the PCR fragment characteristic for *An. quadrianuulatus* and four specimens fom Muheza also identified chromosomally as *An. merus*, producing the fragments characteristics for *An. quadriannulatus* (Rensburg *et al.* 1996). The authors of this study explain that it is probable that DNA degradation led to misidentification of *An. merus* specimens as *An. quadrianuulatus*. This could potentially be an additional reasons that *An. quadriannulatus* was recorded in six of the collecting sites and *An. merus* were not found to occur.

In further research could be interesting to use the technique describe by Cornel *et al.* (1997), which discriminates between *An. quadrianuulatus* and *An. merus*.

A total of 440 *An. funestus* group specimens from 24 localities, were identified by PCR and 100% identified as *An. funestus* s.s. No other species of the *An. funestus* group was identified. The numbers of mosquitoes identified by locality are given in Appendix 4 and the distribution is shown in Figure 6. The results indicate that *An. funestus* s.s. is widely distributed through the country. *An. funestus* s.s. was found at all collection localities, except one locality (Tete) where only two species of the *An. gambiae* complex were.

Mendis *et al.* (2000) and Aranda *et al.* (2002) stated that in Matola and Mahiça, southern Mozambique, *An. funestus* transmits malaria perennially. In Manhiça, *An. funestus* is the most abundant species during all season (dry and rainy season). Its density increase in February, after the peak January rainfall, and reaches it's maximum in April, coinciding with the end of the rainy season, and before temperatures decline (Aranda *et al.* 2002).

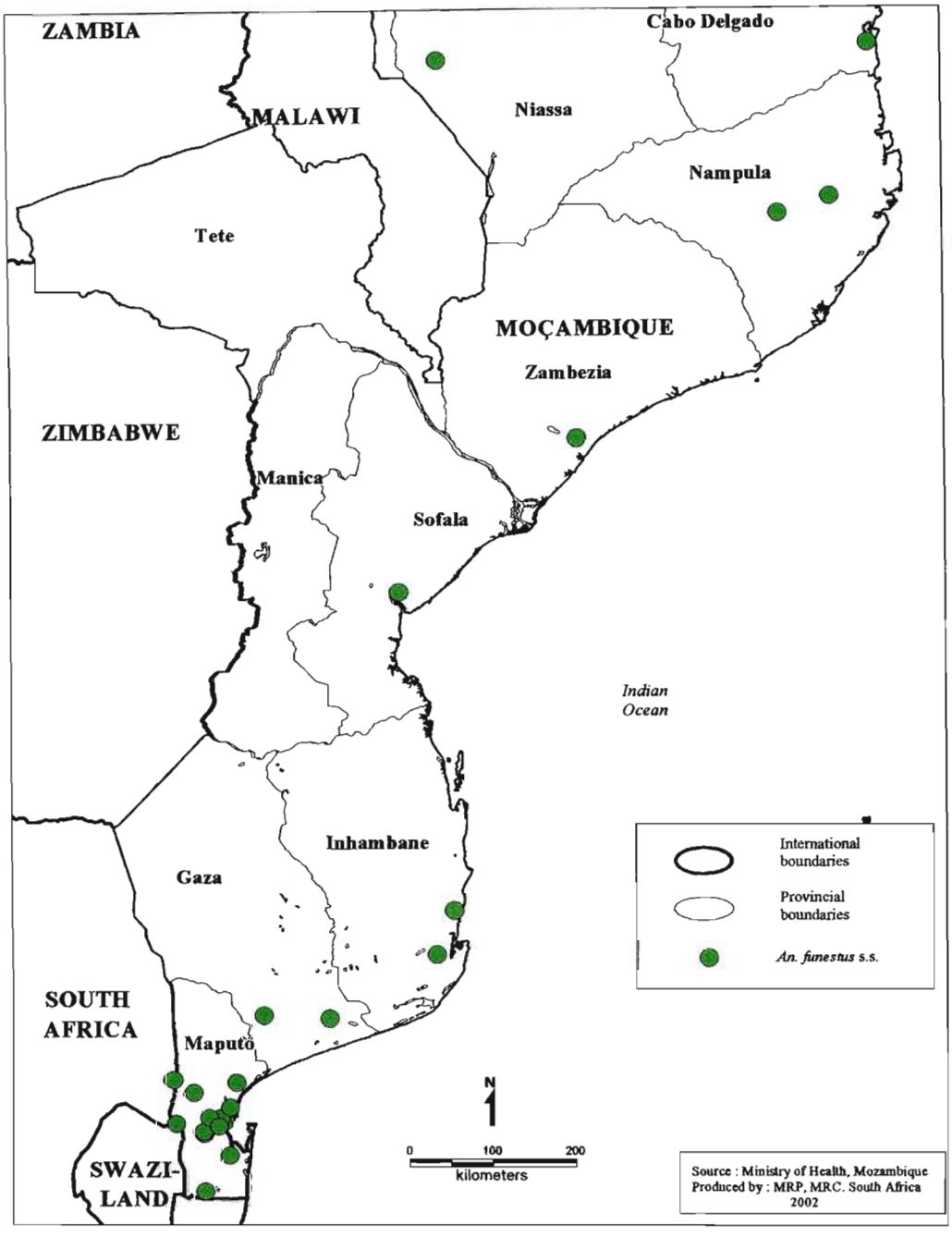

Figure 6. Distribution of *Anopheles funestus* s.s. in Mozambique, based on collections made from 2000-2002.

CHAPTER 3

METHODS TO DETERMINE SUSCEPTIBILITY OR RESISTANCE TO INSECTICIDES

3.1 Introduction

The function of susceptibility-resistance tests is to provide confirmation of resistance to insecticides and to detect the reduction in susceptibility levels and potential emergence of resistance sufficient to cause control failure (Brown 1986).

In the past, the evolution of resistance could only be studied by bioassays, which measure the overall resistance genes present in a population. The Centers for Disease Control and Prevention, Atlanta, Georgia, developed a bottle bioassay method, to undertake time-mortality bioassays which are often more sensitive than dose-mortality bioassays in detecting changes in susceptibility and had a better correlation with micro plate-based biochemical assays for resistance mechanisms (Brogdon & McAllister 1998).

The problems (listed below) with bioassays as the only technique for detecting levels of resistance in natural populations, led to the development of biochemical and molecular techniques to characterize resistance genes in individual mosquitoes (Pasteur & Raymond 1996). These methods allow confirmation of resistance using a small number of insects (Brogdon & McAllister 1998). This is due to the following reasons:

a) While the WHO susceptibility test is a good indication of the degree of insecticide resistance, it can only detect resistance when a mechanism is already at a high frequency in a population.

b) While levels of resistance can be detected, little can be inferred on the type of mechanisms that are operating.

In general, detection methods which utilise molecular technology are better able to distinguish between the different resistance genotypes, i.e. RS (heterozygotes), (SS) homozygous susceptible and RR (homozygous resistant), than traditional bioassay. Because they detect genetic differences, molecular assays can eliminate the environmental components, which often increase the variability

in bioassay and biochemical results (Scott 1995). However molecular techniques have to date only been developed for detection of *Kdr* gene (Martinez-Torres *et al.* 1998)

Martinez-Torres *et al.* (1998) developed a PCR assay to detect a heu-phe sodium channel mutation associated with resistance to DDT and/ or pyrethroid in *An.gambiae.* This technique has been successfully adapted for *An. arabiensis* (Brook *et al.* 1999).

Biochemical assays are not complete substitutes for the standard susceptibility tests which are used to measure resistance as novel mechanisms may be missed (Hemingway 1998). Ideally, a conbination of biochemical, molecular and bioassay tests should be used for resistance detection.

In this study to detect resistance in malaria vector populations in Mozambique, two techniques were employed: Standard WHO (World Health Organization) susceptibility tests and biochemical assays.

3.2 Material and methods

3.2.1 Laboratory procedures

Bloodfed females of the *An. funestus* group and the *An. gambiae* complex were collected as outlined in Chapter 2 and were directly assayed using WHO discriminating dosages of insecticides or were induced to lay eggs and subsequent F1 families were reared through to adults in an insectary at a temperature and relative humidity of 26°C +/- 2°C and 70-80% respectively.

Small bowls (11 cm diameter and 8 cm depth) were prepared with a 3.5cm depth of water. A ring of about 4.25 cm in diameter of waxed paper was cut and placed on the surface of the water. After mosquitoes laid eggs on the surface of filter paper placed in breeding tubes, the eggs were then washed into these rings. The waxed ring kept the eggs in the water otherwise they tend to move to the edge of the container and dry out. Generally, the eggs started to hatch after 24 hours depending on temperature.

The larvae were fed daily with cat food. Small pieces of food were suspended on floats made from thin polystyrene and mesh. The food-float was washed daily and replaced with new food. The water was changed every third day.

When the larvae began to pupate, the bowls were covered with nylon mesh to avoid emerging adults escaping. The newly emerged adults were transferred into paper cups and provided with cotton wool soaked with sugar water (5% solution), which was placed on top of the cup.
This technique used in the insectary at the Institute National of Health, Ministry of Health-Mozambique, was taught to me at the Medical Research Council insectary in Durban.

The F1 progeny from each family were subdivided for susceptibility testing. If the number of F1 progeny from each family were >10, the mosquitoes were used in the susceptibility tests and if less, the mosquitoes were frozen in Eppendorf tubes and transferred to the MRC – Durban, where biochemical analysis was undertaken.

3.2.2 Insecticide Bioassays

All insecticide bioassays described in this work were carried out according to the standard WHO specification using WHO insecticide susceptibility test kits and insecticide treated filter papers (WHO 1998).

The insecticides used were:
Pyrethroids –lambda-cyhalothrin (0.05%), deltamethrin (0.05%) and permethrin (0.75%)
Carbamates - bendiocarb (0.1%) and propoxur (0.1%)
Organophosphate - malathion (5%)
Organochloride - DDT (4%)

Between 5 and 25 adult F1 males and females (one-three day old, non-bloodfed) were aspirated carefully and transferred into the exposure tube which contained an insecticide impregnated paper. The exposure tubes were placed in the vertical position and mosquitoes were exposed for one hour, after this time they were transferred to the holding tube and maintained for 24 hours. A sugar solution was made available during this holding period. For each susceptibility test a control was included with the mosquitoes exposed to untreated papers.

Knock down was recorded every 10 minutes during the exposure time and mortality was recorded after the 24 hour holding period.

Results from each test were recorded on MIM WHO Insecticide Susceptibility Test forms (Appendix 5).

All mosquito survivors used in the controls were stored in the freezer for subsequent analyses (biochemical assays and PCR for species identification).

3.3 Results and discussion

A total of 9500 mosquitoes were tested during the study of which 1193 were wild caught adult *An. funestus* s.l, 6077 *An. funestus* s.s. F1 generation and 2230 *An. gambiae* s.l F1 generation.

WHO diagnostic dosages and test data for F1 *An. gambiae* s.l. from 13 localities are presented in Table II. High levels of pyrethroid resistance were observed in Manjacaze (25% to deltamethrin). There is a need for more collections in this locality to confirm this level of pyrethroid resistance, because the number of mosquitoes tested was low (16). Low levels of mosquito survival followed exposure to pyrethroid were observed in Chokwe (4.25% to lambdacyhalothrin); Catuane (2.0% to lambdacy-halothin), Moamba (0.9 to lambda-cyhalothrin and 4.5 % to deltamethrin). In Bela-Vista the lowest percentage mortality (89 % to lambda-cyhalothrin) was observed, this might be due to the very small sample size (9).

A very low percentage of survival to the carbamate propoxur was observed in two localities: Catembe (2.56%) and Ressano Garcia (2.38%). This low percentage of survival followed exposure to pyrethroids and the carbamates was observed only in *An.arabiensis*. In other areas, complete susceptibility to all insecticides tested was found.

Generally very limited resistance was detected in *An. arabiensis*, this may be due to several factors, including exophily, exophagy and due excito-repellency behaviour of *An. arabiensis* (Sharp & Le Sueur 1990).

Table III shows susceptibility data for wild collected *An. funestus* s.l. of unknown age. High levels of pyrethroid resistance were observed from all four localities in the southern region, (Chokwe, Catuane, Mozal and Boane). In Chokwe and Catembe there was also evidence of cabarmate resistance.

Table II. WHO suceptibility tests data on one-three day old F1 *Anopheles gambiae* species from different collection sites in Mozambique (2000-2002)

Locality	sp	Insecticides % (no of mosquito dead/no of mosquito tested)						
		Lambda cyhalothrin (0.05%)	Deltamethrin (0.05%)	Permethrin (0.75%)	Bendiocarb (0.1%)	Propoxur (0.1%)	Malathion (5.0%)	DDT (4.0%)
Nampula	AR	-	-	-	100 (40/40)	-	-	-
Namialo	AG	100 (23/23)	100 (18/18)	-	-	100 (14/14)	100 (11/11)	100 (11/11)
Mafambissa	AR	100 (12/12)	-	-	100 (12/12)	100 (19/19)	-	-
Tete	AR	-	100 (8/8/)	-	-	-	-	-
	QD	100 (8/8)	100 (6/6)	100 (5/5)	100 (20/20)	-	100 (18/18)	-
Homoine	AG	100 (77/77)	100 (127/127)	-	100 (43/43)	100 (63/63)	100 (46/46)	100 (50/50)
	AR	100 (36/36)	100 (40/40)	-	100 (61/61)	100 (63/63)	100 (9/9)	100 (37/37)
Chokwe	AR	98 (47/48)	100 (10/10)	-	100 (10/10)	100 (14/14)	-	100 (45/45)
Manjacaze	AR	100 (10/10)	75 (12/16)	-	-	-	-	-
Polana Canico	AR	100 (19/19)	100 (17/17)	100 (25/25)	100 (114/114)	100 (10/10)	100 (70/70)	100 (22/22)
Catuane	AR	98 (47/48)	100 (37/37)	100 (28/28)	100 (40/40)	-	100 (18/18)	-
Bela Vista	AR	89 (8/9)	-	-	-	100 (9/9)	-	100 (8/8)
Catembe	AR	100 (8/8)	100 (22/22)	-	-	97 (38/39)	100 (10/10)	100 (8/8)
Ressano Garcia	AR	100 (43/43)	100 (43/43)	-	100 (64/64)	98 (41/42)	100 (21/21)	100 (29/29)
Moamba	AR	99 (108/109)	95 (63/66)	100 (8/8)	100 (58/58)	100 (35/35)	100 (36/36)	-
Marracuene	AR	100 (18/18)	100 (8/8/)	-	100 (19/19)	100 (12/12)	-	100 (13/13)
Manhica	AR	100 (9/9)	100 (18/18)	100 (12/12)	-	-	100 (13/13)	

- = untested; AG = *An. gambiae* s.s.; AR = *An. Arabiensis*; QD = *An. quadriannulatus*

Table III. WHO susceptibility test on wild caught female adults *Anopheles funestus* s.l. from six localities in Mozambique.

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Cabo-Delgado	Lambda-cyhalothrin (0.05%)	66	0	3
	Deltamethrin (0.05%)	48	0	3
	Permethrin (0.75%)	61	0	3
	Bendiocarb (0.1%)	49	0	2
	DDT (4%)	50	0	2
Quelimane	Lambda-cyhalothrin (0.05%)	100	0	4
	Deltamethrin (0.05%)	51	0	2
Chokwe	Lambda-cyhalothrin (0.05%)	66	46.9	3
	Deltamethrin (0.05%)	23	4.3	1
	Permethrin (0.75%)	22	0	1
	Bendiocarb (0.01%)	21	9.5	1
	Propoxur (0.01%)	20	15	1
Catuane	Lambda-cyhalothrin (0.05%)	100	5	4
	Deltamethrin (0.05%)	49	20.4	2
	Permethrin (0.75%)	100	5	4
	Bendiocarb (0.01%)	46	4.34	3
	Propoxur (0.01%)	27	19.14	2
	DDT (4%)	59	0	3
Mozal	Lambda-cyhalothrin (0.05%)	74	50	8
	Deltamethrin (0.05%)	33	15.1	3
	Bendiocarb (0.01%)	39	2.5	2
	Malathion (5%)	18	0	1
Boane – Bairro 2	Lambda-cyhalothrin (0.05%)	71	76.06	3

Chokwe was one of the localities most affected by floods during the first quarter of 2000 and as part of the rehabilitation efforts for flood-affected families, the MOH and UNICEF initiated a programme of ITN distribution in conjunction with community capacity building for malaria prevention and control through participatory approaches and indoor/extra residual spraying. After these interventions, three collections were made, but only a small number of anophelines was collected.

Tables IV and V show WHO susceptibility test data for *An. funestus* s.s.from the north and central regions of Mozambique. Very low levels of survival followed exposure to pyrethroids at only one locality, Namialo (3.1 %). In the other localities, complete susceptibility was observed for all the insecticides tested.

The collections made in Namialo and Mafambissa were in areas with a previous history of house spraying with a pyrethroid (lambda-cyhalothrin). In Nampula, Lichinga and Quelimane, mosquitoes were colleted from previously unsprayed areas.

Table IV. WHO susceptibility test results on one-three day old F1 *Anopheles funestus* s.s. from four localities in the northern region of Mozambique

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Pemba	Lambda-cyhalothrin (0.05%)	66	0	6
	Deltamethrin (0.05%)	41	0	5
	Propoxur (0.1%)	27	0	3
	Malathion (5%)	9	0	2
	DDT (4%)	52	0	6
Namialo	Lambda-cyhalothrin (0.05%)	95	3.1	15
	Deltamethrin (0.05%)	55	0	11
	Propoxur (0.1%)	68	0	10
	Malathion (5.0%)	70	0	11
	DDT (4%)	48	0	10
Nampula	Lambda-cyhalothrin (0.05%)	9	0	1
	Deltamethrin (0.05%)	26	0	4
	Bendiocarb (0.01%)	50	0	9
	Propoxur (0.1%)	10	0	1
Lichinga	Lambda-cyhalothrin (0.05%)	16	0	2
	Deltamethrin (0.05%)	12		1
	Bendiocarb (0.01%)	11	0	1

Table V. WHO susceptibility test results on one-three day old F1 *Anopheles funestus* s.s. from two localities in the central region of Mozambique

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Mafambissa	Lambda-cyhalothrin (0.05%)	23	0	2
	Deltamethrin (0.05%)	11	0	1
	Bendiocarb (0.1%)	22	0	2
	Propoxur (0.1%)	10	0	1
Quelimane	Lambda-cyhalothrin (0.05%)	50	0	7
	Deltamethrin (0.05%)	28	0	3
	Bendiocarb (0.1%)	27	0	2
	DDT (4%)	18	0	2

These results suggest that the current intervention, IRHS with lambda-cyhalothrin, is still effective for malaria control in the north and central regions. However continued monitoring of the resistance status of *An. funestus* is essential.

In Table VI, WHO susceptibility test results for one-three day old F1 *Anopheles funestus* s.s. from 16 localities in the southern region of Mozambique are presented. The results shows complete susceptibility of *An. funestus* s.s. from five localities (Homoine, Manjacaze, Manhica, Polana Canico and Boane-Bairro Manguisa), for all insecticides tested.

According to the criteria outlined by WHO (1998), < 80% mortality 24h post-exposure indicates resistance. Using this definition, pyrethroid resistance in the *An. funestus* populations was observed in seven localities in the southern region, namely: Chokwe, Benfica, Catembe, Bela-Vista, Catuane, Moamba and Boane-Bairro 2.

The distribution of pyrethroid resistance in this area (southern Mozambique) is shown in Figure 7. Red circles indicate resistance to lambda-cyhalothrin, green circles indicate resistance to deltamethrin, blue circles indicate resistance to both pyrethroids (lambadacyhalothrin and deltamethrin) yellow circles indicate complete susceptibility to pyrethroids and pink circles indicate increased tolerance to pyrethroids (10% of survival).

Table VI: WHO susceptibility test results on one to three- day old F1 *Anopheles funestus* s.s. of 16 localities in southern region of Mozambique

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Homoine	Lambdacyhalothrin (0.05%)	43	0	6
	Deltamethrin (0.05%)	23	0	4
	Propoxur (0.1%)	15	0	2
Chokwe	Lambdacyhalothrin (0.05%)	12	50	3
	Deltamethrin (0.05%)	15	0	3
	Permethrin (0.75%)	6	0	1
	Bendiocarb (0.1%)	16	0	3
Manjacaze	Lambdacyhalothrin (0.05%)	40	0	3
	Deltamethrin (0.05%)	8	0	1
	Permethrin (0.75%)	22	0	2
	Propoxur (0.1%)	8	0	1
	Malathion (5%)	24	0	2
	DDT (4%)	25	0	3
Manhiça	Lambdacyhalothrin (0.05%)	56	0	9
	Deltamethrin (0.05%)	48	0	9
	Permethrin (0.75%)	18	0	2
	Propoxur (0.1%)	50	0	8
	Bendiocarb (0.1%)	72	0	12
	Malathion (5 %)	23	0	4
	DDT (4%)	16	0	3
Benfica	Lambdacyhalothrin (0.05%)	19	0	4
	Deltamethrin (0.05%)	16	**56.25**	4
	Permethrin (0.75%)	27	**18.5**	2
	Bendiocarb (0.1%)	16	0	2
	Propoxur (0.01%)	4	0	1
	Malathion (5%)	19	0	3
	DDT (4%)	30	0	5
Polana Caniço	Lambdacyhalothrin (0.05%)	9	0	2
	Deltamethrin (0.05%)	13	0	2
	Permethrin	14	0	2
	Bendiocarb (0.1%)	30	0	4
	Malathion (5%)	8	0	1
	DDT (4%)	26	0	3

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Catembe	Lambdacyhalothrin (0.05%)	177	**40.7**	25
	Deltamethrin (0.05%)	221	**44.8**	32
	Permethrin	15	0	2
	Propoxur (0.1%)	159	13.8	24
	Bendiocarb (0.1%)	232	1.7	34
	Malathion (5%)	88	0	13
	DDT (4%)	71	0	11
Bela-Vista	Lambdacyhalothrin (0.05%)	54	**16.7**	7
	Deltamethrin (0.05%)	11	**40**	2
	Bendiocarb (0.01%	15	13.3	2
	Propoxur (0.1%)	8	0	1
Catuane	Lambdacyhalothrin (0.05%)	44	**27.3**	8
	Deltamethrin (0.05%)	31	**41.9**	5
	Permethrin (0.75%)	83	1.2	6
	Malathion (5%)	24	0	4
	DDT (4%)	13	0	3
	Bendiocarb (0.01%)	19	5.3	3
Mozal	Lambdacyhalothrin (0.05%)	40	10	5
	Deltamethrin (0.05%)	15	0	3
	Bendiocarb (0.1%)	486	0	58
	Propoxur (0.1%)	30	0	4
Moamba	Lambdacyhalothrin (0.05%)	87	**25.3**	14
	Deltamethrin (0.05%)	109	**16.5**	18
	Propoxur (0.1%)	131	4.6	15
	Bendiocarb (0.1%)	116	0.9	15
	Malathion (5%)	23	0	5
	DDT (4%)	32	0	7
Boane-Bairro2	Lambdacyhalothrin (0.05%)	689	57.5	74
	Deltamethrin (0.05%)	223	1.79	25
	Permethrin (0.75%)	121	0	8
	Propoxur (0.1%)	220	0	23
	Bendiocarb (0.1%)	323	1.85	36
	Malathion (5%)	44	0	5

Locality	Insecticide	N Mosquitoes	% Survival	N Bioassays
Boane-Manguisa	Lambdacyhalothrin (0.05%)	22	0	4
	Deltamethrin (0.05%)	30	0	5
	Permethrin (0.75%)	5	0	1
	Propoxur (0.1%)	6	0	1
	Bendiocarb (0.1%)	40	0	6
	Malathion (5%)	6	0	1
Boane – Djimo	Lambdacyhalothrin (0.05%)	30	10	2
	Deltamethrin (0.05%)	49	2.0	5
	Propoxur (0.1%)	57	5.2	9
	Bendiocarb (0.1%)	86	6.9	12
	Malathion (5%)	13	3	3
Ressano Garcia	Lambdacyhalothrin (0.05%)	12	0	2
	Deltamethrin (0.05%)	7	0	1
	Bendiocarb (0.1%)	29	0	4
	Propoxur (0.01%)	32	3.1	6

A high level of lambda-cyhalothrin and deltamethrin resistance in the same locality was observed in Catembe (40 % to lambda-cyhalothrin and 44.8 to deltamethrin) and Catuane (27.3% to lambda-cyhalothrin and 41.9% to deltamethrin). In Catembe *An. funestus* was collected in an area previously sprayed with deltamethrin but in Catuane *An. funestus* was collected in an area with no previous history of insecticide use for more than 20 years.

Chokwe, Boane-Bairro 2 and Moamba had high levels of lambda-cyhalothrin resistance with 50%, 57.5% and 25.3 % survival respectively. A high level of deltamethrin resistance was recorded at two localities: Benfica (56.25%) and Bela-Vista (40.0%). Increase tolerance to pyrethroids was detected in two localities: Boane- Bairro Djimo and Mozal.

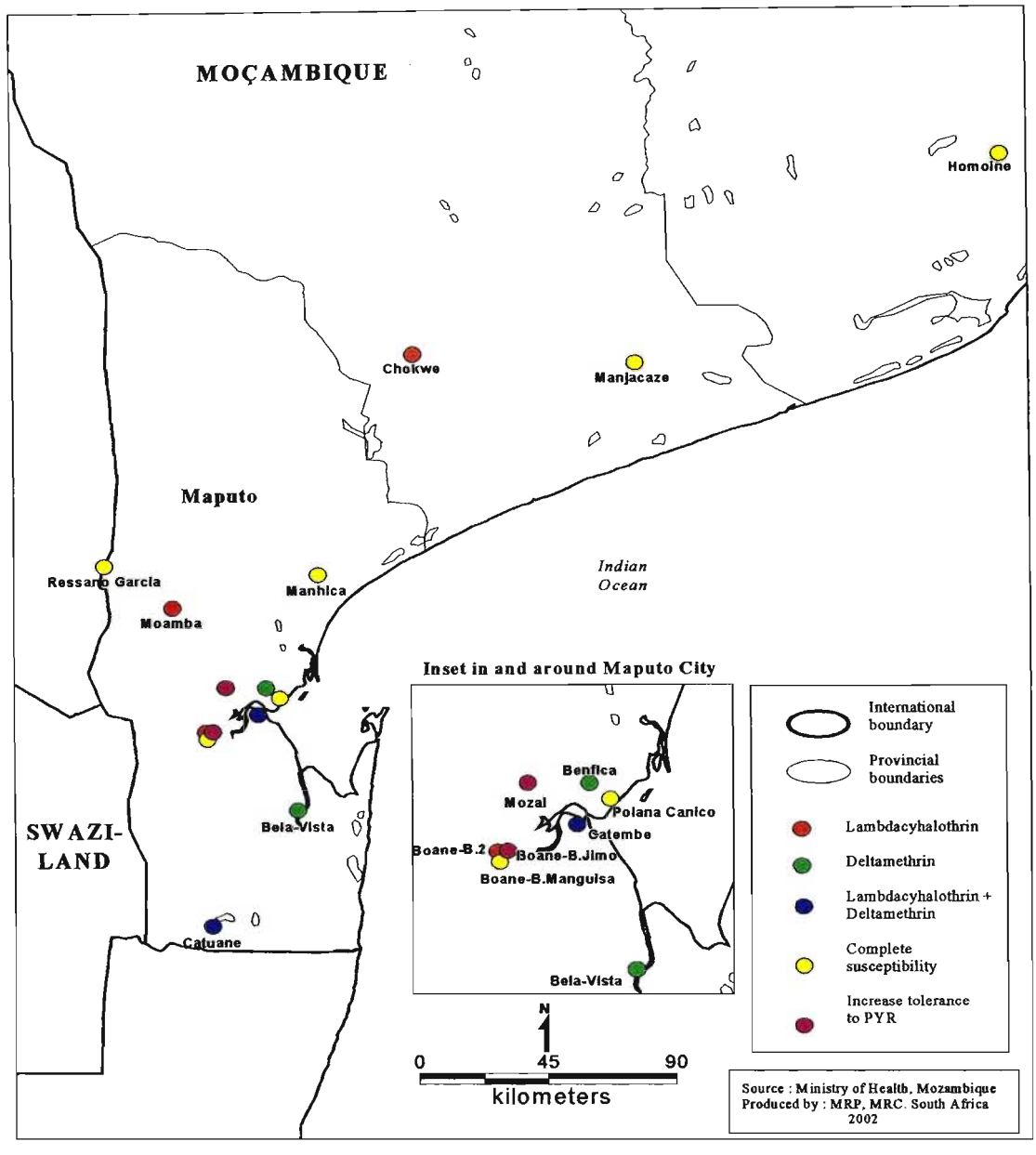

Figure 7. Distribution of pyrethroid resistance in *Anopheles funestus* s.s. in southern Mozambique: 2000-2002.

Evidence of propoxur resistance was observed at four localities: Catembe, Boane-Bairro Djimo, Moamba and Ressano-Garcia. The highest percentage of propuxur survival was observed in Catembe (13.8%). Low levels of bendiocarb survival were observed in Catembe (1.7%), Moamba (0.9%), Boane – Bairro 2 (1.85%), Boane-Bairro Djimo (6.9%) and Bela-Vista (13.3%). There is a need to continue monitoring of the carbamate resistance status of *An. funestus* at these localities.

Brooke *et al.* (2001) showed high levels of propoxur survival in *An. funestus* populations collected in southern Mozambique (Beluluane). However collections made in this study, in the same area of the collection by Brook *et al.* (2001), showed no resistance to bendiocarb.

Prior to the spraying campaign with carbamates in Mozal, Bela-Vista, Catuane and Boane it was possible to collect large numbers of *An. funestus* resting in huts, but following application of the insecticide, it was no longer possible to do so. For example, after spraying, many huts were searched twice a month and only one male *An. funestus* was found in the Mozal area.

If we compare the results from the susceptibility tests on wild adult females (Table III) and the F1 generation (Table VI) from the same species and area, the results shows that the populations of *An. funestus s.s.* from Cabo-Delgado (Pemba) and Quelimane are 100 % susceptible to all insecticides tested.

In Chokwe, the level of survival following exposure to lamba-cyahalothrin was similar in both F1 (50%) and the wild caught adult females (46.9%). These results confirm the resistance of *An. funestus* to lambda-cyhalothrin in this area.

In the Mozal area, the level of survival to pyrethroid (lambda-cyahalothrin) was much higher in the wild caught adult females (50%) than the F1 (10%). There are a number of factors that may have influenced these results, include insecticide pre exposure and age.

In Catuane the level of survival to Deltamethrin on wild caught adult females was 20.4% (Table III) while in the F1 it was markedly higher at 41.9% (Table VI). These findings are in contrast to that from the Mozal area and the results confirm the resistance of *An. funestus* to Deltamethrin. In this area the level of survival of wild caught adult females exposed to Bendiocarb was (4.3%) and in the F1 (5.3%), which both reflect an increase of tolerance to carbamates in this area.

The susceptibility test results from this study, indicated pyrethroid resistance to be widespread in the southern Mozambique province of Maputo. There was also evidence of carbamate resistance at six localities. This situation has implications for the sustainability of indoor residual spraying using this family of insecticides. No resistance to organophosphates or DDT was registrated in any of the localities in the study area.

Organophosphates, carbamates and DDT may present alternatives to be used for IRS, especially in areas of pyrethroid resistance. Although given the evidence of carbamate resistance at six localities within the study area, the alternative insecticide available to vector control would be reduced to organophosphates and DDT in these areas.

There has been much concern about the toxicity of organophosphates over recent years, but it is important to emphasize that organophosphates cover a wide range of acetylcholinesterase inhibitors, ranging from nerve gases to chemicals used as pharmaceuticals or for treatment of human dwellings, grain stores and drinking water (Gallo & Lawryk 1991 [cited by Fanello *et al.* 1999]; Karczmar 1998 [cited by Fanello *et al.* 1999]).

DDT remains generally effective when used for IRS against most species of *Anopheles*, due to excito-repellency as well as direct insecticidal effects. A 1990 cost comparison by WHO found DDT to be considerable less expensive than other insecticides, which cost two to 23 times more on the basis of cost per house per six months of control (Walker 2000).

Since the use of DDT has not been approved by the Ministry of Health in Mozambique, a rotational method of spraying, using different insecticides could be an alternative for implementation in Mozambique.

Rotational strategies to retard insecticide resistance development are based on the principle that there is a reduction in the biotic fitness associated with resistance and that resistance to compound A will decline while compound B is in use (Rodriguez *et al.* 2002).
The NMCP effort in Mozambique is based on three main strategies: Early recognition/diagnosis (both clinical and laboratory) and adequate treatment of malaria, vector control and health education (Ministério da Saúde 2001). Although all components of the programme are very important for

control of the disease, overall success is dependent on the reduction of transmission brought about by the control of the vector mosquitoes. This in turn is dependent on the availability of effective and safe insecticides that can be used in close association with the human population at risk (Hargreaves *et al.* 2000).

The evaluation of insecticide rotation and mosaic applications against *Anopheles albimanus* as resistance management, in Mexico showed that both mosaic and rotational spraying were prophylactic in regard to insecticide resistance in the Mexico vector population of *An. albimanus* (Rodriguez *et al.* 2002).

The long term implications for malaria control in southern Mozambique, as is the case elsewhere, are that continued insecticide susceptibility of the vector species is vital to ensure effective chemical control. Strategies such as rotational spraying and insecticide mixtures need to be evaluated against African vectorial systems.

CHAPTER 4

INSECTICIDE RESISTANCE MECHANISMS

4.1 Introduction

The identification of resistance mechanisms is very important because it helps to determine the cross-resistance spectrum, facilitates the choice of alternative insecticides, and allows detailed mapping of areas with resistant populations (Brogdon & McAllister 1998).

The mechanisms selected in the insects to bring about resistance can broadly be classified as follows:

- Cuticular resistance (reduced penetration)
- Metabolic resistance
- Altered target sites

Of these three categories, the last two are by far the most important (Hemingway 1998; Pasteur & Raymond 1996; Karunaratne 1998). Although resistance due to changes in behaviour (Lockwood *et al.* 1984) and decreased penetration (Priester & Georghiou 1980) have been described in mosquitoes, no recent genetic studies have been undertaken.

The enzyme groups involved in metabolic resistance are esterases for organophosphates, carbamates and pyrethroids, monooxygenases for pyrethroids, organophosphates, carbamates and organoclorines, and glutathione S-transferases for organochlorines, organophosphates and to some extent for pyrethroids. Resistance can be conferred by increased metabolism or sequestration, or an alteration in the catalytic centre activity of the enzyme (Hemingway 1998).

Mutations conferring metabolic resistance are considered to be rare and unique events (Pasteur & Raymond 1996). At present, there are very few insect species in which resistance genes have been identified in single individuals, as is the case of OP- resistance genes in the *Culex pipiens* complex (Pasteur & Raymond 1996).

Pasteur & Raymond (1996) suggest migration and selection as the two major factors in the evolution of resistance in natural populations. Their work showed that the passive migration of *Culex pipiens* associated with commercial transport plays an important role in the dispersal of resistance-associated mutations. The macro- and micro-geographic sampling method to be employed in this study will give an insight into the migration of the detected resistance genes and this will be accompanied by the documentation of the insecticides used in the agricultural areas.

Resistance may decline with the age of the mosquitoes, for example, DDT resistance in *An.gambiae* s.s (Lines & Nassor 1991). Their work showed that mixed-age wild samples of both fed and unfed, DDT resistant *An.gambiae* s.s. from Kikobweni, Zanzibar, gave a higher mortality after exposure to 5% DDT than newly emerged insects.

4.1.1 Cuticular resistance (reduced penetration)

By itself, this mechanism confers low levels (less than 5-fold) of resistance and only becomes important when found in combination with other resistant mechanisms. However, it does seem to provide protection to a wide variety on insecticides (Scott 1989). The simplest way to determine the rate of penetration involves exposing the insect to insecticide and then comparing the amount absorbed over time in resistant and susceptible strains. The amount of insecticide absorbed can be quantified by analysing the insect homogenate for insecticide and its metabolites.

4.1.2 Metabolic resistance

This type of mechanism involves qualitative or quantitative changes in the enzymes which metabolise or sequester the insecticides before they reach their target sites. There are three groups of enzymes involved: esterases, glutathione S-transferase and monooxygenases.

4.1.2.1 Esterases

Perhaps the most common resistance mechanisms in insects are modified levels or activities of esterase enzymes that metabolise a wide range of insecticides (Brogdon & McAllister 1998). Resistance can occur through quantitative and qualitative changes in esterases. When increased quantities occur, sequestration is generally the primary mechanism. Qualitatively changed

carboxylesterases can hydrolyse insecticides at faster rates than their counterparts in susceptible insects (Karunaratne 1998).

Changes in carboxylesterase activity have been associated with resistance to OP insecticides in certain species of mosquitoes, aphids, blowflies and houseflies (Claudianos *et al.* 1999). Esterase levels can be elevated by either gene amplification or altered gene expression (Scott 1995) or a combination of both (Hawkes *et al.* 2001).

4.1.2.2 Glutathione S-transferase

Glutathione S-transferases are a major family of detoxification enzymes found in most organisms. All eukaryotes possess multiple GSTs (gluthathione S-transferase) with different substrate especificities to accommodate the wide range of catalytic function of this enzyme family (Ranson *et al.* 1998, 2001). They catalyse the nucleophilic attack of the endogenous tripeptide glutathione on a variety of reactive substrates. In early literatures a subset of GSTs are referred to as DDT dehydrochlorinases (DDT ases) because of their involvement in dehydrochlorination of DDT to DDE (Prapanthadara *et al.* 1993; Karunaratne 1998).

In mosquitoes, GSTs commonly confer resistance to DDT (Prapanthandara *et al.* 1993, 1995). Insect GSTs are now classified into five classes, but previously only two such classes were recognised (Ranson *et al.* 2002). Class I GSTs are most closely related at the amino acid level to mammalian theta class GSTs, while class II GSTs are related to the pi class, this relationship between insect and mammalian classes does not extend to their substrate specificities (Hemingway 2000). In *An. gambiae* seven GSTs have been partially purified which possess 100% of the DDTase activity (Prapanthadara *et al.* 1995).

4.1.2.3 Monooxygenases

The monooxygenases, also termed cytochrome P450 oxidaes or MFOs (mixed function oxidases) metabolise insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester-oxidation, and nitrogen and thioether oxidation (Wilkinson 1976 [cited by Brogdon McAllister 1998]).

The cytochrome P450s belongs to a vast superfamily of enzymes. Of the 62 families of P450s recognized in animals and plants, at least four families (4, 6, 9, 18) have been isolated from insects. Sequencing of the *Drosophila* and *An. gambiae* genome has now expanded this and over 90 p450s are known in *An. gambiae* (Ranson *et al.* 2002). The insect P450s responsible for resistance primarily belong to family six, which, like esterases, occur in Diptera as a cluster of genes (Maitra *et al.* 1996). The cytochrome P450 monooxygenases are involved in many cases of resistance of insects to insecticides. Resistance is often associated with an increase in monooxygenases activities however, this increase does not always account for all of the resistance (Bergé *et al.* 1999).

4.1.3 Altered target sites

In this category of resistance mechanism, an alteration in the target-site prevents insecticide interaction with target. These changes must be highly specific, so that the normal physiological functions of the target site are largely unaffected. Most of these changes are due to a substitution of a single amino acid in the protein sequence of the target site. There are three target sites: AChE (acetylcholinestarase), Na+ channel proteins and GABA (gamma amino butyric acid) receptors for the four main insecticide families used for vector control, which can develop alterations to bring about insecticide resistance (Karunaratne 1998).

4.1.3.1 Altered acetylcholinesterase

Acetylcholinesterase is the target site for OP and carbamate insecticides and point mutations in the Ace gene are associated with resistance in *Drosophila melanogaster* and *Musca domestica* (Liu *et al.* 1988).

Acetylcholinesterase catalyses the hydroloysis of the neurotransmitter, acetylcholine, thereby ending transmission of nerve impulses at synapses of cholinergic neurones in central and peripheral nervous systems (Baxter & Barker 1999). Quantative and qualitative changes in AChE confer resistance to insecticides (Fournier *et al.* 1992, 1993). In resistant insects the enzyme is altered such that it has reduced sensitivity to insecticide inhibition while maintaining its normal function at levels at least adequate for survival (Raymond *et al.*1985).

Vaughan *et al.*, (1997) demonstrated that the same mutations that cause insecticide resistance in *Drosophila melanogaster* AChE also confer resistance in *Aedes aegypti.*

4.1.3.2 Altered gamma amino butyric acid receptors

Altered gamma amino butyric acid receptors are the primary target of cyclodiene insecticides (Scott 1995). Most cases of cyclodiene resistance appear to be due to decreased sensitivity of the GABA subtype A receptor (Ffrench-Constant *et al.* 1991).

4.1.3.3 Altered Na+ channel proteins

The Na+ channel proteins in the insect nervous system are the target site for pyrethroids and DDT. Insects with altered Na^+ channel proteins are resistant to the rapid knock-down effect of pyrethroids and are called "*kdr*" (knock-down resistance) or "super *kdr*" (highly resistant). These mechanisms have been observed in houseflies (*Musca domestica*) (Sawicki 1987; Rossingnol 1988; Grubs *et al.* 1988) and *Aedes aegypti* (Malcolm & Wood 1982; Hemingway *et al.* 1989) and many other insects.

In *Anopheles gambiae* s.s. , *kdr* has been reported in the Ivory Coast, Burkina Faso (Martinez-Torres *et al.* 1998; Chandre *et al.*1999) and Kenya (Ranson *et al.* 2000).

4.2 Material and methods

4.2.1 Study area

Biochemical assays were undertaken on *An. funestus* s.s. F1 adults from females collected during 2000-2001, from 11 localities: Bela-Vista; Boane, Catembe, Catuane, Homoine, Massinga, Moamba, Mozal, Namaacha, Pemba and Quelimane; and *An. arabiensis* (F1 adults from females collected during 2000-2001) from seven localities: Bela-Vista; Boane, Catembe, Catuane, Moamba, Polana Canico and Homoine. For *An. gambiae* s.s. assays were only undertaken on mosquitoes collected in Homoine.

4.2.2 Insect strain

In this study, data from the Panama strain of *An. albimanus* was used as the standard susceptible strain, because no susceptible laboratory colony of *An. funestus* existed. The Panama strain has been maintained in the laboratory (in Mexico) without insecticide selection for 20 years and prior to this was collected from an area with no history of insecticide use. These data were communicated by Dr. Rodriguez (Rodriguez *et al.* 2002). The Durban laboratory strain of *An. arabiensis* was used to determine the levels of different enzymes compared to the Panama strain. The Durban strain was collected in 1993 from Mamfene, an area with a history of indoor residual spraying with DDT.

Appendix 6 contains summary statistics for the different enzyme baselines in the Panama susceptible strain.

4.2.3 Biochemical assays

Biochemical assays were used to determine the presence of an AChE and to quantify levels of GST and, non-specific esterase activity, plus monooxygenases titres in individual mosquitoes. Batches of 47 one to three-day old mosquitoes were assayed per microtitre plate according to the method described by Penilla *et al.* (1998).

For these assays, only one to three-day old males and females (unfed) of the F1 generation were used. It was not practical to analyse mosquitoes directly from the field, because pre-exposure of some of the mosquitoes to insecticides could affect the enzyme titres. The F1 generation mosquitoes also allow us to standardize age, physiological state and test conditions for the assays (Penilla *et al.* 1998).

Mosquitoes were individually homogenised in 200 µl of distilled water in Eppendorf tubes (1.5µl). The homogenization was carried out on ice. For the AChE assay, two replicates of 25 µl of crude homogenate were transferred to a clean microtitre plate. The remaining homogenates were spun separately at 14K for 10 minutes in a microfuge. Replicates of 20 µl of the supernatants from each sample were transferred to a clean micrititre plate for the elevated esterase naphthyl acetate assays

and for the monooxygenase assay. For GSTs, esterase and protein assays, 10µl of the supernatants were used.

4.2.3.1 Acetylcholinesterase assay

The AChE in the homogenates was solubilized by adding 145µl of Triton phosphate buffer (1% Triton X-100 in 0.1 M phosphate buffer pH 7.8) to each replicate. Ten microlitres of DTNB solution (0.01 M dithiobis 2-nitrobenzoic acid in 0.1 M phosphate buffer pH 7.0) and 25µl of the substrate ASCHI (0.01 M acetythiocholine iodide) were added to one replicate to initiate the reaction. To the second replicate were added 25µl of the substrate ASCHI (acetylthiocholine iodide) containing 0.2% of an 0.1 M solution of the inhibitor propoxur. Control wells contained 25µl of distilled water, 145µl Triton buffer, 10µl DTNB solution and 25µl ASCHI solution without and with propoxur respectively. The kinetics of the enzyme reaction was monitored continuously at 405 nm for five minutes in a microtitre plate reader.

The percentages of propoxur inhibited AChE activity in the test compared to uninhibited wells were calculated by dividing the value for the well with propoxur by that without propoxur for the same insect multiplied by 100 to switch this to percentage inhibition. The assay conditions were set so that individuals without an altered AChE-based resistance mechanism had > 60% inhibition of activity. Resistance gene frequencies were then calculated from the resultant data, assuming the mosquito population was in Hardy-Weinberg equilibrium.

4.2.3.2 Esterase assays

Naphtyl acetate assays

Two hundred µl of α-naphthyl acetate solution (100µl of 30mM α-NA in acetone in 10 ml of 0.02 M phosphate buffer pH7.2) and 200µl of β–NA solution (100µl of 30mM β-Na in acetone in 10 ml of 0.02 M phosphate buffer pH7.2) were added to one replicate of homogenate. The enzyme reaction ran for 30 mins at room temperature before the addition of 50µl of Fast blue stain solution (22.5mg Fast blue in 2.25ml distilled water, then 5.25ml of 5% sodium lauryl sulphate diluted in 0.1M phosphate buffer, pH7.0) was added to each well to stop the reaction. Replicate blanks

contained 20µl distilled water, 200µl α-NA or β–NA solution and 50 µl of stain. Enzyme activity was read at 570nm as an end point. Absorbance levels for individual mosquitoes were compared with standard curves of absorbance for known concentrations of α-naphthol and β-naphthol, respectively. The results were reported as µmoles of the product formed/min/mg protein.

p-Nitrophenyl acetate esterase assay

Two hundred µl of *p*NPA (*p*-Nitrophenyl acetate esterase) working solution (100 mM *p*NPA in acetonitrile: 50mM sodium phosphate buffer pH7.4, 1:100) were added to each replicate. Two blanks were prepared for each plate with 10 µl distilled water and 200µl *p*NPA working solution. Enzyme rates were measured at 405nm for two mins. The pNPA activity per individual was reported as µmoles of product/min/mg protein.

4.2.3.3 Glutathione S-transferase assay

Two hundred µl of GST/CDNB working solution (10 mM reduced glutathione prepared in 0.1M phosphate buffer pH6.5 and 63 mM chlorodinitrobenzene dissolved in methanol). Two blanks were prepared for each plate with 10µl distilled water and 200µl of GST/CDNB working solution. Enzyme rates were measured at 340nm for five min. The GST activity per individual was reported as nmol CDNB conjugated/min/min/mg protein, using published extinction coefficients corrected for the path length of the reaction mixture.

4.2.3.4 Monooxygenase

Eighty µl of 0.625M potassium phosphate buffer pH7.2 were added to the aliquots of the homogenates and 200µl of the solution 3,3'.5,5'-tetramethyl benzindine diluted in absolute methanol (0.01g in 5ml, respectively) and then mixed with 15ml of 0.25M sodium acetate buffer pH5.0. Twenty-five µl of 3% H_2O_2 (hydrogen peroxide) was added and the mixture left for 2 hours at room temperature. Samples and two controls per plate, were prepared with 20µl of distilled water, 200µl of the solution TMBZ (tetramethyl benzindine) and 25µ l of 3% H_2O_2. Absorbance was read at 650nm and values compared with a standard curve of absorbance for known

concentrations of cytochrome C followed by correction for difference in the known haem content of cytochrome C and P450.

4.2.3.5 Protein assay

Three hundred µl of BIO RAD protein reagent solution were added to 10µl of the crude homogenate. Two blanks were prepared for each plate with 10µl of distilled water and 300 µl of BIO RAD solution. The reaction was read at 570nm after five min at room temperature. Protein values in mg/ml were calculated for individual mosquitoes from a standard curve of absorbance of known concentrations of bovine serum albumin.

4.3 Statistical analysis

Mosquitoes on each microtitre plate were categorized by collection site, species (as identified by PCR), sex and the values of absorbance for different enzymes. These values were collected from the plate reader using a SOFTMAX PRO 2.6.1 program and the data then transferred via an Excel spreadsheet for analysis in SPSS 10.0 for Windows. Independent-Samples T-Test were used to detect any significant differences in resistance gene frequencies between sex and One-Way-ANOVA was use to compare means of different enzyme activities between the standard susceptible strain (Panama strain) and the specimens from the study area.

4.4 Results and discussion

The numbers of *An. funestus* s.s. *An. arabiensis* and *An. gambiae* s.s used for the respective assays: the percentage of AChE inhibition by propoxur, elevated esterase activities with substrates α, β and pNPA, monooxygenases and GST, are represented by locality in Appendices 7 and 8.

Altered acetlycholinesterase

The frequency distribution of AChE inhibition by propoxur for the Panama and Durban laboratory populations and for F1 adult progeny from wild-caught *An. funestus* females are represented in Figure 8 and the mean percentage inhibition values of AChE by propoxur are given in Table VII.

The inhibition values in the Panama strain ranged from 100% to 60%, indicating that none of the individuals in this population have the altered AChE gene. The inhibition values for the Durban strain range from 100 % to 79.15%, indicating that this resistance mechanism was not present in this *An. arabiensis* strain. The range for F1 adult progeny from wild-caught *An. funestus* females from the 11 localities was much broader (100 to 14.84 %), indicating the presence of altered AChE within the study populations, which should confer resistance to both carbamates and organosphophates. The lowest mean percentage inhibition values of AChE were registered in Namaacha (51.9%) while the highest mean was observed in Massinga (92.3%). There was no significant difference in % inhibition of AChE by propoxur between collections.

The frequency of the AChE resistance mechanism for *An. funestus* s.s. varied among the different collections sites (Table VIII). The highest altered AChE gene frequencies were registered in Namaacha (0.8) and the lowest in Moamba (0.08), but all values were in the range 0.008 to 0.21. The altered AChE gene frequencies registered in collections sites in this study area were higher than those values documented in *An. albimanus* from Mexico (Penilla *et al.* 1998). This mechanism was not detected in either the Durban strain or in collections from Massinga.

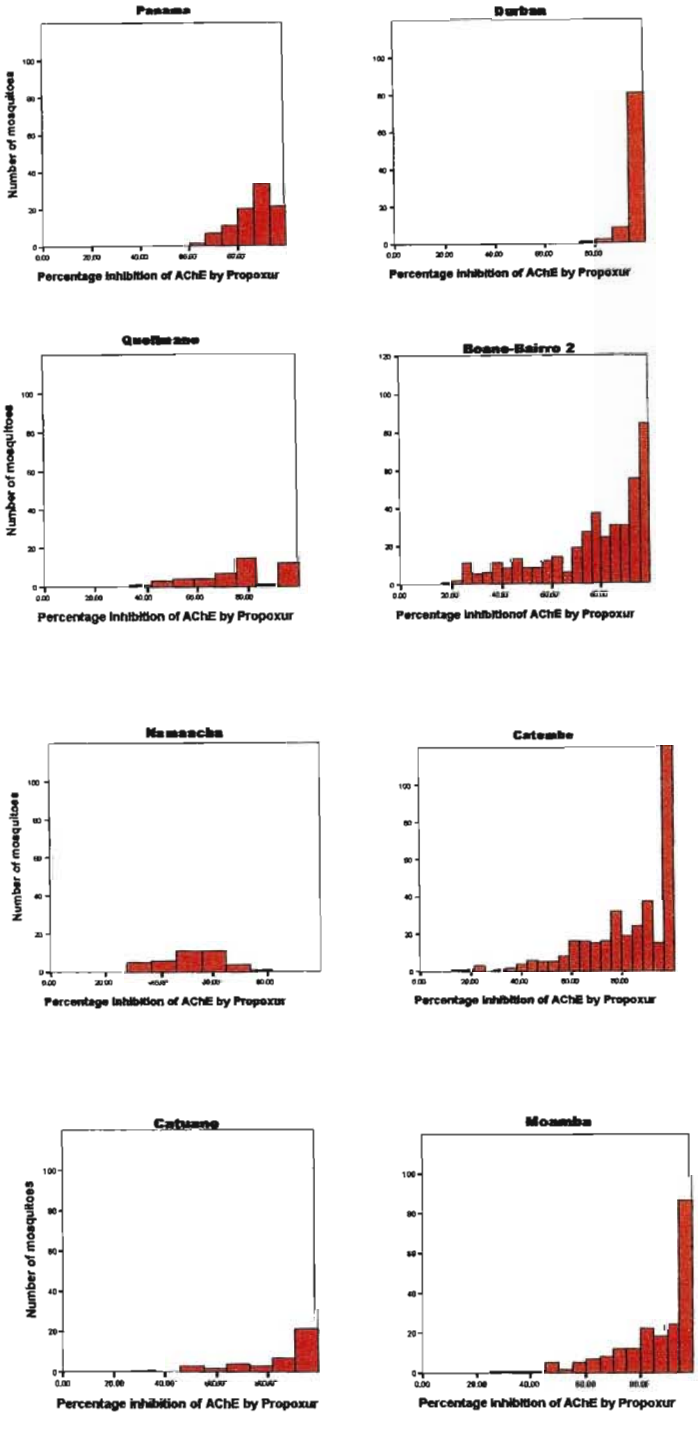

Figure 8. Acetylcholinesterase (AChE) inhibition range in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from six localities in Mozambique, compared to the Durban strain and to the standard susceptible Panama strain.

Table VII. Inhibition of AChE activity by propoxur in one-day-old *Anopheles albimanus* of Panama and *An.arabiensis* of the Durban strains and F1 generations (*Anopheles funestus* s.s.) from 12 study areas in Mozambique, 2000-2001.

Strains and Localities	% inhibition Mean ± SD	n
Panama	86.6 ± 8.3	94
Durban	97.7 ± 4.2	91
Bela Vista	89.6 ± 18.8	47
Boane-Bairro2	77.5 ± 21.2	411
Catembe	82.7 ± 18.2	352
Catuane	84.6 ± 17.8	41
Homoine	80.4 ± 15.9	24
Massinga	92.3 ± 11.2	29
Moamba	86.6 ± 15.5	205
Mozal	89.5 ± 13.8	11
Namaacha	51.9 ± 11.3	38
Pemba	83.2 ± 20.0	23
Quelimane	76.7 ± 17.5	46
Mean	81.0 ± 19.6	1227

Table VIII. Resistance gene frequencies for the altered AChE-based resistance mechanism in the Panama and Durban strains and F1s *Anopheles funestus* s.s. from the different collection sites in Mozambique.

Strain and locality	Resistant gene	Susceptible gene	N
Panama	-	1	94
Durban	-	1	91
Bela Vista	0.09	0.91	47
Catembe	0.11	0.89	352
Catuane	0.12	0.88	41
Boane	0.21	0.79	411
Homoine	0.08	0.92	24
Massinga	-	1	29
Moamba	0.08	0.92	205
Mozal	0.1	0.9	13
Namaacha	0.8	0.2	38
Pemba	0.09	0.91	23
Quelimane	0.17	0.83	46

Massinga (92.3%). There was no significant difference in % inhibition of AChE by propoxur between collections.

The frequency of the AChE resistance mechanism for *An. funestus* s.s. varied among the different collections sites (Table VII). The highest altered AChE gene frequencies were registered in Namaacha (0.8) and the lowest in Moamba (0.08), but all values were in the range 0.008 to 0.21. The altered AChE gene frequencies registered in collections sites in this study area were higher than those values documented in *An. albimanus* from Mexico (Penilla *et al.* 1998). This mechanism was not detected in either the Durban strain or in collections from Massinga.

Gender comparisons of the inhibition of AChE by propoxur in Panama and Durban strains and F1 *An. funestus* from the collections sites are given in Table IX. Only Bela-Vista and Pemba showed significant differences in the levels of propoxur inhibition of AChE between the sexes. In both localities males had higher mean inhibition levels than females ($p< 0.018$ and $p< 0.027$ respectively in Bela-Vista and Pemba), however at the other nine comparison sites there was no statiscal difference between male and female. This is as expected, as the AChE resistance gene is not sex-linked [Kaiser *el al.* 1978 cited by (Penilla *et al.* 1998)]. Malcolm *et al.* (1998) have cloned a sex-linked AchE gene from *Culex* and *Aedes* mosquitoes, hence some variation in total AChE content may occur between sex. The recent publication of the *Anopheles gambiae* genome has now clarified the situation, showing that there are two AChE-like genes. One of these is sex-linked and unaffected by pesticide selection and resistance (Ranson *et al.* 2002).

Table X lists the mean percentage propoxur inhibition values of AChE for the two laboratory strains and *An. gambiae* s.s. from one locality and *An. arabiensis* from seven localities. The lowest mean was observed in Catuane (69.8%).

The frequencies of resistance AChE genes in *An. gambiae* s.s. and *An. arabiensis* are given in Table XI. The highest altered AChE gene frequencies were found in Catuane (0.29), but all values were in the range 0.10 to 0.29. The altered AChE gene was not dected in Bela-Vista.

There was no significant difference in the level of propoxur inhibition of AChE between sexes in any collection localities (Table XII).

Table IX. Gender comparisons of inhibition of acetylcholinesterase by propoxur in one-day-old e Panama and Durban laboratory strains (*Anopheles arabiensis*) and the F1 generation from 2000-2001 collection of *Anopheles funestus*.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. p
	Males	n	Females	n	
Panama	86.5 ± 9.1	48	86.7 ± 7.4	46	NS*
Durban	98.1 ± 3.5	47	97.3 ± 4.9	44	NS
Bela Vista	93.6 ± 9.9	20	86.6 ± 23.0	27	0.018
Boane-Bairro2	77.6 ± 21.1	216	77.3 ± 21.3	195	NS
Catembe	81.5 ± 18.1	149	83.6 ± 18.3	203	NS
Catuane	85.9 ± 15.6	23	83.0 ± 20.7	18	NS
Homoine	77.1 ± 14.9	16	87.2 ± 16.9	8	NS
Massinga	93.5 ± 9.8	20	89.6 ± 14.2	9	NS
Moamba	85.4 ± 15.1	95	87.6 ± 15.9	110	NS
Mozal	86.8 ± 15.2	7	94.3 ± 20.2	4	NS
Namaacha	53.2 ± 11.7	19	50.6 ± 11.1	19	NS
Pemba	83.5 ± 16.4	19	81.8 ± 36.4	4	0.027
Quelimane	78.7 ± 16.8	24	74.5 ± 18.4	24	NS
Mean	80.7 ± 19.0	608	81.3 ± 20.2	619	NS

NS*= Not significant

Table X. Inhibition of acetylcholinesterase activity by propoxur in Panama (*Anopheles albimanus*) and Durban (*Anopheles arabiensis*) strains and F1 generations of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from seven collection localities in Mozambique, 2000-2001.

Strains and localities	% inhibition	n
	Mean ± SD	
Panama	86.6 ± 8.3	94
Durban	97.7 ± 4.2	91
Bela Vista	91.8 ± 3.5	15
Boane-Bairro2	74.2 ± 11.3	139
Catembe	77.2 ± 15.3	51
Catuane	69.8 ± 20.5	14
Homoine	75.9 ± 18.4	79
Homoine*	73.7 ± 17.2	125
Moamba	78.5 ± 19.3	80
Polana-Canico	78.1 ± 18.2	47
Mean	75.9 ± 16.5	550

Homoine* = *An. gambiae* s.s.

Table XI. Resistance gene frequencies for the altered acetylcholinesterase-based resistance mechanism in the Panama (*Anopheles albimanus*) and Durban (*Anopheles arabiensis*) strains and F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from the collection sites in Mozambique.

Strain and locality	Resistance gene	Susceptible gene	n
Panama	-	1	94
Durban	-	1	91
Bela Vista	-	1	15
Catembe	0.16	0.84	51
Catuane	0.29	0.71	14
Boane	0.10	0.90	139
Homoine	0.20	0.20	100
Homoine*	0.19	0.81	125
Moamba	0.18	0.82	80
Polana-Canico	0.17	0.83	47

* *An. gambiae* s.s.

Tabela XII. Gender comparisons of inhibition of AChE by propoxur of the Panama and Durban (*Anopheles albimanus*) and Durban (*Anopheles arabiensis*) strains and the F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. p
	Males	n	Females	n	
Panama	86.5 ± 9.1	48	86.7 ± 7.4	46	NS*
Durban	98.1 ± 3.5	47	97.3 ± 4.9	44	NS
Bela Vista	90.7 ± 2.6	7	92.7 ± 4.0	8	NS
Boane-Bairro2	74.1 ± 11.7	72	74.8 ± 11.4	67	NS
Catembe	73.4 ± 13.7	30	82.5 ± 16.1	21	NS
Catuane	71.4 ± 15.8	7	68.0 ± 25.5	7	NS
Homoine	74.4 ± 20.0	33	76.9 ± 17.3	46	NS
Homoine*	72.8 ± 17.8	67	74.7 ± 16.6	58	NS
Moamba	78.9 ± 19.6	39	78.2 ± 19.2	41	NS
Polana -Canico	80.1 ± 18.9	28	75.1 ± 17.2	19	NS
Mean	75.3 ± 16.7	283	76.6 ± 16.3	267	NS

*NS= Not significant, Homoine * = *An. gambiae* s.s.

Esterase activity

Figures 9, 9a, 10 and 10a give the range of esterase activity with the substrates α- and β-naphthyl acetate in the Panama (*An. albimanus*) and Durban (*An. arabiensis*) strains and F1 progeny from *An. funestus* s.s. from the different collections localities. Elevated esterase activity with α-naphthyl acetate was detected in two localities: Boane-Bairro 2 and Catembe. In others localities esterase activity was very low with the substrates α- and β-naphthyl acetate. Very low esterase activity with α-and β-naphthyl acetate was also detected in the Durban strain.

Mean values of esterase activity are given in Table XIII. The highest means of esterase activity with α-naphthyl acetate were observed in Boane (0.001), Massinga (0.001) and Mozal (0.001), although these values are below the levels seen in *An. albimanus* populations from Mexico (Penilla *et al.* 1998).

Differences in the levels of esterase activity with α-naphthyl acetate between males and females were observed in Boane-Bairro 2 and Namaacha where females were significantly higher than males ($p < 0.05$) in both localities (Table XIV), while with β-naphthyl acetate were observed in three localities (Catuane, Massinga and Namaacha), and also females were significantly higher than males ($p < 0.05$) in all localities (Table XV).

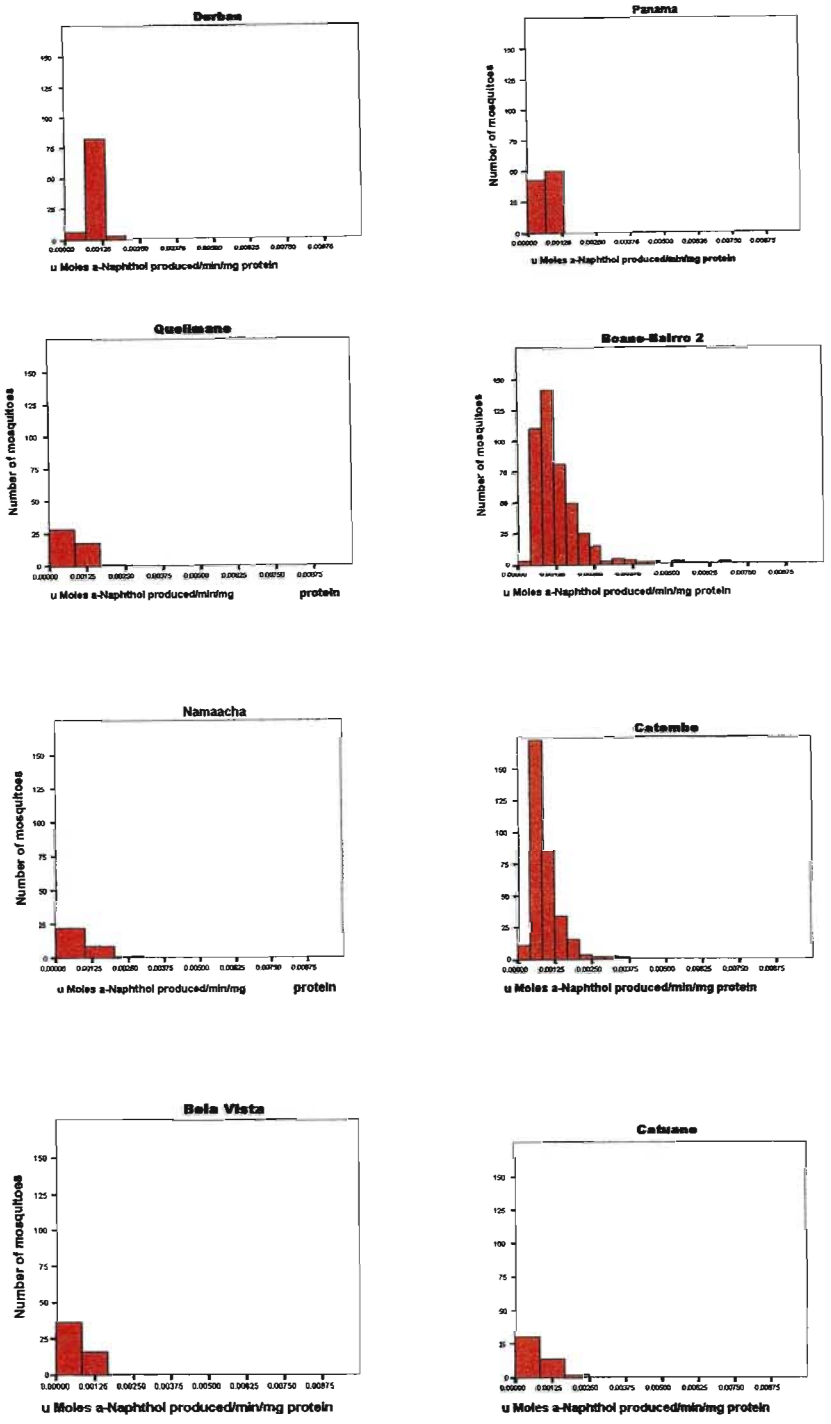

Figure 9. Range of esterase activity with α-naphthyl acetate in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected during 2000-2001) from 10 collection localities in Mozambique, compared to the Panama and Durban strains.

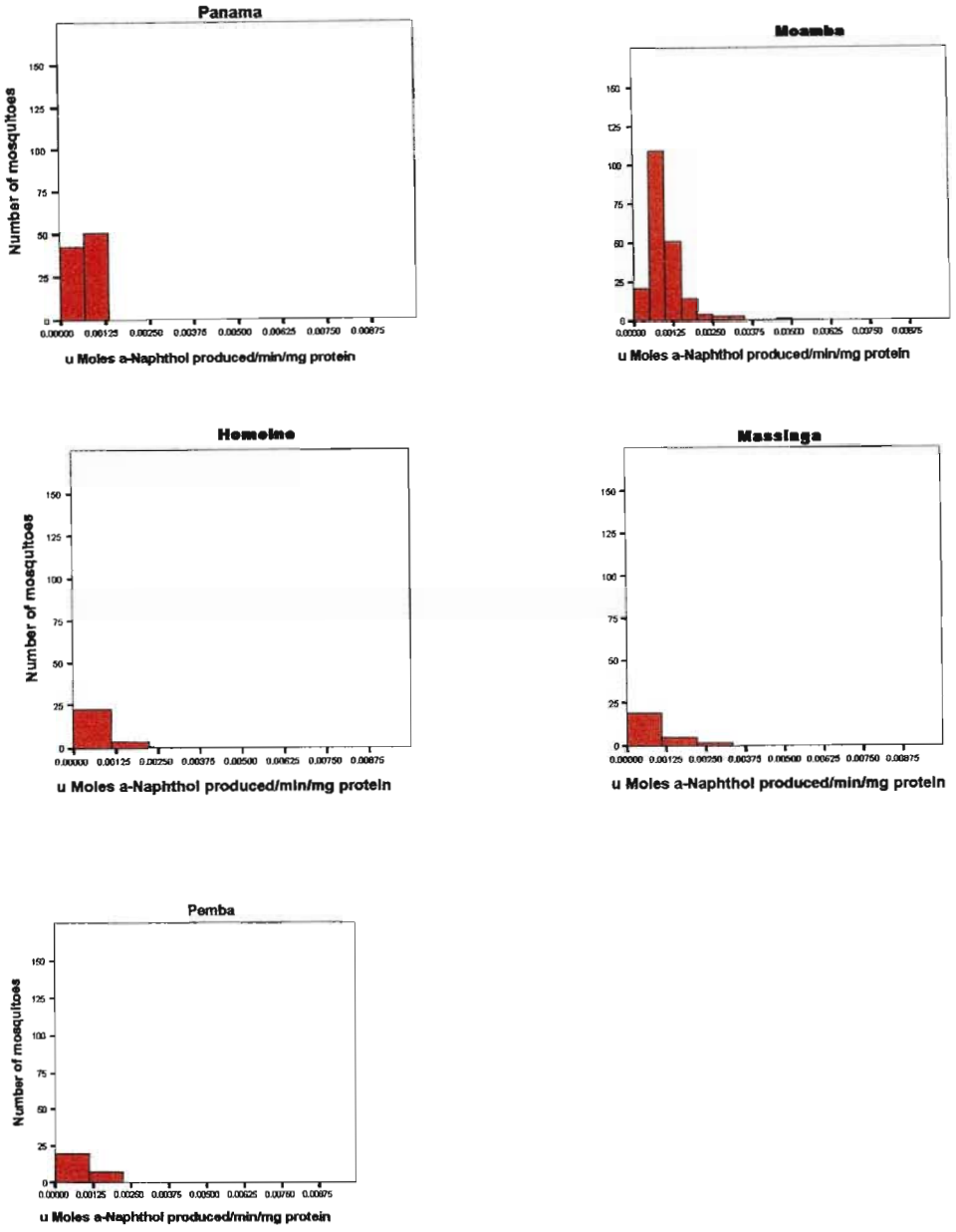

Figure 9a. Range of esterase activity with α-naphthyl acetate in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected during 2000-2001) from four collection localities in Mozambique, compared to the Panama strain.

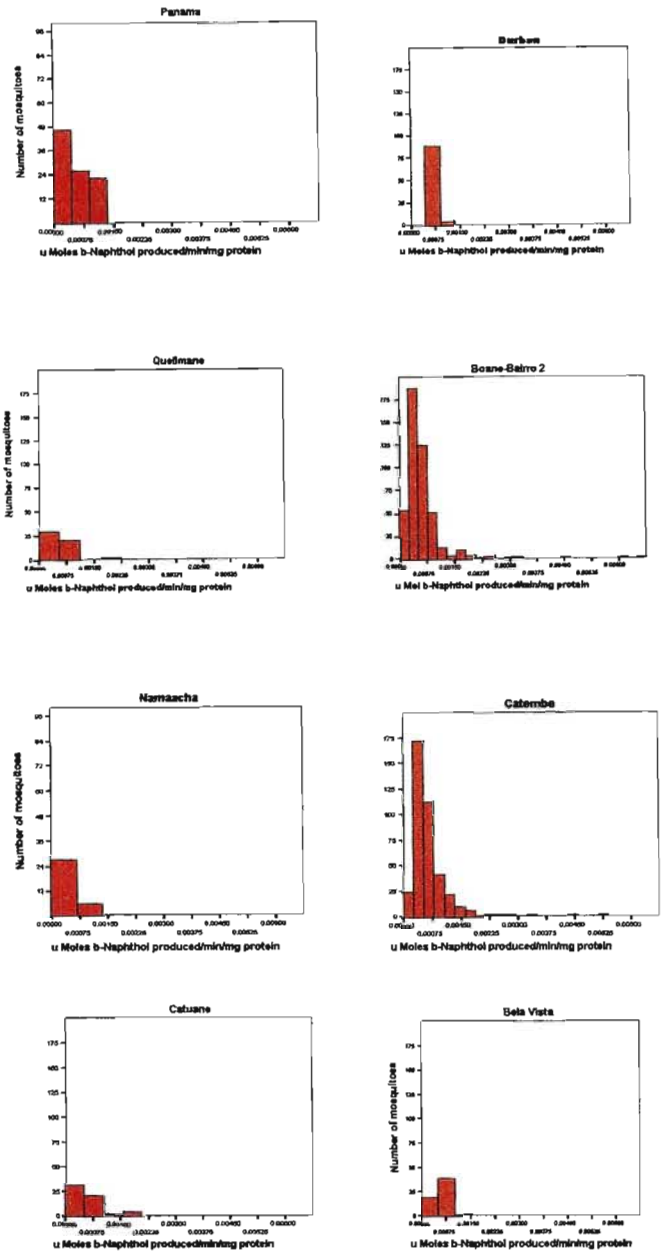

Figure 10. Range of esterase activity with β-naphthyl acetate in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected during 2000-2001) from six collection localities in Mozambique and the Durban strain compared to the Panama strain.

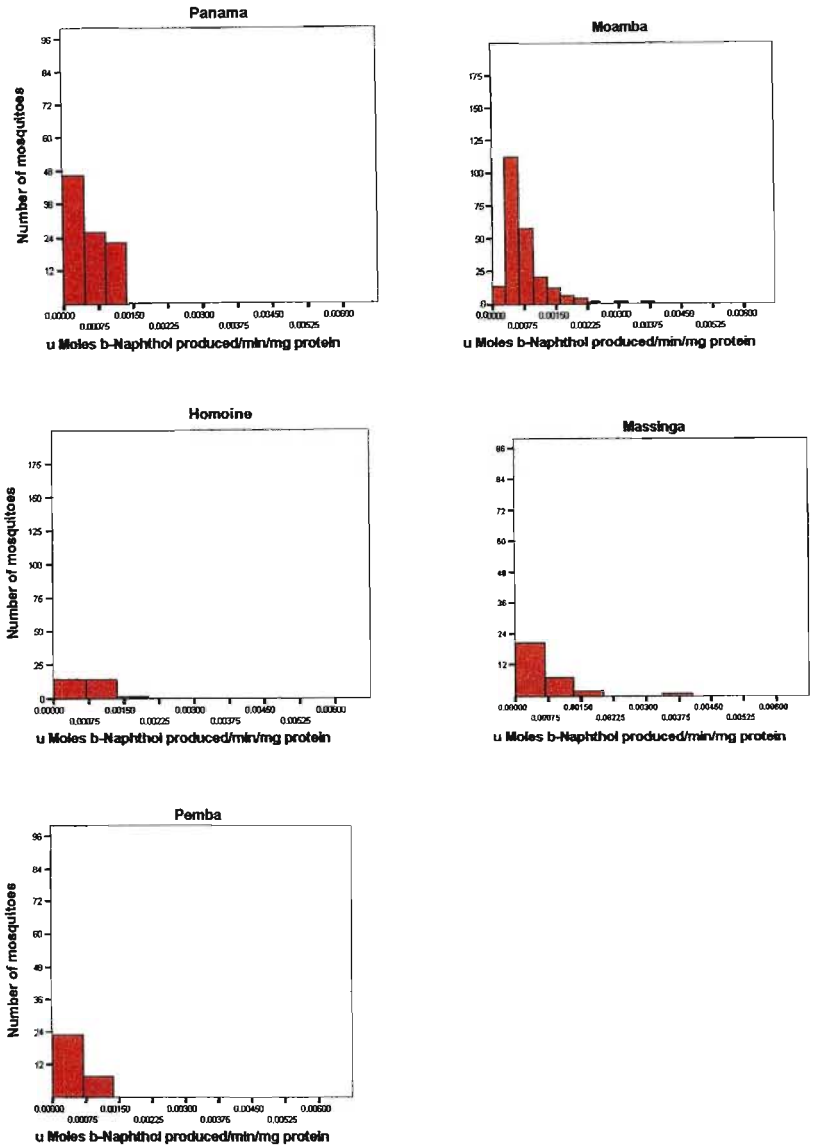

Figure 10a. Range of esterase activity with β-naphthyl acetate in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected during 2000-2001) from four collection localities in Mozambique, compared to the Panama strain.

Table XIII. Esterase activity in one-day-old Panama (*Anopheles albimanus*) and Durban (*Anopheles arabiensis*) strains and the F1 generation of *Anopheles funestus* s.s. from 2000-2001 collections. Activity was measured with the substrates α- and β-naphthyl acetate.

Strains and localities	Means SD ± nmol of product/min/mg protein			
	μ moles α-naphthol produced	n	μ moles β-naphthol produced	n
Panama	0.0009 ± 0.0002	94	0.0007 ± 0.0001	94
Durban	0.0009 ± 0.0002	94	0.0007 ± 0.0001	94
Bela Vista	0.0008 ± 0.0003	52	0.0006 ± 0.0002	57
Boane-Bairro2	0.001 ± 0.0009	451	0.0006 ± 0.0005	456
Catembe	0.0009 ± 0.0005	334	0.0007 ± 0.0006	402
Catuane	0.0008 ± 0.0004	46	0.0007 ± 0.001	59
Homoine	0.0009 ± 0.0004	28	0.0007 ± 0.0003	29
Massinga	0.001 ± 0.0006	26	0.0008 ± 0.0007	30
Moamba	0.0009 ± 0.0005	206	0.0008 ± 0.0008	232
Mozal	0.001 ± 0.0005	13	0.0007 ± 0.0004	13
Namaacha	0.0009 ± 0.0004	32	0.0005 ± 0.0002	33
Pemba	0.0009 ± 0.0004	26	0.0009 ± 0.0001	32
Quelimane	0.0008 ± 0.0003	47	0.0006 ± 0.0003	51
Mean	0.001 ± 0.0004	1261	0.0007 ± 0.0007	1394

Table XIV. Gender comparisons of esterase activity: amount of α-naphthol produced per mosquito of the Panama and Durban strains and F1 generation of *Anopheles funestus* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.0007 ± 0.0002	43	0.0008 ± 0.0003	51	0.008
Durban	0.0009 ± 0.0002	48	0.0008 ± 0.0002	46	NS*
Bela Vista	0.0009 ± 0.0003	23	0.0007 ± 0.0002	29	NS
Boane-Bairro2	0.0012 ± 0.0008	229	0.0014 ± 0.001	222	0.038
Catembe	0.0009 ± 0.0005	159	0.0009 ± 0.0006	175	NS
Catuane	0.0008 ± 0.0004	30	0.0008 ± 0.004	16	NS
Homoine	0.0009 ± 0.0004	19	0.0008 ± 0.0002	9	NS
Massinga	0.0009 ± 0.0006	18	0.0012 ± 0.0007	8	NS
Moamba	0.0011 ± 0.0005	104	0.0009 ± 0.0006	102	NS
Mozal	0.0012 ± 0.0005	9	0.0007 ± 0.0001	4	NS
Namaacha	0.0006 ± 0.0002	14	0.0011 ± 0.0004	18	0.01
Pemba	0.0009 ± 0.0004	21	0.0007 ± 0.0004	5	NS
Quelimane	0.0009 ± 0.0004	20	0.0008 ± 0.0002	27	NS
Mean	0.0011 ± 0.0006	646	0.0011 ± 0.0008	615	0.003

NS*= Not significant

Table XV. Gender comparisons of eserase activity: amount of β-naphthol produced per mosquito of the Panama and Durban strains and F1 generation of *Anopheles funestus* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.0006 ± 0.0003	43	0.0006 ± 0.0003	51	NS
Durban	0.0007 ± 0.0001	48	0.0006 ± 0.0001	46	NS*
Bela Vista	0.0006 ± 0.0002	27	0.0006 ± 0.0002	30	NS
Boane-Bairro2	0.0006 ± 0.0006	231	0.0006 ± 0.0006	225	NS
Catembe	0.0008 ± 0.0009	179	0.0007 ± 0.0005	223	NS
Catuane	0.0006 ± 0.0003	30	0.001 ± 0.001	9	0.012
Homoine	0.0007 ± 0.0003	20	0.0007 ± 0.0002	9	NS
Massinga	0.0007 ± 0.0003	21	0.001 ± 0.001	9	0.012
Moamba	0.0009 ± 0.001	114	0.0007 ± 0.0005	118	NS
Mozal	0.0008 ± 0.0004	9	0.0005 ± 0.0001	4	NS
Namaacha	0.0004 ± 0.0002	15	0.0005 ± 0.0003	18	0.034
Pemba	0.0009 ± 0.0002	25	0.0006 ± 0.0004	7	NS
Quelimane	0.0006 ± 0.0004	25	0.0005 ± 0.0002	26	NS
Mean	0.0007 ± 0.0008	696	0.0007 ± 0.0006	698	NS

NS*= Not significant

Mean esterase activities were measured with the substrates α- and β-naphthyl acetate in one day-old Panama (*An. albimanus*) and Durban (*An. arabiensis*) strains and the F1 generation of *An. arabiensis* and *An. gambiae* are given in Table XVI.

There was a significant difference in esterase activity with α- naphthyl acetate between the collection localities and between two species of *An. gambiae* complex: *An. gambiae* s.s. and *An. arabiensis*.

Esterase activities with α- and β- naphthly acetate are well below the levels seen in *An. arabiensis* population from Komhororo village of Gogwe district, Zimbabwe (Murahwa & Manokore 1995).

Differences in the levels of esterase activity with α and β-NA between males and females were observed in only one locality (Catembe) where females had significantly higher activities than males for both substrates, p=<0.016 and p=0.034 respectively for α and β-NA (Table XVII and Table XVIII)

Table XVI. Esterase activity in one-day-old Panama (*Anopheles albimanus*) and Durban (*Anopheles arabiensis*) strains and the F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* from 2000-2001 collection. Activity was measured with the substrates α- and β-naphthyl acetate.

Strains and localities	Means SD ± nmol of product/min/mg protein			
	μ moles α-naphthol produced	n	μ moles β-naphthol produced	n
Panama	0.0009 ± 0.0002	94	0.0007 ± 0.0001	94
Durban	0.0009 ± 0.0002	94	0.0007 ± 0.0001	94
Bela Vista	0.0006 ± 0.00009	6	0.0004 ± 0.0002	8
Boane-Bairro2	0.002 ± 0.0008	157	0.0009 ± 0.004	142
Catembe	0.0008 ± 0.001	75	0.0006 ± 0.001	83
Catuane	0.0008 ± 0.0005	8	0.0004 ± 0.0002	16
Homoine	0.0007 ± 0.0003	113	0.0005 ± 0.0002	125
Homoine*	0.0007 ± 0.0002	114	0.0005 ± 0.0002	132
Moamba	0.0007 ± 0.0002	85	0.0005 ± 0.0003	107
Polana-Canico	0.0007 ± 0.0002	48	0.0006 ± 0.0002	49
Mean	0.001 ± 0.004	606	0.0006 ± 0.002	662

Homoine* = *An. gambiae* s.s.

Table XVII. Gender comparisons of esterase activity: amount of α-naphthol produced per mosquito in Panama and Durban strains and F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.0007 ± 0.0002	43	0.0008 ± 0.0003	51	0.008
Durban	0.0009 ± 0.0002	48	0.0008 ± 0.0002	46	NS*
Bela Vista	0.0006 ± 0.00001	2	0.0006 ± 0.0001	4	NS
Boane-Bairro2	0.001 ± 0.0004	81	0.003 ± 0.01	76	NS
Catembe	0.0006 ± 0.0002	46	0.0010 ± 0.002	29	0.016
Catuane	0.0009 ± 0.0008	4	0.0006 ± 0.0001	4	NS
Homoine	0.0007 ± 0.0003	48	0.0007 ± 0.0003	65	NS
Homoine*	0.0007 ± 0.0003	58	0.0007 ± 0.0002	56	NS
Moamba	0.0007 ± 0.0002	40	0.0007 ± 0.0003	45	NS
Polana-Canico	0.0007 ± 0.0003	28	0.0007 ± 0.0002	19	NS
Mean	0.0007 ± 0.0003	307	0.001 ± 0.006	229	NS

NS= Not significant Homoine- *An. gambiae* s.s.

Table XVIII. Gender comparisons of esterase activity: amount of β-naphthol produced per mosquito in Panama and Durban strains and F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s.from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.0006 ± 0.0003	43	0.0006 ± 0.0003	51	NS
Durban	0.0007 ± 0.0001	48	0.0006 ± 0.0001	46	NS*
Bela Vista	0.0006 ± 0.0002	27	0.0006 ± 0.0002	30	NS
Boane-Bairro2	0.0005 ± 0.0002	76	0.001 ± 0.006	66	NS
Catembe	0.0005± 0.0001	51	0.0008 ± 0.002	32	0.034
Catuane	0.0004 ± 0.0003	9	0.0004 ± 0.0001	7	NS
Homoine	0.0005 ± 0.0002	56	0.0005 ± 0.0002	69	NS
Homoine*	0.0005 ± 0.0002	66	0.0005 ± 0.0002	66	NS
Moamba	0.0006 ± 0.0003	51	0.0005 ± 0.0002	56	NS
Polana- Canico	0.0006 ± 0.0002	28	0.0005 ± 0.0002	20	NS
Mean	0.0007 ± 0.0008	696	0.0007 ± 0.0006	698	NS

*NS= Not significant

pNPA activity

The mean values of *p*NPA esterase activity are given in table XIX and shown in Figures 11 & 11a. The mean value of *p*NPA esterase activity in one to three-day old *An. funestus* in all the collections localities were 1.75-3.25 fold higher than those for the Panama strain. All values were significantly lower than those in *Culex* mosquitoes with amplified resistance- associated esterases (Karunaratne *et al.* 1995).

Insects from Boane-Bairro 2 and Quelimane had the highest *p*NPA activity, which was 3.25 fold (for both localities) higher than the Panama strain. The lowest *p*NPA activity was observed in Massinga (1.75). Results for the Durban strain showed activity in this strain to be 2.5 fold higher than the Panama strain, showing that this mechanism of resistance was still present in the Durban strain several years after collection and maintainence without insecticide selection pressure or this could reflect a species differences.

There were significant differences in *P*NPA activity of one to three-day old *An. funestus* s.s. between collection localities ($p < 0.000$). The mean diference between the collection localities and Panama strain were significantly different in 6 of 11 collection localities: Homoine ($p < 0.031$), Boane ($p < 0.00$), Bela-Vista ($p < 0.000$), Moamba ($p < 0.00$), Catuane ($p < 0.019$) and Catembe ($p < 0.000$)

The gender comparisons for *p*NPA-activity in the Panama and Durban strains and F1s from 11 collection localities are given in Table XX. The difference in *p*NPA activity between males and females was observed in Moamba and Quelimane $p< 0.032$ and $p< 0.017$ respectively. In both localities, males had higher *p*NPA activity than females.

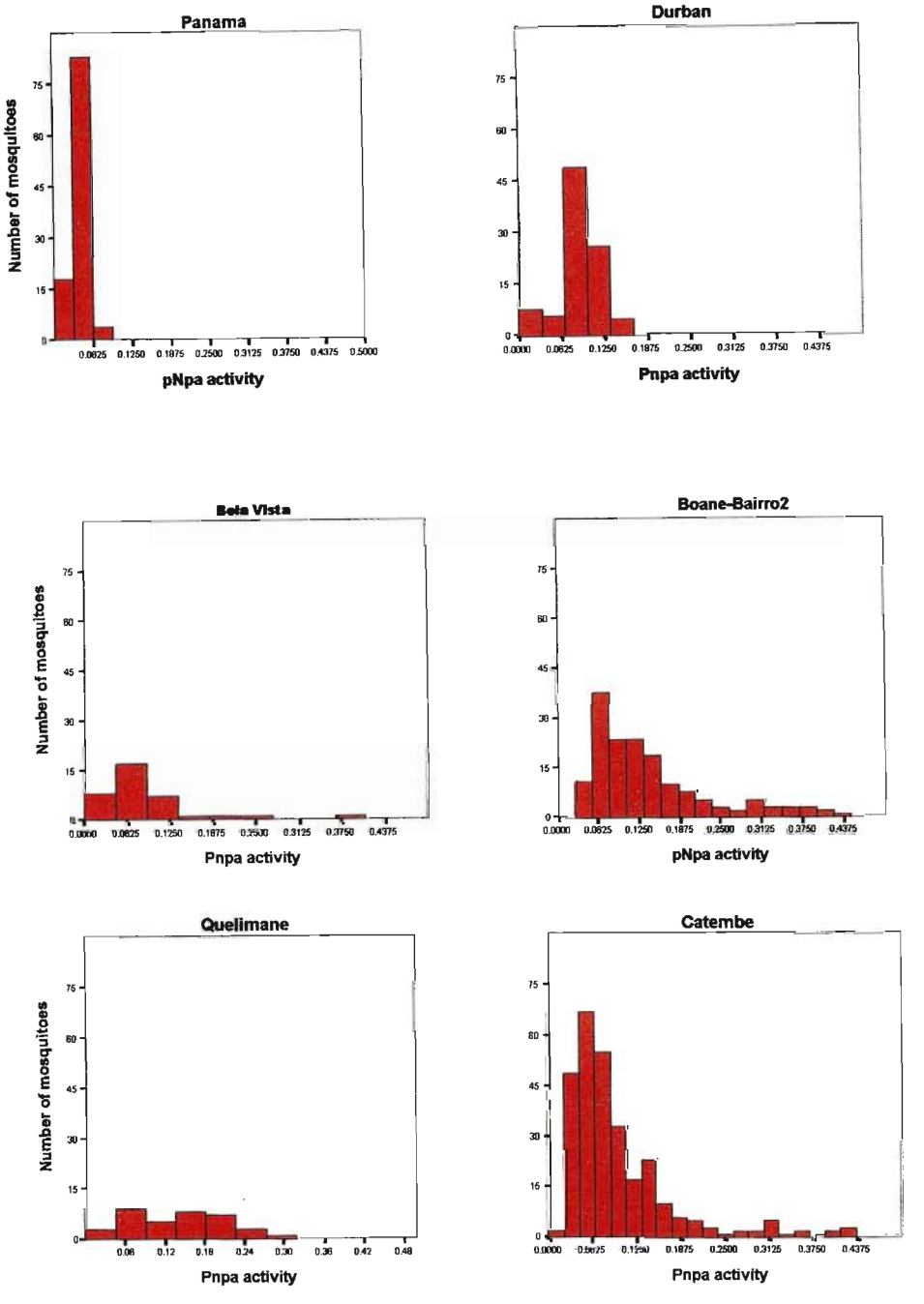

Figure 11. Range of esterase activity with p-nitrophenyl acetate (*p*NPA) in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from four study sites in Mozambique, compared to the Durban strain and to the standard susceptible Panama strain.

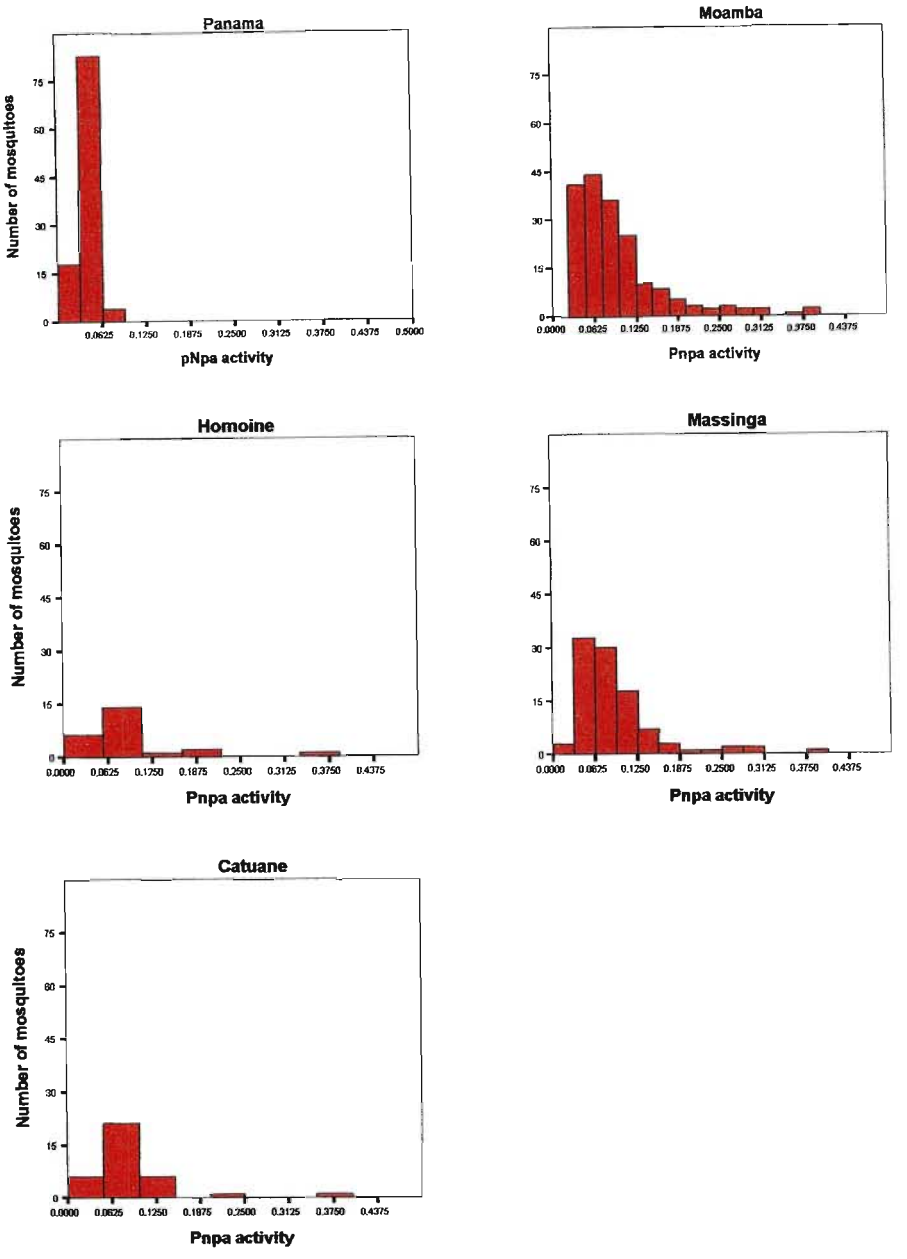

Figure11a. Range of esterase activity with p-nitrophenyl acetate (*p*NPA) in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from four study sites in Mozambique, compared to the standard susceptible Panama strain.

Table XIX. Esterase substrate *p*NPA activity in one-day-old Panama and Durban mosquitoes and F1 generation of *Anopheles funestus* s.s. from 2000-2001 collections

Strains And localities	n	μ Mol Mean ± SD Produced/min/mg protein	Higher than Panama strain
Panama	105	0.04 ± 0.02	-
Durban	94	0.09 ± 0.03	2.25
Bela Vista	36	0.09 ± 0.07	2.25
Boane-Bairro2	161	0.13 ± 0.09	3.25
Catembe	288	0.1 ± 0.08	2.50
Catuane	35	0.09 ± 0.06	2.25
Homoine	24	0.09 ± 0.07	2.25
Massinga	19	0.07 ± 0.02	1.75
Moamba	184	0.1 ± 0.07	2.50
Mozal	8	0.09 ± 0.06	2.25
Namaacha	6	0.12 ± 0.02	3.00
Pemba	20	0.09 ± 0.06	2.25
Quelimane	36	0.13 ± 0.07	3.25
Mean	817	0.11 ± 0.08	2.75

Table XX. Gender comparisons of *p*NPA-activity in one-day-old Panama and Durban mosquitoes and F1 generation *Anopheles funestus* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.04 ± 0.001	61	0.03 ± 0.02	44	0.000
Durban	0.009 ± 0.04	48	0.08 ± 0.03	46	NS*
Bela Vista	0.13 ± 0.09	18	0.06 ± 0.04	18	NS
Boane-Bairro2	0.13 ± 0.09	96	0.14 ± 0.09	65	NS
Catembe	0.10 ± 0.07	147	0.09 ± 0.08	141	NS
Catuane	0.09 ± 0.06	27	0.09 ± 0.06	8	NS
Homoine	0.11 ± 0.07	16	0.06 ± 0.02	8	NS
Massinga	0.07 ± 0.02	14	0.06 ± 0.03	5	NS
Moamba	0.1 ± 0.06	105	0.09 ± 0.08	79	0.032
Mozal	0.09 ± 0.06	7	0.06 ± -	1	-
Namaacha	0.11 ± 0.02	4	0.14 ± 0.014	2	NS
Pemba	0.09 ± 0.05	15	0.11 ± 0.09	5	NS
Quelimane	0.14 ± 0.05	18	0.13 ± 0.08	18	0.017
Mean	0.10 ± 0.07	467	0.10 ± 0.08	350	0.011

NS*= Not significant

The mean value of *p*NPA esterase activity in one to three-day old *An. arabiensis* and *An. gambiae* s.s. from collection localities compared to the Durban strain and to the standard susceptible Panama strain are represented in Table XXI. The mean values of *p*NPA activity in *An.arabiensis* and *An. gambiae* s.s. from different localities were 1.50-6.50 fold higher than those for the susceptible strain. Although all values were significantly lower than the rates seen in *Culex* mosquitoes with amplified resistance-associated esterases (Karunaratne *et al.* 1995).

Differences in *p*NPA activity between males and female were detected only in *An. arabiensis*, in three localities: Catembe ($p < 0.004$), Catuane ($p < 0.019$) and Homoine ($p < 0.012$) (Table XXI)

Table XXI. *p*NPA activity in one-day-old mosquitoes of the Panama and Durban strains and F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains and localities	n	μ Mol Mean ± SD produced/min/mg protein	Fold higher than Panama strain
Panama	105	0.04 ± 0.02	-
Durban	94	0.09 ± 0.03	2.25
Bela Vista	7	0.06 ± 0.02	1.50
Boane-Bairro2	39	0.10 ± 0.06	2.50
Catembe	62	0.26 ± 0.43	6.50
Catuane	12	0.20 ± 0.25	5.00
Homoine	77	0.12 ± 0.27	3.00
Homoine*	106	0.20 ± 0.34	5.00
Moamba	68	0.14 ± 0.3	3.50
Polana-Canico	45	0.09 ± 0.06	2.25
Mean	416	0.16 ± 0.31	4.00

Homoine *= *An. gambiae* s.s.

Table XXII. Gender comparisons of *p*NPA-activity in one-day-old Panama and Durban mosquitoes and F1 generation of *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.04 ± 0.001	61	0.03 ± 0.02	44	0.000
Durban	0.009 ± 0.04	48	0.08 ± 0.03	46	NS*
Bela Vista	0.05 ± 0.02	3	0.06 ± 0.03	4	NS
Boane-Bairro2	0.12 ± 0.07	24	0.08 ± 0.03	15	NS
Catembe	0.21 ± 0.33	39	0.35 ± 0.57	23	0.004
Catuane	0.25 ± 0.31	8	0.11 ± 0.05	4	0.019
Homoine	0.16 ± 0.39	36	0.09 ± 0.10	41	0.012
Homoine*	0.18 ± 0.30	55	0.23 ± 0.38	51	NS
Moamba	0.12 ± 0.25	32	0.15 ± 0.45	36	NS
Polana-Canico	0.09 ± 0.07	27	0.07 ± 0.03	17	NS
Mean	0.16 ± 0.28	224	0.17 ± 0.35	192	NS

NS*= Not significant, Homoine* = *An. gambiae* s.s

Monooxygenases (MFO)

Titres of monooxygenases in *An. funestus* s.s. estimated from bound haem quantification from collection localities and the Durban strain of *An. arabiensis* compared to the susceptible Panama strain are shown in Figure 12. There were only a small number of F1 generation *An. funestus* s.s. from all the collections localities with elevated monooxygenases titres, which suggests that this resistance mechanism is not operating in these areas. Penilla *et al.* (1998) found very low monooxygenase titres in *An. albimanus* populations from Mexico. The Durban *An. arabiensis* strain also had very low MFO titres.

Mosquitoes from Catuane had the highest mean titres of MFO, which were 2.25 fold higher than the Panama strain (Table XXIII). However there were no significant differences in mean titres of MFO between collection sites and the Panama strain.

Table XXIV gives a comparison of cytochrome p450 titres between males and females. Differences in MFO between males and females were detected in four localities. Males in Bela Vista, Homoine, Moamba had high titres of MFO compared to females and females in Namaacha than males. The Panama strain also showed a difference in MFO titres between males and females.

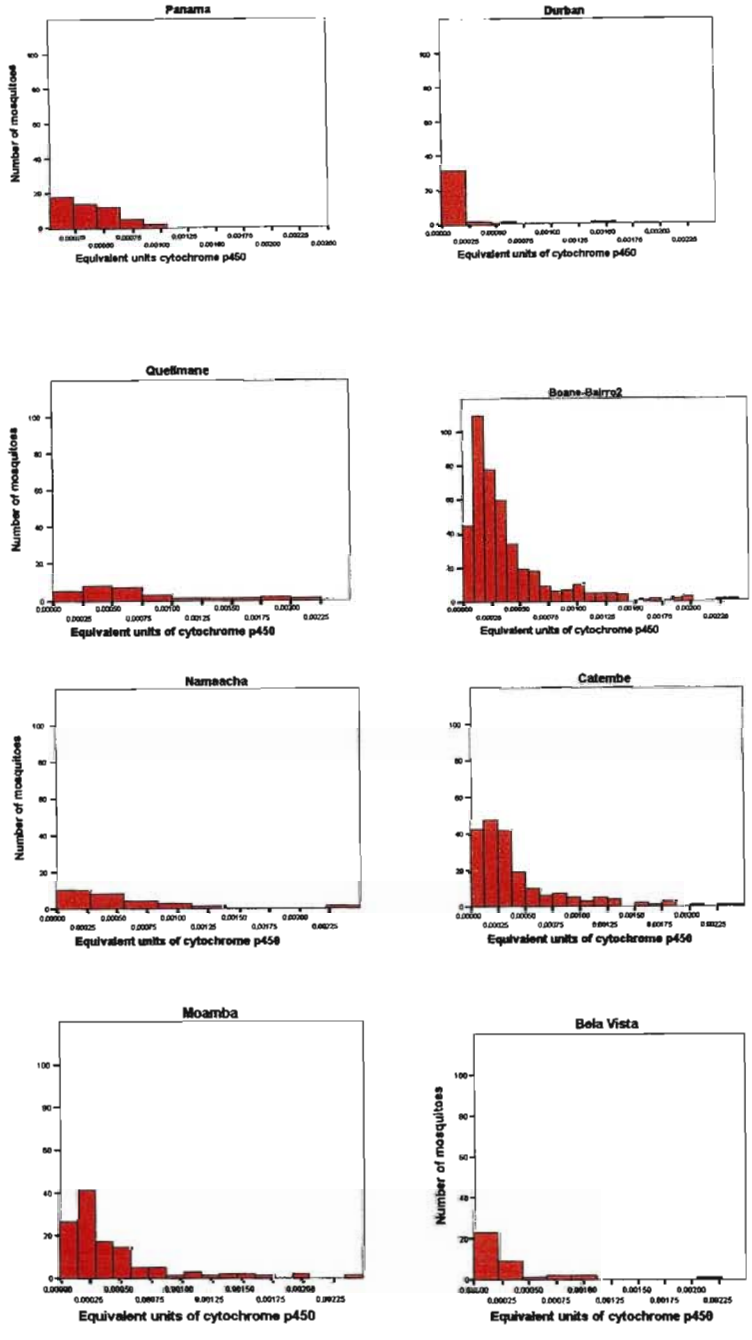

Figure12. Monooxygenase titres in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from six study sites in Mozambique compared to the Durban strain and to the standard susceptible Panama strain.

Table XXIII. Equivalent units of cytochrome p450 (EUC) in one-day-old *Anopheles albimanus* of the Panama and Durban strains and F1 generation (*Anopheles funestus* s.s.) from 2000-2001 collections

Strains And localities	n	µ Mol Mean ± SD Produced/min/mg protein	Higher than Panama strain
Panama	57	0.0004 ± 0.0004	-
Durban	36	0.0002 ± 0.0002	*
Bela Vista	38	0.0003 ± 0.0004	*
Boane-Bairro2	437	0.0007 ± 0.0046	1.75
Catembe	204	0.0005 ± 0.0006	1.25
Catuane	22	0.0009 ± 0.0023	2.25
Homoine	17	0.0002 ± 0.0001	*
Massinga	16	0.0004 ± 0.0004	0
Moamba	126	0.0006 ± 0.0009	1.5
Mozal	5	0.0004 ± 0.0008	0
Namaacha	27	0.0005 ± 0.0005	1.25
Pemba	8	0.0004 ± 0.0005	0
Quelimane	29	0.0007 ± 0.0006	1.75
Mean	929	0.0006 ± 0.003	1.50

* Less than the Panama strain

Table XXIV. Gender comparisons of cytochrome P450 equivalent units representing monooxygenases in one-day-old *Anopheles albimanus* of the Panama and Durban strains and F1 generation *Anopheles funestus* s.s. from the 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.0002 ± 0.0001	14	0.0005± 0.0004	43	0.008
Durban	0.0001 ± 0.0006	17	0.0002 ± 0.0003	19	NS*
Bela Vista	0.0004 ± 0.0005	20	0.0002 ± 0.0002	18	0.025
Boane-Bairro2	0.0008 ± 0.006	233	0.0006 ± 0.001	204	NS
Catembe	0.0004 ± 0.0008	55	0.0005 ± 0.0005	149	NS
Catuane	0.002 ± 0.0004	30	0.0008 ± 0.004	16	NS
Homoine	0.0009 ± 0.004	9	0.0003 ± 0.0003	91	0.017
Massinga	0.0003 ± 0.0003	11	0.0005 ± 0.0004	5	NS
Moamba	0.0007 ± 0.0001	45	0.0005 ± 0.0007	81	0.003
Mozal	0.0002 ± 0.00006	3	0.0008 ± 0.0002	2	NS
Namaacha	0.0004 ± 0.0003	20	0.0007 ± 0.0008	7	0.032
Pemba	0.0003 ± 0.0005	6	0.0007 ± 0.0004	2	NS
Quelimane	0.0008 ± 0.0005	13	0.0006 ± 0.0006	16	NS
Mean	0.0007± 0.004	425	0.0005 ± 0.0009	504	NS

NS*= Not significant

Table XXV gives the mean titres of MFO in *An. gambiae* s.s. and *An. arabiensis* F1 generation females at all collections localities. Mosquitoes from Catuane (*An. arabiensis*) and Homoine (*An. arabiensis* and *An. gambiae* s.s.) had the highest mean titres of MFO, which were 3.50 fold higher than the standard susceptible strain. Because of the small number of mosquitoes used for this assay, the mean titres of MFO between males and females of these species were not compared.

Table XXV. Equivalent units of cytochrome p450 (EUC) in one-day-old *Anopheles albimanus* of the Panama and Durban strains and F1 generation *Anopheles arabiensis* from 2000- 2001 collection.

Strains And localities	n	μ Mol Mean ± SD Produced/min/mg protein	Higher than Panama strain
Panama	57	0.0004 ± 0.0004	-
Durban	36	0.0002 ± 0.0002	*
Bela Vista	2	0.001 ± 0.002	2.50
Boane-Bairro2	86	0.0005 ± 0.002	1.25
Catembe	7	0.0007 ± 0.0003	1.75
Catuane	2	0.0009 ± 0.00006	2.25
Homoine	5	0.001 ± 0.0006	2.50
Homoine*	8	0.001 ± 0.001	2.50
Moamba	12	0.0007 ± 0.0006	1.75
Polana Canico	12	0.0006 ± 0.0003	1.50
Mean	134	0.0006 ± 0.002	1.50

* Less than the Panama strain, Homoine*=*An. gambiae* s.s.

Glutathione S-transferase activity

Elevated GST is a major mechanism of DDT resistance in *Anopheles* mosquitoes and the cause of both DDT and OP resistance in houseflies and *Anopheles subpitcus* Grassi (Clark *et al.* 1984; Hemingway *et al.* 1991). It also confers pyrethroid resistance indirectly by moderating the effects of oxygen free radicals produced by these insecticides (Vontas *et al.* 2001).

Figure 13 & 13a represent the frequency distributions for GST activity of *An. albimanus* (Panama) and *An. arabiensis* (Durban) strains and F1 generation *An. funestus* s.s from different collection localities. Mozal, Moamba, Catembe, Boane and Bela-Vista had high levels of GST activity. Elevated GST activity has been detected in organophosphate resistant houseflies and in DDT resistant *An. albimanus* (Penilla *et al.* 1998). In this study, the high levels of GST activity detected were not correlated with DDT resistance, as WHO susceptibility tests showed complete susceptibility of *An. funestus* to this insecticide in all the study areas. It is possible that these enzymes in the *An. funestus* s.s. populations from southern Mozambique are involved in pyrethroid resistance, but this needs to be confirmed by further biochemical analysis. All localities with high levels of GST activity had high levels of pyrethroid resistance. Vontas *et al.* (2001), implicated elevated GST activity in resistance to pyrethroids in olive fruit flies.

The mean GST activity from different collections ranged from 0.11 (± 0.13) to 0.64 (± 0.49) nmol min^{-1} mg^{-1}protein (Table XXVI). The highest GST activity was observed in Mozal, which had a 4.6- fold higher activity than the Panama strain. This mean is similar to that seen in resistant *An. albimanus* (Penilla *et al.* 1998). These average activity values for all collection localities were higher than the Panama strain. However, the differences in mean GST activity between the collection and the Panama strain were significantly different in only five of the 11 collection sites: Moamba ($p = 0.000$); Catembe ($p = 0.000$); Boane ($p = 0.025$); Bela- Vista ($p = 0.001$) and Mozal ($p = 0.047$)). There was no significant difference in GST activity of one to three-day old *An. funestus* s.s between the collection localities ($p = 0.146$).

Differences in the levels of GST activity between males and females were observed in only one locality (Quelimane) $p < 0.045$ (Table XXVII) where females had a higher levels GST than males. This indicates as expected that this resistance mechanism is not sex-linked.

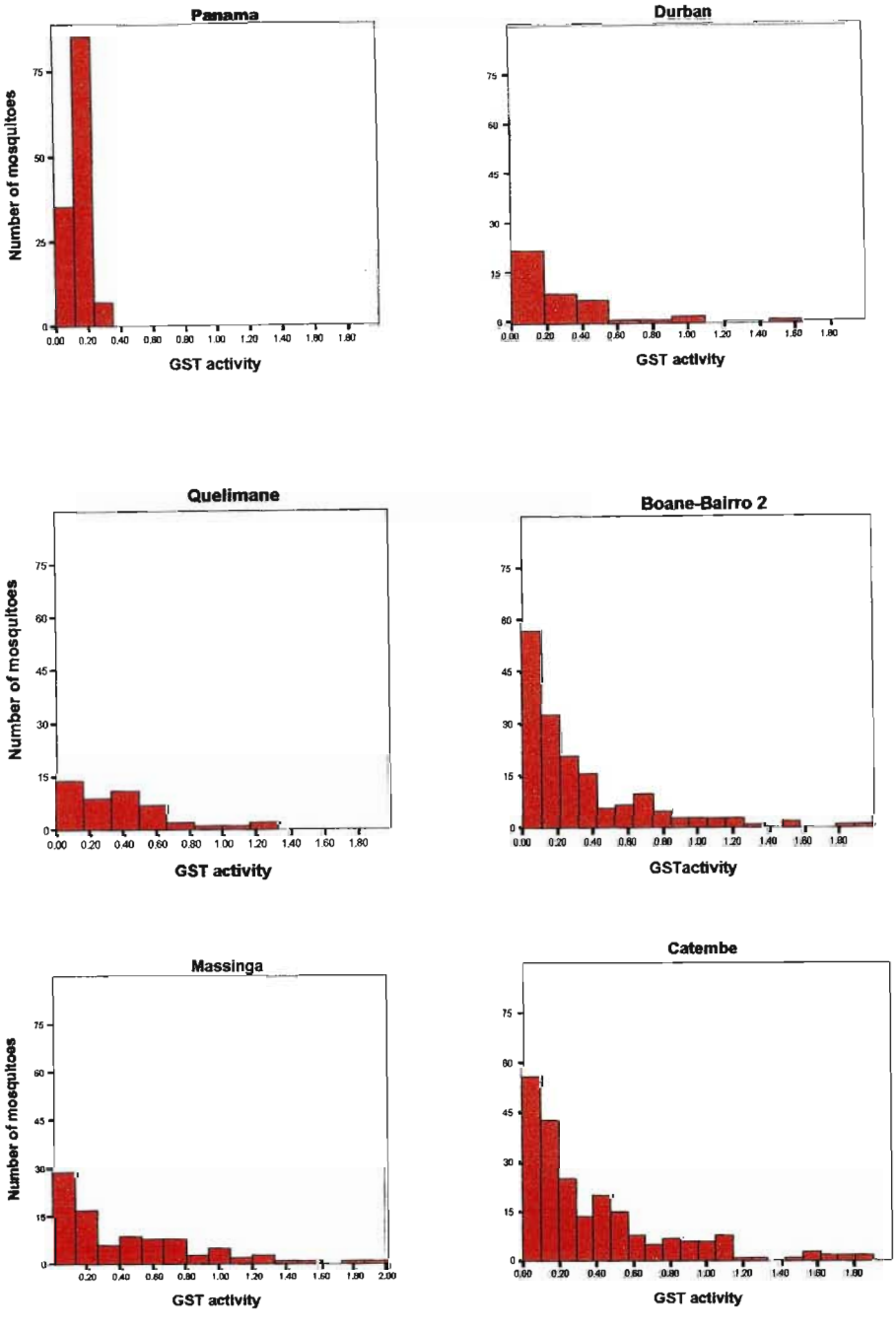

Figure 13. Ranges of glutathione S-transferase (GST) activity with the substrate dichloronitrobenzene in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from four study sites in Mozambique, compared to the Durban strain and to the standard susceptible Panama strain.

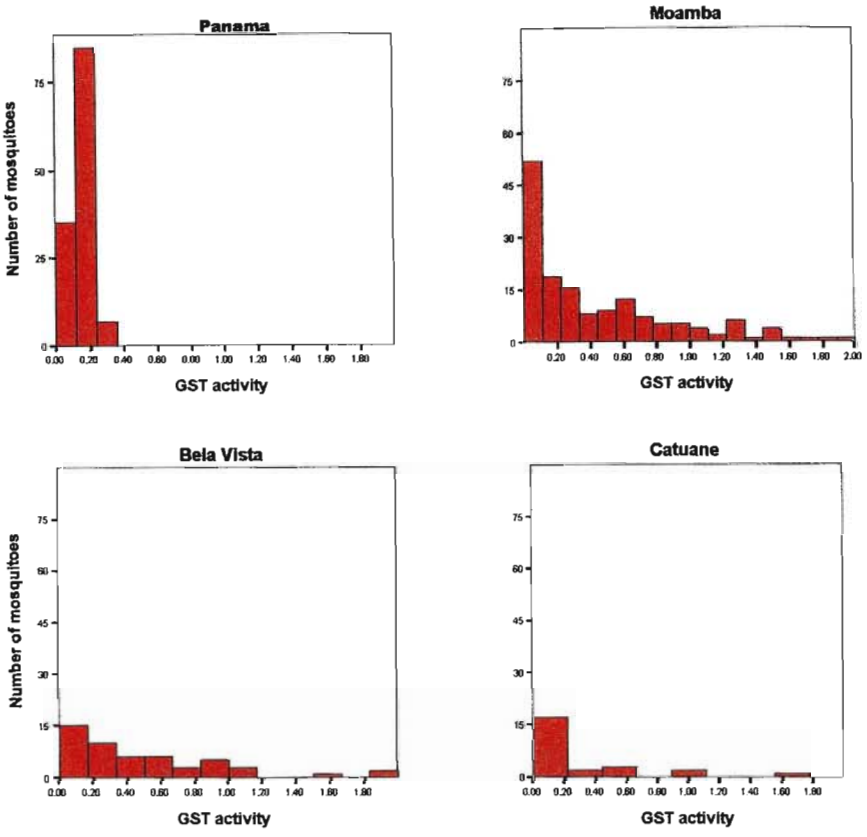

Figure 13a. Ranges of glutathione S-transferase (GST) activity with the substrate dichloronitrobenzene in *Anopheles funestus* s.s. (F1 adult progeny from wild-caught females collected in 2000-2001) from three study sites in Mozambique, compared to the standard susceptible Panama strain.

Table XXVI. GST activity in one-day-old *Anopheles albimanus* of the Panama and *Anopheles arabiensis* of the Durban strains and F1 generation *Anopheles funestus* s.s. from 2000-2001 collection.

Strains And localities	n	GST activity	Higher than Panama strain	P**
Panama	127	0.14 ± 0.06	-	-
Durban	43	0.29 ± 0.31	2.1	NS
Bela Vista	50	0.49 ± 0.47	3.5	0.001
Boane-Bairro2	170	0.34 ± 0.36	2.4	0.025
Catembe	219	0.44 ± 0.71	3.1	0.000
Catuane	23	0.31 ± 0.43	2.2	NS
Homoine	12	0.19 ± 0.28	1.3	NS
Massinga	10	0.39 ± 0.31	2.8	NS
Moamba	150	0.45 ± 0.46	3.2	0.000
Mozal	11	0.64 ± 0.49	4.6	0.047
Namaacha	17	0.41 ± 0.49	2.9	NS
Pemba	10	0.11 ± 0.13	*	NS
Quelimane	46	0.38 ± 0.31	2.7	NS
Mean	718	0.41 ± 0.52	2.9	NS

* Less than the Panama strain ** p value of difference in GST mean between the collections site and the Panama strain.

Table XXVII. Gender comparisons of GST activity in one-day-old *Anopheles albimanus* of the Panama and *Anopheles arabiensis* of the Durban strains and F1 generation *Anopheles funestus* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.16 ± 0.05	64	0.12 ± 0.06	63	NS*
Durban	0.32± 0.36	22	0.25 ± 0.26	21	NS
Bela Vista	0.46 ± 0.44	22	0.50 ± 0.49	28	NS
Boane-Bairro2	0.32 ± 0.33	85	0.35 ± 0.39	85	NS
Catembe	0.44 ± 0.96	94	0.45 ± 0.43	125	NS
Catuane	0.25 ± 0.36	16	0.43 ± 0.58	7	NS
Homoine	0.22 ± 0.37	7	0.16 ± 0.07	5	NS
Massinga	0.43 ± 0.32	6	0.32 ± 0.31	4	NS
Moamba	0.48 ± 0.48	65	0.42 ± 0.44	84	NS
Mozal	0.81 ± 0.49	7	0.32 ± 0.33	4	NS
Namaacha	0.37 ± 0.43	12	0.5 ± 0.65	5	NS
Pemba	0.12 ± 0.14	8	0.08 ± 0.09	2	NS
Quelimane	0.31 ± 0.22	23	0.45 ± 0.37	23	0.045
Mean	0.39 ± 0.61	346	0.41 ± 0.43	372	NS

*NS= Not significant

The GST activity results for *An. gambiae* s.s. and *An. arabiensis* are shown in Table XXVIII. Average activity values for all collections localities were higher than the Panama strain. The highest average GST activity was in Catuane which had a 4.36 fold higher activity than the Panama strain.

Differences in GST activity between males and females were detected in three collection localities: Catuane (p=0.038), Homoine (0.007) and Moamba (p=0.049) (Table XXIX).

Table XXVIII. GST activity in one-day-old *Anopheles albimanus* of the Panama and *Anopheles arabiensis* of the Durban strains and F1 generation *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains And localities	n	GST activity Mean ± SD	Higher than Panama strain
Panama	127	0.14 ± 0.06	-
Durban	43	0.29 ± 0.31	2.10
Bela Vista	12	0.58 ± 0.28	4.14
Boane-Bairro2	68	0.26 ± 0.26	1.86
Catembe	56	0.53 ± 0.59	3.78
Catuane	7	0.61 ± 0.79	4.36
Homoine	60	0.26 ± 0.44	1.86
Homoine*	76	0.32 ± 0.43	2.28
Moamba	63	0.29 ± 0.49	2.07
Polana Canico	27	0.43 ± 0.56	3.07
Mean	369	0.35 ± 0.47	2.50

Homoine * = *An. gambiae* s.s.

Table XXIX. Gender comparisons of GST activity in one-day-old *Anopheles albimanus* of the Panama and *Anopheles arabiensis* of the Durban strains and F1 generation *Anopheles arabiensis* and *Anopheles gambiae* s.s. from 2000-2001 collection.

Strains and localities	% inhibition: Mean ± SD				Gender Diff. P
	Males	n	Females	n	
Panama	0.16 ± 0.05	64	0.12 ± 0.06	63	NS*
Durban	0.32± 0.36	22	0.25 ± 0.26	21	NS
Bela Vista	0.63 ± 0.34	6	0.54 ± 0.22	6	NS
Boane-Bairro2	0.25 ± 0.24	42	0.28 ± 0.28	26	NS
Catembe	0.46 ± 0.53	32	0.63 ± 0.66	24	NS
Catuane	0.84 ± 1.22	3	0.45 ± 0.40	4	0.038
Homoine	0.20 ± 0.26	27	0.31 ± 0.54	33	0.007
Homoine**	0.25 ± 0.31	35	0.39 ± 0.51	41	NS
Moamba	0.36 ± 063	32	0.22 ± 0.26	31	0.049
Polana Canico	0.39 ± 0.49	18	0.50 ± 0.70	9	NS
Mean	0.33 ± 0.45	195	0.37 ± 0.49	174	NS

NS*= Not significant, ** = *An. gambiae* s.s.

Locality resistance comparison between *Anopheles funestus* s.s. and *Anopheles arabiensis*

If we compare the results from the biochemical tests for both species (*An. funestus* s.s. and *An. arabiensis*) from the same areas, the results show that: altered acetylcholinsterase were found for both species tested (Table VII & X).

Acetylcholinesterase is the target site for organophosphate and carbamate (Liu *et al.* 1988). However susceptibility tests showed complete susceptibility in both species to organophosphate (malathion) and very low level of survival to carbamates was observed in *An. funestus*.

Very low monooxygenases titres were registered in both species, which suggest that this resistance mechanism is not operating in these areas (Table XIII & XXV).

There was no locality correlation between elevated GST in the *An. arabbiensis* and *An. funestus*, although elevated levels were found in both *An. funestus* and *An. arabiensis* were from different localities (XXVI & XXVIII).

CHAPTER 5

CONCLUSIONS

Distribution of the *Anopheles gambiae* complex and *Anopheles funestus* group in Mozambique.

- Three species of the *An. gambiae* complex, namely *An. gambiae* s.s, *An. arabiensis* and *An. quadriannulatus* and only one of the *An.funestus* group, *An.funestus* were identified from indoor-collected samples from 25 localities in 10 provinces, using the polymerase chain reaction species- specific identification technique.

- Previous studies have shown *An. merus* to occur in Mozambique as well as *An. gambiae* s.s., *An. arabiensis* and *An. quadiannulatus*.

- *Anopheles arabiensis* is widely distributed in southern Mozambique, and was also identified from the central region.

- *Anopheles quadriannulatus* was only found in the southern region (Catuane, Boane, Moamba, Ressano Garcia and Chokwe) and in the central region of Mozambique (Tete).

- *Anopheles gambiae* s.s. was the only member species of the *An. gambiae* complex identified from the northern region and it was not widely distributed in southern Mozambique.
- *Anopheles funestus* s.s. was found to be widely distributed thoughtout the country. This species was identified from 24 of the 25 collection sites from the 10 provinces sampled.

The susceptibility test results from this study:

- *Anopheles gambiae* s.s., *An. arabiensis* and *An. funestus* s.s. were tested for susceptibility to all four groups of insecticides namely: pyrethroids, organophosphates, carbamates and organochlorines. Only *An. gambiae* s.s. was found to be susceptible to all four groups of insecticides.

- *Anopheles arabiensis* was found to be resistant to only one insecticide, lambda-cyhalothrin, from only one locality, Manjacaze.

- Pyrethroid resistance in *An. funestus* s.s. was widespread in southern Mozambique, in Maputo City (Bairro Benfica), Maputo province (Bela-Vista, Catembe, Catuane, Boane-Bairro 2, Mozal, Moamba) and one locality of Gaza province (Chokwe), but not in central or northern Mozambique.

- There was evidence of carbamate resistance in *An. funestus* s.s. from six localities in Maputo province

- No resistance to organophosphate and DDT was observed from any of the collection sites for any species.

Biochemical test indications:

In regard to *Anopheles funestus* s.s., the following were apparent:

- Presence of altered acetylcholinesterase was found at all collections localities with the exception of Massinga.
- Elevated esterase with α-naphthyl acetate from Boane-Bairro 2.
- Elevated GST from Boane, Moamba and Catembe.
- Very low monooxygenase titres were registered from all the localities sampled.

In regard to *Anopheles arabiensis* and *Anopheles gamiae* s.s., the following were apparent:

- Elevated esterase with substrate α-naphthyl acetate was detected *in An. arabiensis* from only one locality (Boane-Bairro 2).
- Elevated GST were detected in *An.arabiensis* and *An.gambiae* s.s from all localities.
- Very low monooxygenases titres were registered in both species from all the localities sampled.

As expected, the resistance mechanisms were generally not sex-linked. Although there were observed differences in GST activity between males and females from Catuane, Homoine and Moamba.

RESEARCH RECOMMENDATIONS

1. There is a need for further research (biochemical and metabolic studies) to clarify the involvement of each mechanism in insecticide resistance.

2. A sentinel site monitoring system should be put place in Mozambique to evaluate changes in insecticide resistance over time and to monitor geographic distribution of resistance.

3. A rotational spraying programme based on the succeful Mexico trial (Rodriguez *et al.* 2002) needs to be initiated to evaluate its efficacy in vector control and resistance containment in African vectorial systems. This has regional significance given that resistance in *An. funestus* is widespread in southern Mozambique and is also present in South Africa. This situation has compromised vector control by indoor spraying.

4. There is a need to study the selection impact of agricultural pesticide use on the development of vector resistance in malaria – carrying mosquitoes

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Appendix 1

Actual malaria vector control programme in the study area

Nampula province

Namialo: IRS annually with Lambda-cyhalothrin

Nampula City: Unsprayed area

Niassa

Lichinga: Unsprayed area

Cabo-Delgado

Pemba: Since 2000, IRS annually with Lambda-cyhalothrin

Zambezia

Quelimane: Unsprayed area

Sofala

Mafambissa: IRS annually with Lambda-cyhalothrin

Inhambane

Mafambissa: Unsprayed area

Homoine: Unsprayed area

Gaza

Chokwe: IRS annually with Lambda-cyhalothrin and ITN programm

Manjacaze: IRS annually with Lambda-cyhalothrin

Maputo City

Polana-Canico: IRS annually with Lambda-cyhalothrin

Benfica: IRS annually with Lambda-cyhalothrin

Maputo province

Boane (Manguisa, Djimo and Bairro 2): Since December 2001, IRS with Bendiocarb

Bela-Vista: Since 2000, IRS with Bendiocarb

Catuane: Since 2000, IRS with Bendiocarb

Namaacha: Since 2000, IRS with Bendiocarb

Mozal: Since 2001 IRS with Bendiocarb

Ressano Garcia: Unsprayed area

Manhica: Unsprayed area

Marracuene: Unsprayed area

Moamba: Unsprayed area

Appendix 2

Study areas and periods of data collection

Study area		Latitude (S)	Longitude (E)	Data collection period
Province	Locality			
Niassa	Lichinga	25.8952	32.5652	Jun 02
Cabo-Delgado	Pemba	13.10194	40.44806	May 00 & May 01
Nampula	Namialo			June 01 & May 02
	Nampula	15.11972	39.35111	May 02
Sofala	Mafambissa	19.613	34.735	May 01 and June 02
Tete	Tete	15.91472	33.15881	Julho, Oct.01 & Feb.02
Zambezia	Quelimane	17.7889	36.89722	May,Sep.01 & Feb.02
Inhambane	Homoine	23.94194	35.17944	April & August 01
	Massinga	23.40944	35.37861	April2001
Gaza	Chokwe	24.66722	33.09389	May, Jun00; May 01, &Feb,May 02
Maputo – Province	Boane	26.05806	32.35278	Mach 00 to July 02
	Bela-Vista	26.342778	32.6736111	May 00 to July 02
	Mozal	25.895	32.42278	October 00- July 02
	Catuane	26.77	32.37278	June 00 - July 02
	Ressano Garcia	25.44139	31.995	February-July 02
	Moamba	25.59694	32.23472	January 01 - July 02
	Marracuene	25.77167	32.67222	January -July 2002
	Manhica	25.47611	32.75111	August 2001 -July 02
	Catembe			Nov 00 to Jan 02
	Namaacha	25.965	32.02361	Feb – March 00
Maputo City	Polana Canico	25.9325	32.61361	Nov 01 to Feb 02
	Benfica	25.89578	32.56528	January 01 to July 02

Apendix 3

Distribution records of species of *Anopheles gambiae* complex in Mozambique.

Province	Locality	Specie	Reference
Cabo Delgado	Olumbi	*An. gambiae* s.s. and *An. merus*	Petrarca *et al.* 1984
	Mocimboa da Praia	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Mueda	*An. gambiae* s.s.	Petrarca *et al.* 1984
	N'guri	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Pemba	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Montepuez	*An. gambiae* s.s.	Petrarca *et al.* 1984
Niassa	Metangula	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
	Unango	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
	Meponda	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
	Lichinga	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
	Matama	*An. gambiae* s.s.	Petrarca *et al.* 1984
Nampula	Nampula	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Namapa	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Memba	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Ribaue	*An. gambiae* s.s.	Petrarca *et al.* 1984
	Namialo	*An. gambiae* s.s.	Petrarca *et al.* 1984
Tete	Moatize	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
			Petrarca *et al.* 1984
Sofala	Marromeu	*An. gambiae* s.s. and *An. arabiensis*	Petrarca *et al.* 1984
	Beira	*An. gambiae* s.s and *An. merus*	Petrarca *et al.* 1984
			Petrarca *et al.* 1984
Gaza	Chokwe	*An. arabiensis*	Rungo 1999
		An. merus	
			Petrarca *et al.* 1984
Maputo Province	Palmeira	*An. arabiensis*	Petrarca *et al.* 1984
	Marracuene	*An. arabiensis* and *An. merus*	Petrarca *et al.* 1984

Province	Locality	Specie	Reference
Maputo Province	Bela-Vista	*An. arabiensis*	Petrarca *et al.* 1984, Rungo 1999
		An. merus	Petrarca *et al.* 1984
		An. quadriannulatus	Petrarca *et al.* 1984
	Salamanga	*An. arabiensis* and *An. merus*	Petrarca *et a.*, 1984, Rungo 1999
	Tinonganine	*An. arabiensis* and *An. merus*	Petrarca *et al.* 1984
	Boane	*An. arabiensis; An. merus*	Mnzava *et al.* 1997, Rungo 999, Donnelly *et al*, 1999
	Matola	*An. arabiensis*	Mnzava *et al.* 1997, Donnelly *et al.* 1999, Mendis *et al.* 2000 Thompson *et al.* 1997.
		An. merus	Donnelly *et al.* 1999
Maputo City	Laulane	*An. arabiensis* and *An. merus*	Mnzava *et al.* 1997
	Polana	*An. arabiensis*	Mnzava *et al.* 1997
	Jardim	*An. arabiensis* and *An.merus*	Mnzava *et al.*1997

Appendix 4

Distribution of *Anophele funestus* s.s. indentified by PCR, in collection localities in Mozambique (2000-2002)

Province	Locality	n
Niassa	Lichinga	4
Cabo delgado	Pemba	24
Nampula	Nampula	4
	Namialo	28
Sofala	Mafambissa	5
Zambezia	Quelimane	15
Imhambane	Homoine	8
	Massinga	7
Gaza	Manjacaze	7
	Chokwe	13
Maputo-City	Benfica	6
	Polana-Canico	4
Maputo-Province	Catembe	72
	Bela-Vista	9
	Catuane	16
	Mozal	20
	Boane-Bairro 2	104
	Boane- Jimo	7
	Boane- Manguisa	8
	Namaacha	15
	Ressano Garcia	5
	Moamba	48
	Marracuene	4
	Manhica	7
Total		440

Appendix 5

MIM WHO Insecticide Susceptibility Tests Sheet No.

Mosquito Code Identifier:

Insecticide tested:

Date of expiry:

Batch No.:

Temp.: %RH:

Number of mosquitoes tested:

Time (min)	Knockdown data	
	Test	Control
10		
20		
30		
40		
50		
60		

Scores after 24 hours

No. of test mosquitoes dead: % test mosquitoes dead

No. of control mosquitoes dead: % control mosquitoes dead:

If control dead is between 5-20%, adjusted % test mosquitoes dead using Abbot's formula

Observations:

Apendix 6

Summary statistics for the different enzyme baselines in the Panama susceptible strain of *Anopheles albimanus*

Mechanism	n	Mean	SE	Min	Max
AChE inhibition	94	86.6	8.3	64.78	100
α- esterase activity	94	0.0008	0.0003	0.0039	0.0123
β- esterase activity	94	0.0006	0.0003	0.0017	0.0118
Eq. Units of cytochrome p450	132	0.0004	0.0002	0.0002	0.02
PNPA activity	105	0.04	0.02	0.0008	0.7
GST activity	127	0.14	0.06	0.01	0.32

Appendix 7

Number of *An. funestus* s.s. by collection locality used for percentage inhibition of AChE by propoxur, GST, monooxygenase (MFO), elevated esterases with substrates α and β- naphthyl acetate and p-nitrophenyl acetate (PNPA)

Locality	AChE	α	β	MFO's	GST	PNPA
Bela-Vista	47	52	56	38	50	36
Boane	411	451	456	437	170	161
Catembe	352	334	402	204	219	288
Catuane	41	46	59	22	23	35
Homoine	24	28	29	17	12	24
Massinga	29	26	30	16	10	19
Moamba	205	206	232	126	150	184
Mozal	11	13	13	5	11	8
Namaacha	38	32	33	27	17	6
Pemba	23	26	32	8	10	20
Quelimane	46	47	51	29	46	36
Total	1224	1261	1394	929	718	817

Appendix 8

Number of *Anopheles arabiensis* and *Anopheles gambiae* s.s. by collection locality used for percentage inhibition of AChE by propoxur, GST, monooxygenase (MFO), elevated esterases with substrates α and β- naphthyl acetate and p-nitrophenyl acetate (PNPA)

Locality	AChE	α	β	MFO's	GST	*P*NPA
Bela-Vista	15	6	8	2	12	7
Boane	139	157	142	86	68	39
Catembe	51	75	83	7	56	62
Catuane	14	8	16	2	7	12
Homoine	79	113	125	5	60	77
Homoine*	125	114	132	8	76	106
Moamba	80	85	102	12	63	68
Polana-Canico	47	84	49	12	27	45
Total	552	606	662	134	364	416

* *An. gambiae* s.s.

UNIVERSITY OF NATAL

SUSCEPTIBILITY AND RESISTANCE TO INSECTICIDES AMONG MALARIA VECTOR MOSQUITOES IN MOZAMBIQUE

2003

SÓNIA LINA RODRIGUES CASIMIRO

SUSCEPTIBILITY AND RESISTANCE TO INSECTICIDES AMONG MALARIA VECTOR MOSQUITOES IN MOZAMBIQUE

By

SÓNIA LINA RODRIGUES CASIMIRO

Submitted in fulfilment of the requirement for the degree of
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Durban

March 2003

ABSTRACT

Insecticide resistance in malaria vector mosquitoes reduces the efficacy of insecticide in killing and can therefore cause a major problem for malaria vector control by insecticides. In Mozambique, pyrethroid resistance in *Anopheles funestus* was first detected in December 1999 in the southern corner of Maputo Province. Since then, various collections have been made at selected sites through out the country and WHO standard susceptibility tests and biochemical assays were conducted to determine the susceptibility status and the major resistance mechanisms, in the F1 generation of field collected mosquitoes. Three malaria vector species: *Anopheles funestus* s.s., *Anopheles gambiae* s.s. and *Anopheles arabiensis* were identified in this study by Polymerase Chain Reaction (PCR) and their distributions plotted. The susceptibility data indicate that the *Anopheles funestus* s.s population in southern Mozambique is widely resistant to pyrethroid and with low levels of carbamate resistance evident at six localities. No resistance to organophosphate and DDT was observed at any study sites. Biochemical tests indicate the presence of an altered acetylcholinesterase in all collection localities with the exception of Massinga district. Elevated esterase activity with substrate α-naphthyl acetate were detect in Boane with a probable role in organophosphate resistance. Elevated GST were detected in Boane, Moamba and Catembe. Very low levels monooxygenase titres were registered in all the localities in Mozambique, which suggest that this resistance mechanism is not operating in these areas. Pyrethroid resistance in the *Anopheles gambiae* complex was detected only in *Anopheles arabiensis* from one locality. No resistant to other groups of insecticide were observed. Altered acetylcholinesterases were registered in all collection localities and in both species: *Anopheles gambiae* s.s. and *Anopheles arabiensis*. Elevated esterase with substrate α-naphthyl acetate were detected in *Anopheles arabiensis* at only one locality. Elevated GSTs were detected at all localities and in both species.

The implications of the findings for malaria vector control in Mozambique are discussed.

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I dedicate this work to memory of my father Feliciano Casimiro.

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ABREVIATIONS

WHO	World Health Organization
DDT	Dichloro-diphenyl-trichloethane
HCH	Hexachlorocyclohexane
BHC	Benzene hexachloride
An.gambiae s.s.	*Anopheles gambiae sensu strictu*
An.gambiae s.l.	*Anopheles gambiae sensu lato*
An.arabiensis	*Anopheles arabiensis*
An. merus	*Anopheles merus*
An. melas	*Anopheles melas*
An. quadriannulatus	*Anopheles quadriannulatus*
An. albimanus	*Anopheles albimanus*
An. funestus	*Anopheles funestus*
An. rivulorum	*Aopheles rivulorum*
ITNs	Insecticide treated nets
NMCP	National Malaria Control Programme
IRS	Indoor residual spraying
PCR	Polymerase Chain Reaction
MRC	Medical Research Council
SW	Salt water
FW	Fresh water
DNA	Deoxyribonucleic acid
GABA	Gamma amino butyric acid
OP	Organophosphate
GST	Glutathion S-Transferase
AChE	Acetylcholinesterase
MFO	Mixed Function Oxidase
DNTB	Dithiobis 2-nitrobenzoic acid
ASCHI	Acetylthiocholine iodide
*P*NPA	Para nitrophenyl acetate
SSCP	Single Strand Conformation Polymorphism

PREFACE

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This study represents original work by the author and has not been submitted in any form to another University. Where use has been made of the work of others it is duly acknowledged in the text

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