Insecticide Resistance and Vector Status of *Anopheles funestus* and *An. gambiae* Populations at a Sugar Estate in Mozambique

By

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DECLARATION

I, RONALD GRAHAM KLOKE, declare that this dissertation is my own, unaided work. It is being submitted for the Degree Master of Science, at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

11 November 2009

ABSTRACT

Malaria is on the increase in Mozambique since 2001 and impacts primarily on children < 5 years of age. Insecticide resistance in the malaria vector mosquitoes is on the increase in Mozambique and Africa and is cause for serious concern. Maragra sugar estate is situated in close proximity to the nKomati river floodplain in a rural area in Mozambique and requires intense irrigation for cane growing and as a result provides extensive breeding sites for *An. funestus* and other mosquitoes. In the areas surrounding the estate there are two important vectors of malaria, *Anopheles funestus* group and *An. gambiae* complex. There is intense malaria transmission in the areas surrounding the sugar estate and the last entomological study on the vectors in the Manhica area was done in 1998. It was becoming increasingly urgent to identify to species level the vectors in this area and to monitor the insecticide resistance status of these vectors. Due to leakage (theft) of insecticides and a change by the National Malaria Control Programme (NMCP) to an insecticide to which the predominant vector is resistant, an entomological survey was carried out in this area from January 2009 to March 2009 to ascertain by Polymerase chain reaction (PCR) what species of malaria vectors were present inside and outside of the Maragra vector control area, their population levels and their vectorial status in these two areas. Insecticide resistance studies by insecticide exposure and the synergist piperonyl butoxide (pbo) were carried out using the World Health Organisation (WHO) bioassay method on collected *An. funestus* mosquitoes. This was done to establish this species resistance status to the four classes of insecticides recommended by the WHO for malaria vector control. The collections of *An. arabiensis* and *An. merus* that were identified were too few to carry out insecticide resistance tests on these two species. Enzyme linked

immuno-sorbent assay (ELISA) tests were undertaken to establish the vectorial capacity of *Anopheles funestus* and *An. gambiae* complex in this area. The predominant malaria vector species in this area is *An. funestus s.s.*, with the secondary vector being *An. arabiensis*. *An. funestus* has a high vectorial capacity in this area and found to have a *Plasmodium falciparum* sporozoite rate of 6.02%. This is an increase in the sporozoite rate of 1.2% from 1998 when the last survey in this regard was carried out. Coupled with this increase is an increase in the *An. funestus* populations in this area since this time. One *An. gambiae* complex sample was found to be positive but the species is not known as this particular sample did not amplify on PCR. *Anopheles funestus* is highly resistant to synthetic pyrethroids and exhibits a lower level of resistance to bendiocarb, a carbamate insecticide in use at Maragra sugar estate. The synergist pbo mediates the resistance mechanism in both these insecticides indicating that the metabolic resistance mechanism present in this mosquito is strongly mediated by monooxygenase detoxification. The role of the medical entomologist is increasingly necessary and important in the monitoring of this resistance phenomenon in malaria vector mosquitoes, as is the role of the vector control programme manager in implementing and managing vector control programmes. The implication of cane sugar farming and its impact on vector production and malaria transmission is discussed. Insecticide resistance and the change by the NMCP to a synthetic pyrethroid to which the predominant vector of malaria is resistant is discussed.

DEDICATION

I dedicate this dissertation to my parents Ronald (Ronnie) Trevor and Phyllis Kloke, who always encouraged my brother Brian and I to strive for our best, and to my children Jehanne, Calie and Jared who are an inspiration to me in themselves. I also dedicate this dissertation to Professor Maureen Coetzee who instilled the idea and confidence in me of ‘going academic’, and to my long time friend and colleague Keith Hargreaves, both of whom have always encouraged me while working in the field and stream of malaria control.

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I would like to take this opportunity to thank Maragra (Illovo) Sugar Estate General Manager, Mr. Mike Buchanan for his support to me in undertaking this study of their malaria vector control programme and its impact on the malaria profile in their area of operations and residence. Special mention and thanks must also be made to Mr. Tony Currie, the previous GM of Maragra who supported malaria control and enabled me to continue this work, and to the residents of Maragra Estate for their friendship and consideration to me over the past years, despite the inconvenient invasion of their homes at times.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1. Background

Malaria in the south of Mozambique is mesoendemic to hyperendemic. It is a major medical and socio-economic burden to the country and is the primary cause of clinic outpatient attendance (World Health Organization, 2008). It impacts particularly on the morbidity and mortality of children < 5yr of age. Virtually all the population of Mozambique (99%) of some 20,881,109 people are at risk of malaria, with 3,425,399 children < 5 yrs being the most vulnerable (World Health Organization, 2008). Malaria is on the increase in Mozambique with 4 million cases in 2001 and 6 million cases in 2006. *Plasmodium falciparum* the fatal form of malaria infection, accounts for 90% of parasite infections and the normally non-fatal infections of *P. malariae* and *P. ovale* account for 9% and 1% respectively of other malaria infections (World Health Organization, 2008).

1.2. History of the use of insecticides for vector control in Mozambique.

Use of chemicals for indoor insect control was first experimented with in 1927, by spraying pyrethrum and other reagents mixed with kerosene and using a hand sprayer, directly onto as many insects as possible. This was carried out at the South African Institute for Medical Research in Johannesburg, South Africa (Ingram & De Meillon, 1927). Indoor spraying utilising a kerosene-pyrethrum mixture for the control of malaria vectors in South Africa, was first successfully tried in 1932-1933 in the Letsitele Valley in the then Transvaal province, and continued in Eshowe, Natal, Zululand (De Meillon, 1986). Dichloro-diphenyl-trichloroethane (DDT) was

implemented in 1946 in Mozambique and South Africa, with good results in killing vector mosquitoes and reducing transmission of malaria in both countries (Soeiro, 1956).

Malaria control in Mozambique started in 1946 with indoor residual spraying (IRS) using DDT and Benzene Hexachloride (BHC) and stopped in 1956 with good results in reducing parasite and spleen rates in children <5 yrs during that period (Soeiro, 1956). Control actions were initiated again in 1960 utilising DDT as part of a malaria eradication programme and continued through to 1971, when malaria control operations were limited to main towns due to civil war. By 1980 malaria control actions were limited to the Maputo area (Martinenko *et al.,* 1989). A limited control action was again initiated in 1994 to evaluate lambdacyhalothrin, deltamethrin, baythroid and cyfluthrin insecticides, all synthetic pyrethroids or derivatives of pyrethrum. Lambdacyhalothrin was selected as the insecticide of choice at that time (Cuambo & Dambo, 1994).

In October 1999 the first commercial integrated malaria vector control (IMVC) programme in Mozambique was implemented at Mozal aluminium smelter in the Beluluane district of Maputo, initially using deltamethrin, a synthetic pyrethroid (Kloke, 2000.). In early 2000 an insecticide resistance study was carried out on *An. funestus* at the Mozal site due to suspected resistance of *An. funestus* to deltamethrin (Brooke *et al.,* 2001). This proved to be the case with both deltamethrin and lambdacyhalothrin. The high levels of pyrethroid resistance eliminated the possibility of using this group of insecticides in this area. The carbamate insecticide bendiocarb replaced deltamethrin on the basis of vector susceptibility to this insecticide, it's

acceptability for use in both western and traditional structures, and its non-repellency of mosquitoes.

Shortly thereafter, following this work at Mozal, the Mozambique National Malaria Control Programme (NMCP) under the auspices of the Lubombo Spatial Development Initiative (LSDI), also changed from pyrethroid insecticides and adopted the use of bendiocarb for vector control by Indoor Residual Spraying (IRS) in this area of Mozambique in late 2000 and has continued to do so, with the addition of DDT for IRS in 2006 (Casimiro *et al.,* 2007, Coleman *et al.*, 2008).

1.3. Malaria Transmission in Mozambique and at Maragra Sugar Estate

The number of Maragra estate employees fluctuates between 1,250 and 4,500 personnel per month through the year, with an increase in personnel from April through to November/December, when the harvesting and milling of the cane takes place (Fig. 1.1.a). There is an expatriate population of between 20-22 consisting of cane farmers and administration personnel employed by Maragra estate (Maragra Human Resources Department).

There is year round transmission of malaria with a peak during and after the rainy season from November/December to April (World Health Organization, 2008; Abellana *et al.,* 2008), which can extend into June (Kloke, Maragra reports, unpublished data; Figs. 1.1.b,c) depending on rainfall and the encroachment of winter and cooler temperatures.

Fig. 1.1.a Maragra total of permanent and temporary employees Jan. 2003 – Apr. 2009

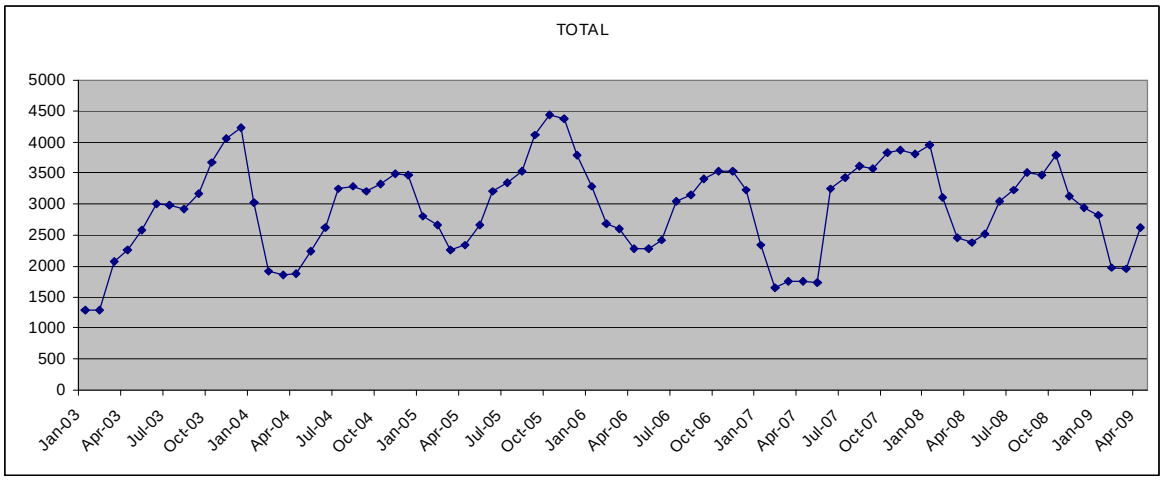


Fig. 1.1.b Maragra malaria cases Jan. 2003 – Apr. 2009

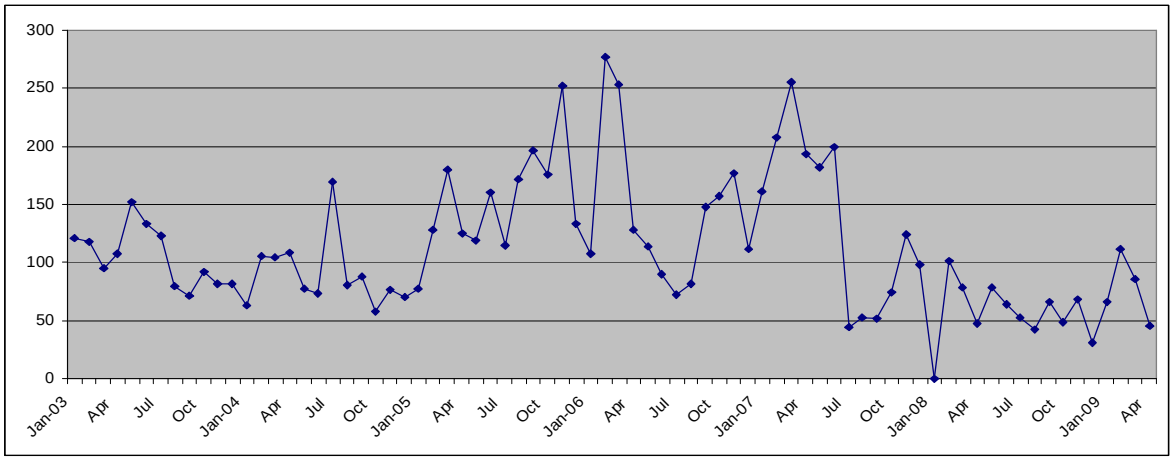
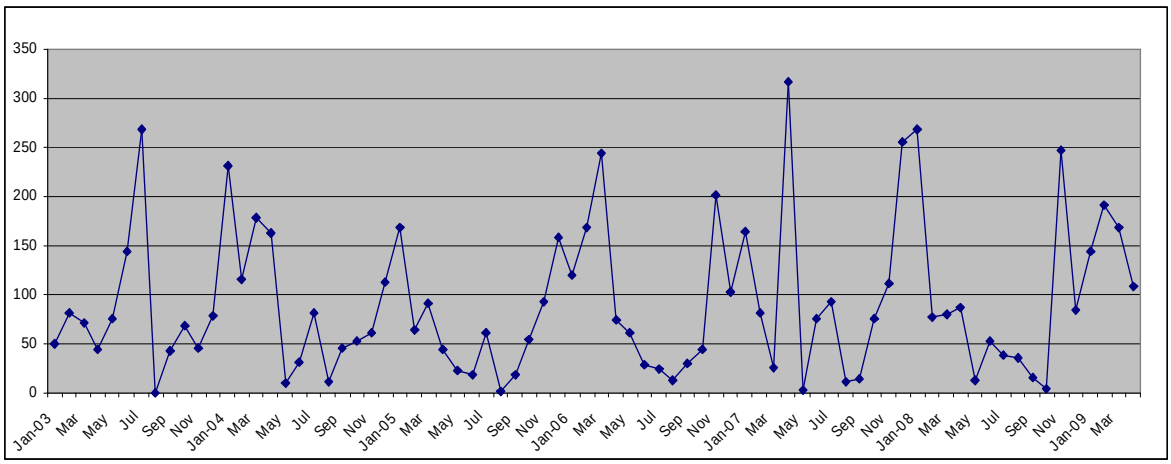


Fig. 1.1.c Maragra rainfall Jan. 2003 – Apr. 2009



Seasonal climatic conditions can vary the spatial conditions and distribution of malaria. Abellana *et al*. (2008) make the point that the Manhica area presents a spatial pattern which is independent of the seasonal climatic conditions, and that neighbourhoods with a higher incidence of malaria, maintain their higher incidence over the annual climatic season. Though reducing in the dry season, they still remain the areas of highest incidence throughout the climatic seasons (Abellana *et al.*, 2008). It has also been demonstrated that close proximity to breeding sites of the vector mosquito *An. funestus* increases the incidence of malaria of those residents, with incidence decreasing with distance from breeding sites (Aranda *et al.*, 2005; Charlwood *et al.*, 1998).

Prior to the initiation of house spraying, the incidence of malaria was the highest cause of out-patient and in-patient admission to the clinic on the Maragra estate. From January to September 2000 after extensive flooding in the district in February 2000, akin to an inland sea at Maragra, there were 208 malaria cases. From January to September 2001, there were 765 cases, a 367% increase in case numbers over one year. The period May to September 2000 had 90 cases compared to the same period in 2001 when there was 521 cases, an increase of 578% (Maragra malaria audit. Kloke, unpublished data) (Fig. 1.2).

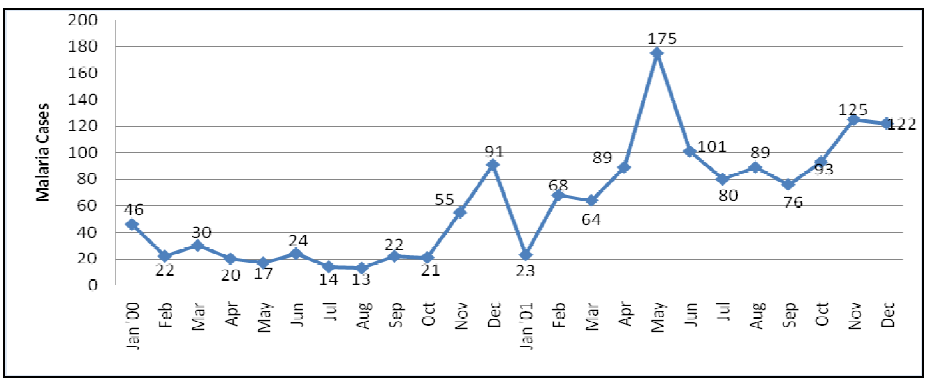


Fig. 1.2 Maragra malaria cases 2000 - 2001

This was significant as this is normally the low season of malaria transmission, and the malaria case numbers should have been reducing, not increasing over this drier period of the malaria season. The Maragra vector control programme has since been very effective in reducing malaria transmission among the employees living on the estate and in surrounding villages, to an average annual incidence rate of between 5-6% (Fig. 1.1.b, Kloke, unpublished data) of an annual total of 30,000 to 42,000 personnel (Maragra Human Resources Department/Kloke, unpublished data).

1.4. The malaria vectors

The principle vector of malaria in Maputo Province and on the sugar estate is *Anopheles funestus s.s.*, a highly efficient vector and a stream and vegetated pool breeding mosquito (Gillies & De Meillon, 1968). In 1997 to 1998 it comprised about 72.3% of the *Anopheles* population in this area, with an estimated sporozoite rate of 1.2% (Aranda *et al.*, 2005). Substantial variation in anopheline collections from house to house occurred, with a peak in numbers in April towards the end of the warm and rainy season.

Anopheles funestus is a member of a group of 9 species; *An. funestus, An. aruni, An. brucei, An. confusus, An. fluviatilis, An. fuscivenosus, An. leesoni, An.parensis,* and *An. rivulorum* (Gillies & De Meillon, 1968). All, except *An. funestus s.s.* are zoophagic, and they have no formal malaria vector status. *Anopheles funestus* s.s. is endophagic and anthropophagic and feeds almost exclusively on humans (Gillies & De Meillon, 1968). It is also endophilic in its habit rendering it amenable to IRS.

Due to the nature of the agricultural industry and permanent breeding sites of *An. funestus* on the estate (Figs. 1.3), there is continual malaria transmission with a seasonal increase of vectors during and shortly after the rains from November to April. Cohuet *et al*, (2004) have shown that *An. funestus* can be the major vector in areas of high agricultural activity and have shown that it was responsible for 88% of the total malaria transmission in this Cameroon study area. This may apply even more in areas of very high water availability and sugar cane production such as at Maragra sugar estate.

Fig. 1.3 Potential *Anopheles funestus* breeding site in permanent irrigation canals and swamps at Maragra Sugar Estate.

Anopheles arabiensis is also a vector of malaria in southern Mozambique and is a member of the *An. gambiae* complex of species which consists of seven members: *An. gambiae; An. arabiensis*; *An. bwambae*; *An. merus*; *An. melas*; *An. quadriannulatus;* (Gillies & Coetzee, 1987) and *An. quadriannulatus* species B (Hunt *et al*., 1998), of which not all are vectors of malaria. In comparison to *An. funestus*, very few adult members of the *An. gambiae* complex have been found within the estate since the inception of the malaria control programme in 2001, and no active breeding sites of *An. gambiae* have been found despite continual searching.

1.5. Insecticide Resistance in Mozambique

The first indication of insecticide resistance in *An. funestus* to synthetic pyrethroids was reported from the Ndumu area of northern KwaZulu-Natal (KZN) bordering southern Mozambique in 1999 (Hargreaves *et al.*, 2000). This was as a result of an increasingly severe outbreak of malaria that occurred in that area, culminating in over 27,000 cases in 1999, a doubling of malaria cases from 1998, with a total over the period 1995 to 1999 of over 67,000 cases reported, with *An. funestus* implicated as the principle vector (Hargreaves *et al.*, 2000: Maharaj *et al.*, 2005). The scale of the epidemic was reminiscent of the 1932 epidemic of malaria in Natal where there were tens of thousands of reported cases and deaths (Le Sueur *et al.*, 1993). It was this 1932 epidemic that saw the early beginnings of intra-domiciliary spraying as a malaria control strategy using liquid pyrethrum and kerosene and later, with the advent of longer acting residual insecticides, referred to as Indoor Residual Spraying (IRS) (Le Sueur *et al.*, 1993).

The resistance status of *An. funestus* within Mozambique was confirmed with insecticide resistance studies in the Beluluane district of southern Mozambique. This study revealed that this vector was resistant to pyrethroids and the carbamate propoxur, but susceptible to DDT, organophosphates and the carbamate insecticide bendiocarb, although there was a low level of resistance to this latter insecticide as well. This was also the first study in Mozambique on insecticide resistance and the first record of a metabolic mechanism of resistance in *An. funestus* to pyrethroids (Brooke *et al.*, 2001).

Subsequent resistance studies carried out on *An. funestus* and *An. arabiensis* have revealed varying levels of insecticide resistance in both these vectors throughout Mozambique, including the areas bordering KZN (Casimiro *et al.*, 2007: Coleman *et al.*, 2008).

1.6. Project Site and Industry

Maragra Sugar Estate is situated in the Maputo province of Mozambique at 25 º 27'S, 32 º 46'E, 90 km north of Maputo city and 3km south of Manhica town and is surrounded by rural residential areas on all but the east side (Figs. 1.5-1.7). Maragra Sugar Estate and a portion of its surrounding villages are situated in close proximity to Manhica and the nKomati River. The mill and residential areas of the Maragra estate itself and the rural population housing, are situated 28m above sea level on an elevated plane above the nKomati floodplain, in which cane farming takes place.

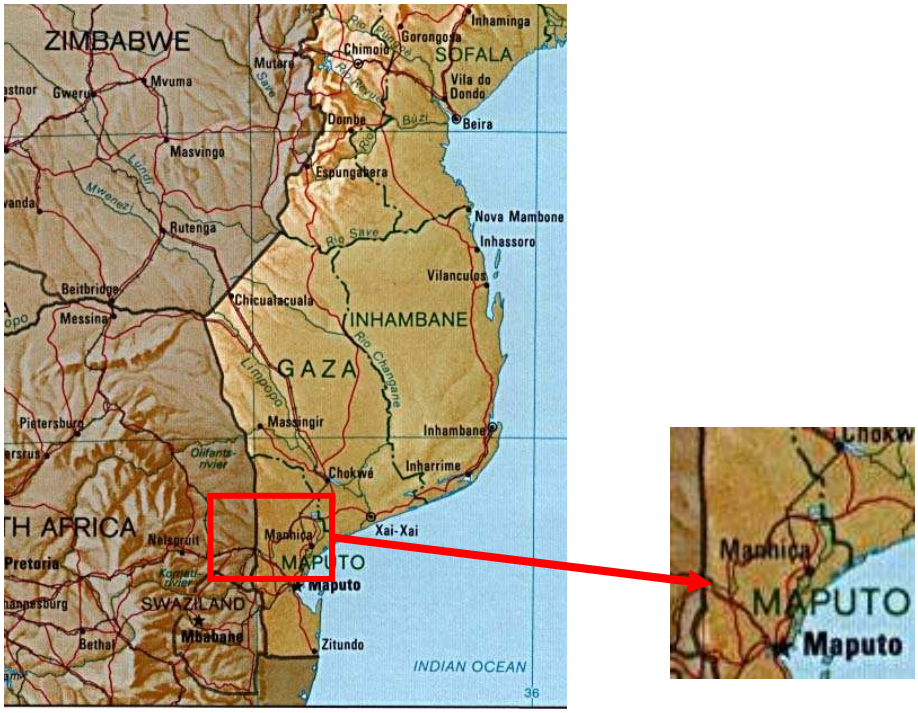


Fig 1.4 Map of Mozambique and Maragra study area near to Manhica

Fig. 1.5 Aerial photo showing proximity of Maragra sugar estate and Manhica to nKomati river, sugar cane fields and irrigation system.

Fig. 1.6 Aerial photograph showing Maragra sugar estate and Mill and proximity to local housing.

The local population in and around Maragra and Manhica is approximately 80,000 (Guinovart *et al.,* 2008). On the western boundary of the sugar estate there are 3 large villages forming one single residential area with a population of approximately 20,000

inhabitants. Employees are drawn from these villages for work on the estate and mill. These village areas border the sugar estate residential area and the mill, and are the principle reservoir of malaria affecting the estate employees.

Overhead spray irrigation is carried out to the fields by pumping water from the nKomati river into an extensive system of permanent irrigation canals ranging from deep to shallow and based on a grid system to the fields. These irrigation canals cover the entire cane growing area and are permanently filled with water. They support aquatic vegetation and range in depth from 0,25m - 2m deep. These are ideal breeding sites for the malaria vector *An. funestus* which breeds in vegetated streams and pools of fresh water and maintains malaria transmission on an endemic basis (Gillies & De Meillon, 1968). *Anopheles gambiae s.l.* on the other hand prefers temporary sunlit shallow pools of fresh water with no or very little vegetation and escalates the transmission of malaria during the summer and autumn months during and shortly after the rains (Gillies & De Meillon, 1968). As the terrain outside of the prepared cane fields is primarily very sandy and water drains away within two days, it is extremely difficult to find *An. gambiae s.l.* breeding sites in this area.

1.7. Vector Control at Maragra Sugar Estate

Adult vector control utilising IRS has been carried out on the sugar estate since early 2002. All housing on the estate and the surrounding village housing within a radius of 1, 6 km from the centre of the housing estate is included (Kloke, unpublished data). This creates a buffer zone or *cordon sanitaire* between the villages and the estate residential and mill area as recommended by others (Charlwood *et al.,* 1998; Mendis *et al*., 2000). The insecticide in use for IRS since 2002 has been bendiocarb. The

remaining portions of the surrounding villages beyond the 1,6 km zone have been sprayed by the NMCP/LSDI with the same insecticide and also with DDT (Presidents Malaria Initiative: Malaria Operational Plan–FY 09).

Ultra-Low-Volume (ULV) spray application with a synthetic pyrethroid is carried out in targeted areas at Maragra where there is accumulation of large numbers of nuisance mosquitoes, and at outdoor social gatherings in the evening.

No larvaciding is carried out for control of *An. funestus*, due to the extensive and permanent irrigation canals and the vectors amenability to IRS control, due to its endophilic, endophagic and anthropophagic habit (Gillies & De Meillon, 1968). The lack of *An. gambiae* breeding sites found thus far has precluded such control measures being implemented for this vector. Mosquito nets have been provided to the Maragra employees on a subsidised cost recovery basis. There is no formal estate insecticide treated nets (ITN) programme and the use of mosquito nets on a subsidised basis is left to individual employee choice.

1.8. Rational and Objectives

1.8.1. Insecticide resistance in *Anopheles funestus*

Understanding the levels and mechanisms of insecticide resistance in *Anopheles funestus* is of paramount importance in the monitoring and management of insecticide resistance in this principle malaria vector (Coleman *et al.*, 2008). It has great implications for the Maragra sugar estate, and the NMCP control strategy using IRS and ITN's. Of the four classes of public health insecticides recommended by WHO for use in malaria vector control (World Health Organization, 2001) (see Table 2.1),

two are presently in use in the Manhica district. The carbamate bendiocarb is used for IRS in the Maragra vector control zone, and by the LSDI outside of the Maragra control zone. The NMCP have also used bendiocarb and DDT outside of the Maragra control zone in the Manhica district (see Discussion for update on change of policy with NMCP insecticide usage).

Insecticide resistance in the *An. funestus* population at Maragra needed to be investigated to assist in proactively managing insecticide resistance that may be present now, and may also arise in the future in control programmes. This is necessary knowledge for the IRS programme managers in this area to make informed operational decisions on the choice of insecticides used in national and localised malaria vector control programmes.

1.8.2. Implication of the sugar cane agriculture industry on malaria vector production, malaria transmission and insecticide resistance at Maragra

At present there are five sugar cane farming areas in Mozambique, four situated in Maputo province, southern Mozambique and one in Sofalo province near Beira in the north. These farms are situated in the flat lowland and coastal areas of Mozambique, which are the areas of highest malaria vector abundance and disease transmission (Mabunda *et al.*, 2008). The nature of the industry is highly water dependent and very large areas of land are required for commercial sugar cane farming, running into many thousands of hectares and continually expanding. They are invariably situated on or around major river systems, due to the high demand for water for intensive irrigation. This is highly significant in respect of ecology change and malaria vector population increase, insecticide resistance, malaria transmission and related disease and health

impact on resident and surrounding human community populations. The residential areas are usually surrounded by sugar cane fields and irrigation systems, rendering the residents particularly susceptible to malaria infection due to their close proximity to vector breeding sites (Thompson *et al.*, 1997; Charlwood *et al.*, 1998; Mendis *et al.*, 2000). These intensively irrigated agricultural areas may also impact significantly on local, provincial and country disease profiles in the areas they are situated, if sustainable and monitored vector surveillance and control measures are not implemented.

1.8.3. Aim of Project

The necessary and critical aspect of knowing what vector mosquito species you are attempting to control in a malaria vector and disease control programme, is crucial to ensure that operations are in fact directed at a vector of malaria, and not at a sibling species which is a non-vector of malaria. This will prevent financial and human resources and effort being wasted on controlling a non-vector of disease (Mouatcho *et al.*, 2007).

There is a need to investigate the resistance status of the vectors to the four classes of insecticides. Insecticide resistance of malaria vector species is an increasingly serious problem for vector control programme managers utilising IRS as a means of vector control (Hemingway *et al.*, 2006). The importance of monitoring of insecticide resistance is enhanced by the present necessary reliance on vector control by chemical means and the lack of new public health insecticides (Gratz & Jany, 1994; Hemingway *et al.*, 2006; Tren *et al.*, 2008), and the integrity of such vector control programmes (Townson *et al.*, 2005; Beier *et al.*, 2008).

The Maragra Sugar Estate is surrounded by areas of erratic vector control measures and areas of no vector control. It is akin to an island surrounded by a sea of malaria, and it is becoming increasingly necessary to establish and monitor the levels of insecticide resistance in the malaria vectors in these areas to enable informed decisions by programme managers.

The vector species and their population densities and sporozoite rates in and out of the control area, need to be investigated to establish their vectorial capacities or infection rates in these areas, to establish a baseline to monitor the impact of control measures within the Maragra vector control area (Gillies & De Meillon, 1968; Service, 2000).

1.8.4. Specific Objectives of Project

1. To conduct Polymerase Chain Reaction (PCR) assays to confirm the species identification of the malaria vectors at and around Maragra.
2. To establish the insecticide susceptibility levels to the four classes of public health insecticides approved for malaria control, using WHO standard test kits.
3. To carry out enzyme-linked immunosorbent assays (ELISA) tests for parasite detection in the vectors.
4. To establish *An. funestus* and *An.gambiae* population densities in and out of Maragra control area using different trapping techniques.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Wild Mosquito Collections

The field site at Maragra was visited for two weeks per month from January to March 2009. Wild populations of live female *An. funestus* group and *An. gambiae* complex were collected from areas outside of the control area and transferred into polystyrene cups. The tops of the cups were covered with gauze held in place by elastic rubber bands, to prevent escape of the captured mosquitoes. A small cotton wad soaked in a 10% sugar solution was placed on the tops of the cups and gauze to allow feeding by the captured mosquitoes. These cups and mosquitoes were then kept in a cool box with a freeze block and a damp towel, to maintain a cool temperature and a higher humidity level, to prevent heat exhaustion and death of the mosquitoes and transported back to the Vector Control Reference Unit (VCRU) insectary at the National Institute for Communicable Diseases (NICD) Johannesburg. There PCR and insecticide susceptibility tests were carried out on them and their F1 generations. At the VCRU all collected mosquitoes were kept in separate prepared glass vials containing a wad of damp filter paper to facilitate oviposition, and to keep each captured mosquito family progeny separate. The mosquitoes in the glass vials were fed a 10% sugar water solution soaked into a cotton pad and also blood fed. After they had laid eggs they were again blood fed to encourage further egg laying.

Mosquitoes were collected by using the following techniques (Service, 2000)

2.1.1 Indoor House Searches.

Rooms in houses inside and outside of the Maragra vector control area were physically searched early in the mornings. Using a torch and an aspirator tube, searches were carried out for mosquitoes resting indoors during the morning and day. These searches started at 07h00 and extended in some instances to 15h00 in the afternoon, but generally until 10h00 or 11h00 depending upon numbers found resting indoors during this period. Searches were carried out on walls and under beds and behind furniture. These collections were done for three days once a month for three months.

All collected mosquitoes were identified morphologically on capture (and again later at a holding station at Maragra estate) before being transported back to the VCRU.

2.1.2 Knock Down Catches

These investigations were carried out from 07h00 to 11h00 for three days a week once a month. White sheets were placed over all furniture and floors in rooms of houses and an aerosol insecticide sprayed outside around the eaves and then inside the room in the case of traditional dwellings, to kill any resting mosquitoes. In the case of western type dwellings the windows were closed and sheets placed over all furniture and floors and the room sprayed with a knockdown aerosol insecticide and the door to the room closed. After a period of fifteen minutes the houses were searched for dead and dying vector mosquitoes. Mosquitoes were collected off the sheets using forceps

and were placed in Eppendorf tubes containing silica gel to keep them dry, and taken back to the VCRU laboratory in Johannesburg for PCR and species identification.

2.1.3 Exit Window Traps

Sixteen of these traps were placed on the outside of windows to capture mosquitoes exiting the room during the night or early morning, ensuring that the traps were correctly fitted to the windows to preclude mosquitoes escaping from around the sides of the trap. Eight traps were placed inside the Maragra vector control area and eight outside of the control area to investigate the populations and species of vectors in these two areas. Introduction of these traps was done at the same time for both inside and outside of the control area to ensure all conditions were the same over the collection periods. As far as possible these traps were placed in windows facing in an easterly direction to facilitate observed preferred direction of exit of mosquitoes in the morning towards the light of the sun below the horizon (K. Hargreaves; personal communication). The traps were cleared of any mosquitoes from 06h00 – 10h00 in the morning for three days for one week a month. All collected mosquitoes were identified morphologically on capture (and again later at a holding station at Maragra estate) before being transported back to the VCRU.

2.1.4 Natural Shelters and Pit Traps

Searches for adult mosquitoes were carried out at Maragra in natural shelters such as crevices and holes in a sand bank near to mosquito breeding sites. These natural shelters are in a 15m high sand cliff which rises from the nKomati floodplain, the top of which is the plateau on which the estate and local people live. It is immediately in front of and below sprayed and unsprayed houses, and is approximately 1 km long. It

has been shaped by natural erosion and excavation for soil for road maintenance and faces in a south-east direction towards the coast. Ten artificial pit shelters were also created by digging a number of horizontal shafts measuring approximately 20cms square and approximately 45cms deep into the sand banks. These artificial shelters were dug where they were sheltered from the rising sun in the morning and spread out over an area of approximately 750m in a north to south direction. They were searched in the mornings from 06h00 to 10h00 and cleared of mosquitoes found resting inside.

2.2 Mosquito Colonies

2.2.1 *Anopheles funestus* (FANG) Colony

FANG is an insecticide susceptible colony from Angola, kept at the insectaries at VCRU Johannesburg, and used as a reference strain (control) for experiments performed on wild caught *An. funestus s.l.* and F1 generations for species identification, insecticide resistance tests and sporozoite antibody detection.

2.2.2 *Anopheles gambiae* (SUA) Colony

SUA is a fully insecticide susceptible colony from Liberia and kept at the insectaries at VCRU Johannesburg and used as a reference strain (control) for experiments performed on wild caught *An. gambiae s.l.*

F1 Progeny

Wild caught females were kept in the VCRU insectary and after oviposition their F1 generations were raised from larvae through to adults. The insectary is maintained at a temperature of 25 – 27˚C and a relative humidity of 75 – 80%. The insectary lighting is on a 12 hour cycle of light and dark to mimic the natural cycle of day and night

with a 45 min dusk/dawn period. Once oviposition had taken place the eggs were washed into a small plastic bowl containing distilled water, and the wad of filter paper from the oviposition tube was also placed into this bowl to ensure any attached eggs were also immersed in the water. Emerged larvae were fed on a finely ground mixture of dog biscuits and brewers yeast until pupation and the emergence of adults occurred. The F1 adults were kept separately in their respective families. The 1-4 day old F1 mosquitoes were then standardized in age and physiological state for insecticide resistance bioassays. This eliminated any possibility of wild caught mosquitoes being pre-exposed to insecticides and thus affecting the study.

2.3 Species Identification

Captured vector mosquitoes were initially identified to group by morphology (Gillies & Coetzee, 1987) and then underwent identification by Polymerase Chain Reaction (PCR) assay to determine the species of *An. funestus* (Koekemoer *et al.*, 2002) and *An. gambiae* (Scott *et al.*, 1993) collected in the study area.

PCR identification of *Anopheles funestus* group

PCR using the rDNA method (Koekemoer *et al.*, 2002) was used to identify members of this group. Extraction of DNA was done using the Collins Extraction Method (Collins *et al.*, 1987).

A EDTA grinding buffer solution was made up of 1600µl 1M NaCl, 1.095g Sucrose, 2400µl 0.5M EDTA, 1000µl 10% SDS, 2ml 1M Tris-Cl (pH 8.6) and ±13ml deionised H_2O to make a total final volume of 20ml.

The abdomen and a leg of the wild sample mosquitoes and a known mosquito from the FANG colony held at the VCRU insectary for a positive control, were crushed and ground with a pestle in a 1.5ml microcentrifuge tube and then homogenized with 200µl of the EDTA grinding buffer and kept on ice. When all mosquitoes were homogenized they were then incubated at 70°C for 30 minutes on a heating block. The same procedure was carried out for a negative sample. This sample did not contain any mosquito material. After incubation 28µl 8M KAc was added to all samples and mixed by tapping with the finger. Samples were then incubated on ice for 30 minutes and then centrifuged for 12 minutes @ 13 K.rpm with the hinge facing out. Thereafter all liquidized homogenate was pipetted off without disturbing the pellet and this liquid placed in a new numbered Eppindorff tube with the same number as the original tube. The original tube with the pellet was discarded. Then 400µl of ice cold (-20˚C) 100% ethanol was added to the contents of the new tube and mixed by inverting the tube. The samples were kept overnight in the -20˚C freezer.

The samples were taken out of the freezer and centrifuged for 35 minutes @ 13K.rpm. The 100% ethanol was pipetted off all samples, including the positive and negative controls, so as not to disturb the remaining pellet in the samples to be tested, and the ethanol discarded. Then 200µl of ice cold (-20˚C) 70% ethanol was added to the sample tubes, including both controls, ensuring the pellet was not washed off. The samples were then centrifuged @13k.rpm for 15 minutes. The 70% ethanol was then pipetted off and the pellet allowed to air dry overnight. The samples were re-suspended in 200µl of 1x TE, ensuring the pellet had dissolved, and stored in the fridge until needed.

The PCR reagent Master Mix was made up of 1.25µl of 10x reaction buffer (500mM KCl, 100mM Tris-HCl pH 8.3), 1.25µl of 10x dNTP's, 0.75µl of 1.5 mM $MgCl_2$, 1.0µl Primers each of UV, FUN, VAN, LEES, RIV, PAR, 3.15µl of deionised H_2O and 0.1µl of RTaq, to give a final concentration of 12,5µl per sample to be tested.

Additional numbered 200µl microcentrifuge tubes, corresponding to the initial DNA extracted wild sample tube numbers, and the positive and negative controls and five positive controls of *An. funestus*, *An. vaneedeni*, *An. leesoni*, *An. rivulorum*, *An. parensis* and a 'No DNA', were prepared. One microlitre of DNA from the wild samples, 1µl of the negative control sample and 1µl ofthe positive control were added to their respectively marked 200µl tubes and the five positive controls and the 'No DNA", to their respectively marked 200µl tubes and kept on ice.

The first control was a DNA extraction negative in which extractions are performed without any DNA to check for contamination during homogenising. The second control was a negative control containing all the substances in the PCR mixture except DNA template to check for contamination during the preparation of the Master Mix.

When aliquoting of the respective DNA was completed 12,5µl Master Mix was then aliquoted to all tubes, and the tubes centrifuged briefly to ensure all reagents were at the bottom of the tube and mixed. The samples and the two controls were then placed into the PCR machine and the cycle set to the *An. funestus* amplication programme cycle.

Amplification conditions were as follows: Initial hot start at 94°C for 2 minutes initial denaturation, followed by a further 40 cycles of denaturation at 94°C for 30 seconds,

annealing of the primers at 50°C for 30 seconds and extension at 72°C for 40 seconds and a final extension at 72°C for 10 minutes.

Four microlitres of Ficol dye were added to all samples and controls and centrifuged briefly to ensure contents mixed. Five microlitres of molecular weight marker was placed in the first and last wells of the 2.5% agarose gel to identify species with the base pair ladder, followed by 10 µl each of the 'No DNA' control in well 2 and the positive control amplicons of *An. vaneedeni*, *An. funestus*, *An. leesoni*, *An. rivulorum* and *An. parensis* in wells 3 to 7 of the gel, and the positive and negative controls in wells 8 and 9. The amplicons to be identified followed in their respective wells after that.

Visualisation and identity of amplified product was done by electrophoresis on a 2.5% agarose gel, stained with ethidium bromide and immersed in an electrophoresis bath containing a 1x TAE buffer and electrophoresed at 100V/40mA for approximately 45 minutes.

The agarose gels were visualized under UV light using the GeneSnap cabinet (Vacutec G-Box from Syngene, sydr 4/1152) and the samples identified by comparing the amplicons to the molecular weight marker ladders. The gels were photographed and filed on the computer data base.

2.3.1 PCR Identification of *Anopheles gambiae s.l.*

PCR using the rDNA method (Scott *et al*, 1993) was used to identify members of this group, using the scales of a leg of the sample mosquito. Four positive controls of *An. gambiae s.s., An. arabiensis, An. merus* and *An. quadriannulatus* were obtained from

the insectary at the VCRU, where colonies of these species are kept and maintained for many generations. These were treated as for the wild samples tested. The master mix without DNA was used as the negative control.

The PCR Master Mix was made up of the following reagents: 1.25µl 10x reaction buffer (100m M Tris-HCL pH 8.3, 1mM KCl), 1.25µl 10x dNTP, 0.5µl MgCl2 solution, 0.5µl Quad Primer, 1.0µl each of UN, AG, AR, ME and QD primers, 4.9µl deonised H_2O and 0.1µl Rtaq, to give a total volume of 12.5µl per sample.

A leg from each wild caught mosquito was placed into individually numbered 1.5 ml Eppindorff tubes and 12.5 µl of the Master Mix aliquoted into each sample tube. All sample tubes were then centrifuged for 20 seconds at 16K rpm to ensure all contents at the bottom of the tube.

The samples and control were then placed into the PCR machine and the *An. gambiae* programme cycle selected and underwent a one hour cycling, at 95˚C for 2 minutes initial denaturation, 30 cycles of 94˚C for 30 seconds denaturation of DNA, 50˚C for 30 seconds annealing of specific primers, 72˚C for 30 seconds extension and a final auto extension of 72˚C for 5 minutes.

After amplification 4 µl of Ficol dye was aliquoted into all samples and controls for visualization of the samples and controls. A molecular weight marker was placed in the first and last wells and 10 µl of the *An. arabiensis*, *An. gambaie*, *An. merus* and *An. quadriannulatus* positive amplicons were placed into wells 19 to 22 respectively. Then 10 µl of the samples to be identified and of the negative control, were aliquoted into wells 2-18 with the negative control aliquoted into the second last well (23) next to the second molecular weight marker at the end of the gel.

Visualisation and identity of amplified product was done by electrophoresis on a 2.5% agarose gel, stained with ethidium bromide and immersed in an electrophoresis bath containing a 1x TAE buffer and electrphoresed at 100V/40mA for approximately 45 minutes.

After electrophoresis the gel was placed into the GeneSnap cabinet (Vacutec G-Box from Syngene, sydr 4/1152) for visualization of the gel samples and controls and identified by comparing the length of their amplicons to the molecular weight marker ladders. The gel was photographed and filed on computer.

2.4 Insecticide Resistance Studies

Insecticide susceptibility bioassays were carried out on both wild caught and F1 generation *An. funestus* mosquitoes to the four classes of public health insecticides (Table 2.1), according to the standard WHO operating procedure (World Health Organization, 1998). Bioassays were also conducted on the carbamate bendiocarb and synthetic pyrethroid deltamethrin without and with the synergist piperonyl butoxide (PbO) and results evaluated in line with work done at Beluluane (Brooke *et al.*, 2001), the site of the Mozal aluminium smelter and a densely populated residential area a few kilometres south of Maputo.

Due to low numbers of *An. gambaie* complex collected on a daily basis, insufficient numbers survived for raising of F1 generations, and of those that did survive, oviposition was not successful. As a result no insecticide resistance assays could be carried out on this group of species.

Table 2.1. List of the four public health insecticides tested showing insecticide concentrations and to which classes of insecticides they belong

Insecticide Class	**Insecticides and Concentrations**
Organochlorines	DDT (4%)
Organophosphates	Malathion (5%)
Carbamates	Bendiocarb (0.1%); Propoxur (0.1%)
Pyrethroids	Lambdacyhalothrin (0.1%); Deltamethrin (0.05%)

The diagnostic concentration is twice the concentration of insecticide which results in 100% mortality in susceptible mosquitoes and has been set as a standard for testing by WHO.

Between 10 and 19 wild caught, and between 19 and 31 F1 adult *An. funestus* between the ages of 2 to 4 days, depending upon available numbers of newly emerged mosquitoes, were exposed to each of the WHO insecticide treated papers for one hour. Knockdown was recorded at the end of the one hour exposure period. Mosquitoes were then transferred to holding tubes for 24 hours and provided with 10% sugar water solution soaked in a cotton pad. After 24 hours the final mortality of mosquitoes was recorded and the mean percentage mortality calculated and recorded. Between two and nine replicates per insecticide were performed where possible (dependent upon available F1 mosquitoes) with an emphasis on the carbamate bendiocarb, as this is the insecticide in use on the estate at present. Unexposed F1 mosquitoes acted as a control. The WHO insecticide treated papers were tested as to their insecticidal activity by exposing the susceptible reference strain FANG to them in the same manner as for the exposed F1 mosquitoes. According to WHO criteria, 98-100% mortality indicates full susceptibility, 80-97% mortality requires further investigation

and below 80% mortality indicates confirmed resistance (World Health Organization, 1998).

Between 6 and 16 adult *Anopheles funestus* mosquitoes from 6 families and 2 pooled F1 generations between the ages of 2 to 4 days, depending upon available numbers of newly emerged mosquitoes, were exposed to 4% PbO impregnated papers for one hour in a WHO bioassay tube prior to exposure to the bendiocarb and deltamethrin WHO treated papers. One each of the two pooled samples tested against deltamethrin was exposed to PbO papers for an hour prior to exposure to deltamethrin to separate the effect of PbO on the deltamethrin assay. After one hour on the PbO papers, the mosquitoes were then transferred to the holding tube with the insecticide treated papers and held there for one hour. Knockdown was recorded at the end of the one hour insecticide exposure period. Mosquitoes were then transferred to holding tubes with untreated papers for 24 hours and provided with 10% sugar water solution soaked in a cotton pad. After 24 hours, the mortality of mosquitoes was recorded and the mean percentage mortality calculated and recorded. A total of eight replicates of PbO and insecticide were performed on bendiocarb, six *An. funestus* F1 families and two pooled *An. funestus* F1 generations, and two replicates on deltamethrin with pooled F1 generations. Unexposed F1 mosquitoes acted as a control. The WHO insecticide treated papers were tested as to their insecticidal activity by exposing the susceptible reference strain FANG to them in the same manner as for the exposed F1 mosquitoes.

2.5 ELISA assays for *Plasmodium falciparum* parasites in wild caught vectors.

Vector mosquitoes collected from an unsprayed area outside of the Maragra estate were assayed by enzyme-linked immunosorbent assay (ELISA) to investigate their *Plasmodium falciparum* sporozoite rate, to give an indication of their vectorial capacity (Service 2000). This assay entailed measuring the *P. falciparum* circumsporozoite (CS) protein levels in wild mosquitoes (Wirtz *et al.,* 1987). This was achieved by homogenizing the anterior section of head and thorax section of the mosquitoes in a 1,5ml Eppi tube with a pestle in 50µl Blocking Buffer (BB)-NP-40. Once homogenized, the pestle was washed in the tube with 150µl BB to give a total volume of 200µl. Seven female non-infected insectary mosquitoes of the same species were homogenised in the same manner and used as negative controls. A 5ml solution of Phosphate Buffered Saline (PBS) and 40µl stock Monoclonal Antibodies (MAb Pf2A10) was made up and 50µl added per well. The microtitre well plate was covered with aluminium tin foil and incubated overnight at 4°C.

After incubation the well plate was then aspirated and filled with Blocking Buffer and incubated for one hour again at room temperature. The well plate was then aspirated and 50µl of the positive control as supplied by Professor Wirtz (Wirtz *et al.,* 1987) (100pg/50µl) was added to well A1. The seven negative samples were added to the last seven wells of the well plate (H6 – H12) at 50µl each. The samples to be tested were added from well A2 at 50µl each. The well plate was incubated at room temperature for 2 hours. The wells were then aspirated twice with PBS-Tween 20.

A 50µl solution of 5.6ml BB/10µl Peroxidase antibody (pf2A10) was added to each plate well, covered with aluminium foil to eliminate any light and incubated for 1

hour at room temperature. The well plate was washed 5 times with PBS Tween and 100µl/well of the one component substrate was added to each well and again incubated covered with foil, at room temperature for 30 minutes. Observations were then made as to a colour change in the individual wells, to a shade of green if positive for *Plasmodium falciparum* CS. After the 30 minute incubation and initial observation, the well plate was then placed into a Plate Reader for Optical Density (OD) results. Positive results were calculated by determining the average OD value of the 7 negative controls. This averaged value was multiplied by 2 and samples with an OD above this value were scored as positive for *P. falciparum* sporozoites. All positive samples were retested by the same ELISA assay method as above to confirm the positive results.

2.6 *Anopheles funestus* and *An. gambiae* population densities inside and outside of Maragra control area

Estimation of the population densities were made from the following collections: knockdown catches, exit window traps, indoor resting catches and natural shelters and pit traps. The above collections were carried out as described in 2.1.1 to 2.1.4 and any mosquitoes collected were initially identified morphologically and later by PCR as described in 2.3.1 and 2.3.2 above. Species and population densities of *An. funestus* group and *An. gambiae* complex in the different trapping areas were assessed by the numbers and species collected with the above techniques.

2.7 Data Analysis

Standard statistical data analysis (eg. Paired Sample Student's t-test) was carried out on results obtained during the project duration where appropriate.

CHAPTER THREE

RESULTS

3.1 Wild Mosquito Collections

A total of 528 wild caught vector mosquitoes were collected from the study site over the period January to April 2009. *Anopheles funestus* group was the dominant vector species in this area (n = 475) and *An. gambiae* complex was very low in numbers (n = 53) in these collections compared to *An. funestus*.

Table 3.1. *Anopheles funestus* and *An. gambiae s.l.* collection methods and species identifications.

Method	*An. funestus*	*An. rivulorum*	*An. gambiae s.l.*	*An. arabiensis*	*An. merus*
Indoor Resting	439	1	38	25	8
Window Trap	9	0	5	-	-
Knockdown	26	0	10	-	-
Natural Shelters & Pit Traps	0	0	0	0	0

Indoor house searches proved to be the most successful method of the collection of mosquitoes, with both *An. funestus* group and *An. gambiae* complex found to be resting indoors in unsprayed houses during this period. Collections of mosquitoes were carried out in unsprayed and sprayed houses outside and inside of the Maragra control zone. Indoor searches in sprayed houses within the Maragra estate resulted in no collections of vector mosquitoes resting indoors. Only a few *Culex spp*. were

observed resting on sprayed walls indoors in this area. In the unsprayed houses there were many vector mosquitoes resting on walls and clothes, with 508/528 (96.4%) of the total mosquitoes sampled collected solely by this method. *Anopheles funestus* group (n = 462, 90.76%) were collected in significantly greater numbers than the *An. gambiae* complex (n = 46, 10%) by this means.

Knockdown catches were not very successful for the collection of mosquito samples, particularly in sprayed houses. In unsprayed houses results were better but not what was expected. Only 26 (5.5%) *An. funestus* group and 10 (18.9%) *An. gambiae* complex were collected from unsprayed houses in this manner. *Culex spp.* were observed in great numbers in the unsprayed houses and were the predominant mosquitoes observed by this method.

Exit window traps were also not very successful in collecting anopheline mosquitoes. None was collected in any of the sprayed houses and in the unsprayed houses only 5 (9.4%) *An. gambiae* complex and 9 (1.9%) *An. funestus* group were collected by this technique.

Natural shelters and pit traps were entirely unsuccessful in the collection of vector mosquitoes, but highly successful for the collection of *Culex spp.* It was evident from this collection technique that both *An. funestus* group and *An. gambiae* complex do not utilise such refuges in this area.

3.2 Species Identification

Anopheline mosquitoes were initially identified morphologically to groups according to the keys of Gillies & Coetzee (1987) and thereafter by species-specific identification by PCR. Both malaria vectors *An. funestus s.s.* and *An. arabiensis* (Figs 3.1 and 3.2) were identified as species of importance in this area.

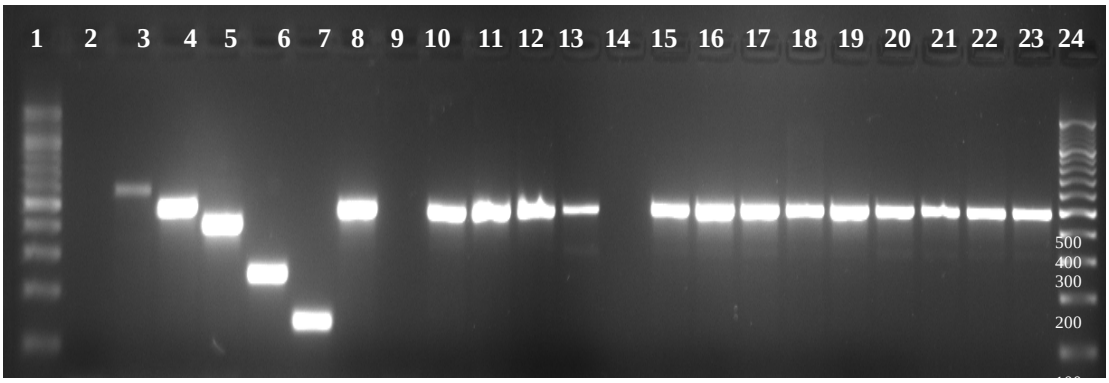


Figure 3.1 PCR identification of *An. funestus* group. Lanes 1 & 24 molecular weight markers, Lane 2 'No DNA', Lanes 3-7 *An. vaneedeni, An. funestus, An. leesoni, An. rivulorum, An. parensis* positive controls, Lane 8 positive control, Lane 9 negative control, Lanes 10-23 wild samples identified as *An. funestus.*

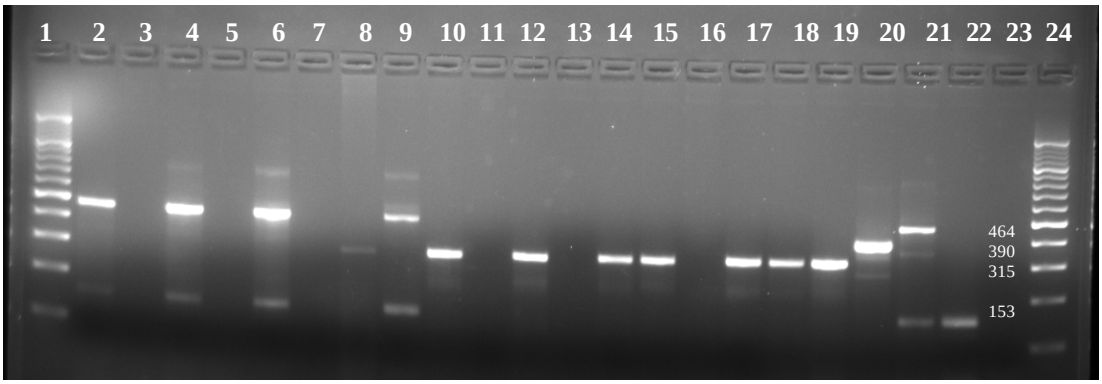


Figure 3.2 PCR identification of *An. gambiae* complex. Lanes 1& 24 molecular weight markers, Lanes 2-18 wild samples of *An. merus* and *An. arabiensis*, Lanes 19-22 *An. arabiensis, An. gambiae, An. merus, An. quadriannulatus* positive controls

A total of 175/475 (36%) *An. funestus* group and 52/53 (98%) *An. gambiae* complex were tested by PCR to establish species-specific identification of these mosquitoes. The majority of the *An. funestus* group samples (n = 167, 95%) were identified as *An.*

funestus s.s., and one specimen identified as *An. rivulorum*. Only 33/52 of the *An. gambiae* complex samples gave amplified products and of these 75.8% were identified as *An. arabiensis* and 24.2% as *An. merus* (Fig. 3.2). The lack of PCR products from 19 samples could have been due to DNA degradation through poor storage in the field.

3.3 Insecticide Resistance

A total of 952 *An. funestus* wild caught and F1 generations (including controls) were tested for resistance to the four classes of insecticides given in Table 2.1, using the WHO standard for bioassays (World Health Organization, 1998). The results are summarised in Tables 3.2 to 3.5. Given that 99.4% of all PCR identified samples were *An. funestus s.s.*, it is assumed that the results from this section pertain to this species only.

A total of 261 wild caught *An. funestus* were tested for susceptibility on the same day they were collected. The highest levels of resistance were found to lambdacyhalothrin and deltamethrin (Table 3.2). According to WHO criteria, resistance to bendiocarb with 71.2% mortality in the wild caught population is now confirmed. Similar results were found for pyrethroids amongst pooled *An. funestus* F1 generations (Tables 3.3 and 3.5). In six *An. funestus* F1 families tested against bendiocarb, the average mortality was 73.7% with a high of 100% and a low of 57% (Table 3.4) with unsynergised samples showing a similar trend in Table 3.5. Both DDT and malathion gave 100% mortality (Table 3.2).

Those F1 families producing enough adults for subsequent analysis were assayed against the monooxygenase inhibitor pbo and the insecticides bendiocarb and deltamethrin. The results are given in Tables 3.4 and 3.5. Six families consisting of pooled F1 progeny showed significant levels of resistance to bendiocarb (P = 0.002 on the Paired Sample Student's t- test) with percentage mortalities 24 h post-exposure ranging from 57% to 83%. In all of these samples 100% susceptibility was achieved with the pbo.

The two tests with pooled F1 generations on deltamethrin also showed high levels of resistance ranging from 7.7% to 83%. In one of the two tests 100% susceptibility was achieved with pbo, the second test achieving 83% susceptibility with pbo, but still within the limit of acceptability by WHO (1998). There was no mortality in control samples exposed to pbo and untreated papers. In all cases bendiocarb resistance was completely nullified using pbo, strongly suggesting that bendiocarb resistance in the wild parent population is mediated by monooxygenase detoxification.

Table 3.2. Insecticide susceptibility of wild caught *Anopheles funestus s.s.* from Village 2000, Maragra study site, Mozambique to the four classes of insecticides.

Insecticide	**No. Exposed**	**% Mortality**
0.05% Deltamethrin	37	32.5
0.05% Lambdacyhalothrin	35	14.6
0.1% Bendiocarb	117	71.2
4% DDT	20	100
5% Malathion	52	100

Table 3.3. Insecticide susceptibility of pooled F1 *Anopheles funestus* from Village 2000, Maragra study site, Mozambique to three classes of insecticides.

Insecticide	No. exposed	% Mortality
0.05% Deltamethrin	114	53.4
0.05% Lambdacyhalothrin	54	33.3
4% DDT	61	100
5% Malathion	52	100

Table 3.4 WHO bendiocarb (carbamate) bioassay results comparing Piperonyl butoxide (pbo) Synergised and unsynergised subsamples of six *Anopheles funestus s.s.* F1 families, Maragra study site, Mozambique

Family No.	4%pbo + 0.1% Bendiocarb	0.1% Bendiocarb
39	100% (n = 7)	57% (n = 7)
32	100% (n = 8)	62.5% (n = 8)
23	100% (n = 9)	100% (n = 6)
58	100% (n = 16)	62.5% (n = 16)
42	100% (n = 12)	83% (n = 12)
19	100% (n = 10)	77.7% (n = 9)
Controls	0% (n = 144)	

Table 3.5 WHO bendiocarb (carbamate) and deltamethrin (pyrethroid) bioassay results comparing mortality of Piperonyl butoxide (pbo) synergised and unsynergised subsamples of pooled *Anopheles funestus s.s.* F1 families, Maragra study site, Mozambique

Sample	4%pbo + 0.1% Bendiocarb	0.1% Bendiocarb	4%pbo + 0.05% Deltamethrin	0.05% deltamethrin
An. funestus	100% (n = 11)	62.5% (n = 8)	83% (n = 12)	30% (n = 10)
An. funestus	100% (n = 8)	80% (n = 10)		7.7% (n = 13)
Control	0% (n = 11)		0% (n = 14)	
An. funestus			100% (n = 15)	83% (n = 6)
An. funestus				92% (n = 13)
Control		(Average = 71.1%)	0% (n = 15)	(Average=53.1%)

3.4 ELISA assays for P. falciparum parasites

A total of 166 *An. funestus* samples were subjected to the ELISA test for *Plasmodium falciparum* circumsporozoite (CS) protein. Ten specimens were confirmed positive after retesting, giving a positivity rate of 6.02% for *An. funestus* in the areas outside of the Maragra control zone.

The ELISA tests conducted on 52 *An. gambiae* complex samples gave one confirmed positive specimen for *Plasmodium falciparum* from 25 PCR identified *An. arabiensis* and 8 *An. merus*. But, the specific vector species is unknown, as this particular vector sample, positive on ELISA testing, did not amplify on PCR.

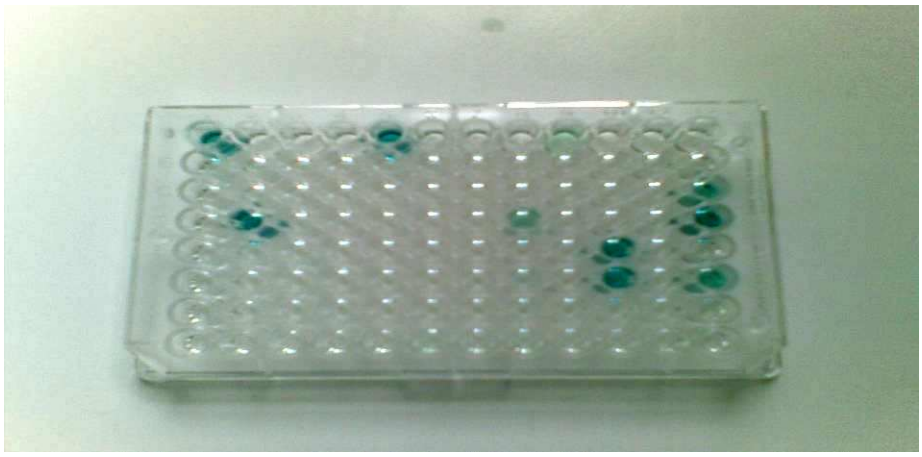


Fig. 3.3 ELISA microtitre plate with positive specimens stained green.

3.5 *Anopheles funestus* and *An. gambiae* species composition and population densities inside and outside of Maragra control area.

Despite utilising the same collection techniques as employed in the areas outside of the malaria control zone, no *An. funestus* and *An. arabiensis* mosquitoes could be

captured or found resting inside houses in the Maragra control zone, which is encouraging in respect of the impact of residual insecticide spraying in this area.

In the unsprayed areas outside of Maragra the malaria vectors are very easily found and in good numbers. Up to 50 anopheline specimens were able to be captured in indoor resting searches in 2-4 houses on some days. A total of 528 anopheline mosquitoes were collected by indoor resting searches, exit window traps and knockdown collections with four different species identified, these being *An. funestus, An.rivulorum, An. arabiensis* and *An.merus* (Figs. 3.1 and 3.2).

CHAPTER FOUR

DISCUSSION

Malaria is the scourge of Africa with more than a million deaths due to this disease every year with 350-550 million cases (Snow *et al*., 2005; World Health Organization, 2005), causing untold suffering and economic loss on an individual, family and country basis. These case numbers are recognised as an estimation, as many cases and deaths are not reported to a health institution due to remoteness, lack of transport, poverty, political upheaval and lack of a close and functioning health institution. Malaria is on the increase in Mozambique affecting children < 5 years of age to the greatest extent (World Health Organization, 2008).

At Maragra sugar estate the vector control programme is effecting control of the vectors and transmission of malaria within the estate. There is good correlation between rainfall and malaria cases, particularly in the peak of the rainy season from November to March when there was a concomitant rise in malaria cases. The period from January 2003 to November 2005 was a relatively quiet period of malaria transmission except for the period of August/September when there was a late season increase in cases due to unusual late rains in May/June of that year. November 2005 to April/May 2006 thereafter and the same period for 2006/2007 were a particularly severe two years for malaria in this area. The malaria cases since then however, have reduced again from November 2008 to date, despite the good rains during this period. There is a substantial increase in employees from May through to December when harvesting and milling of the cane begins, and this correlates quite well with the

increase in malaria cases. The great majority of these temporary employees are from outside of the Maragra vector control area and undergo a medical examination prior to employment. A large number of the malaria cases recorded for Maragra are from these temporary employees, and tend to distort the Maragra case figures as to transmission within the control area during this period. The correlation of malaria cases to employees is quite consistent from April/May to December when their contracts end for that year, and the malaria cases drop dramatically in January.

In the Maragra area the principle vector of malaria is *Anopheles funestus s.s.*, an indoor feeding and dwelling malaria vector, with the secondary vector being *An. arabiensis,* an outdoor and indoor feeding vector of malaria. Whilst IRS at Maragra has been very effective in controlling both *An. funestus* and *An. arabiensis, An. arabiensis* is more difficult to control due to its endophilic and exophilic habits. Both of these vectors could not be found within the Maragra vector control areas.

4.1 Species identification of the malaria vectors in the Maragra area

In this study *Anopheles funestus s.s.* was found to be the predominant vector species in the area surrounding Maragra estate and the rural community. This has been reported before in the Manhica area (Aranda *et al*., 2005). This is hardly surprising given the permanent and extensive breeding sites of the irrigation systems and swamp sites preferred by this vector.

Anopheles arabiensis was the second malaria vector identified in this area. The number of *An. arabiensis* and *An. merus* collected and the proportion of *An. merus* to

An. arabiensis (8/25 = 32%) was interesting. Many searches have been carried out to find the breeding sites of these vectors by the author in the past without success.

Anopheles merus prefers brackish or saline water as a breeding site. Breeding of this vector in some of the more shallow irrigation canals and in the random shallow channels in the banana plantations to the south of the cane fields is entirely possible, as this area is close to the Indian Ocean and used to form part of the old sea bed during the Cretaceous, Miocene and Pliocene times, with the base of the Lebombo mountains forming the shoreline (Maud, 1980). Salt may have leached to the surface due to the irrigation techniques in these areas creating suitable breeding sites for this species. However, this may not necessarily be the case, as *An. arabiensis* and *An. merus* have previously been found breeding together in the same fresh water body (Kloke, 1997). Further investigation needs to be done in regard to the breeding sites and role of malaria transmission of these two vectors in this area.

Although there are cases of malaria amongst residents of the estate, it is most likely primarily due to circulating *An. arabiensis* which exhibits exophilic and endophilic behaviour. *Anopheles funestus* is strongly endophilic and may not be responsible for outdoor transmission within the residential area of the estate. On previous landing catches outdoors carried out by the author prior to this study, only *An. gambiae* complex have been found, albeit in very low numbers.

4.2 Insecticide resistance status of *Anopheles funestus*.

From the studies carried out at Maragra on the wild caught populations from the area around the estate, high levels of resistance to the pyrethroids lambdacyhalothrin

(85.4%) and deltamethrin (67.5%) were found in *An. funestus*, and a lower level of resistance to bendiocarb (29%), the insecticide in use at Maragra. On a single pooled F1 *An. funestus* sample tested against lambdacyhalothrin, a 66.7% resistance level was recorded, and for deltamethrin on 2 pooled samples of F1 *An. funestus* a 46.7% resistance level was recorded. In both wild caught and pooled families, lambdacyhalothrin exhibits the highest level of resistance, and has been one of three insecticides in use by the NMCP (lambdacyhalothrin, bendiocarb and DDT) in the district around Maragra for a number of years. Deltamethrin has not been used in this area and this may account for its lower level of resistance to this vector, despite the high level of resistance conferred to the vectors, by belonging to the same family of synthetic pyrethroid insecticides, as that of lambdacyhalothrin.

However, there is an 18.7% and 20.8% difference in the recorded levels of resistance between the *An. funestus* wild caught and F1 pooled families to lambdacyhalothrin and deltamethrin respectively, both samples exhibiting higher levels of resistance in the wild caught populations than in the F1 pooled families samples. This may be due to varying ages of the wild caught populations sampled, their fed and gravid status and extent of previous contact with this insecticide, and is a factor that should be taken into account when determining resistance profiles of malaria vectors in an area. This percentage difference between wild caught and F1 sample results may be significant in monitoring of insecticide bio-efficacy and also deciding what insecticide to use in a vector control programme, in order to adhere to the WHO guidelines on this aspect of vector control (World Health Organization, 1998). Although there is a higher level of resistance exhibited in the wild population of vectors than that of their F1 generations, this is the real situation in the field and is a reliable guide to the levels

of resistance in a vector population. Cognisance must be taken of this, and the necessary planning instituted to control and if possible, reduce or eliminate this gene pool of resistance.

Although the level of resistance in the wild populations of vectors around Maragra to bendiocarb is cause for concern (71.2% susceptibility) in terms of the WHO (1998) guidelines as to levels of resistance status, it must be exerting a control influence on both *An. funestus* and the *An. gambiae* complex, as none of these vectors were found resting indoors or collected in window traps or knockdown catches in houses from the sprayed areas. The low level of malaria cases amongst Maragra employees is further evidence of the success of this insecticide in vector control programme.

Ideally, a change of insecticide from bendiocarb inside the Maragra control area and a change from the recently introduced synthetic pyrethroid outside of Maragra for IRS is indicated. But what insecticide is the one of choice in this situation? Only two options are available at present and they present difficulties in procurement and implementation, these being DDT and Malathion, to which *An. funestus* is fully susceptible. Both these insecticides are not popular with residents of most western type and traditional housing, due to their high visibility on walls and furniture etc., (DDT) and their strong odour and staining properties (Malathion). DDT was being used in the areas outside of Maragra, but has been discontinued this year and replaced by lambdacyhalothrin,, to which *An. funestus* is highly resistant. As bendiocarb is controlling the vectors of malaria within its control area, for the present it is necessary to continue with this insecticide until a viable alternative can be found to replace it. Continual monitoring of the situation in this regard is of crucial importance in

maintaining the present levels of control, and in making informed decisions based on scientific fact on future actions to take to reduce the levels of resistance to this insecticide and synthetic pyrethroids.

Due to the difficulty of rearing *An. funestus* F1 generation mosquitoes in the laboratory because of their longer egg to adult development, only two series of bioassays were carried out to determine if there was a biochemical mechanism of resistance in *An. funestus* to bendiocarb and deltamethrin. These assays revealed that the resistance mechanism was strongly mediated by the synergist pbo, a synergist that specifically inhibits monooxygenase activity. Between synergised and unsynergised samples, bendiocarb resistance was completely nullified using pbo, strongly suggesting that bendiocarb resistance in the wild parent population is mediated by monooxygenase (P450) detoxification. Although one of the deltamethrin samples of the two synergized and unsynergised samples tested gave a result of 83% susceptibility and the other 100% susceptibility, it is already well known that P450 genes are responsible for pyrethroid resistance in southern Africa *An. funestus* (Amenya *et al.*, 2005; Wondji *et al.*, 2009).

Due to the small sample size of the *An. gambiae* complex it was not possible to test for insecticide resistance in this study, however further studies on this complex are needed, particularly on *An. merus*. The insecticide resistance status of *An. arabiensis* is discussed in Casimiro *et al.* (2006a,b), with specimens collected from an area approximately 40 km north of the Maragra study area and they report low levels of pyrethroid resistance.

Of serious concern to the Maragra vector control programme manager is the cessation of the use of the insecticide bendiocarb in the NMCP IRS programme in the Manhica district and areas surrounding Maragra (except for the LSDI control programme in the Manhica district - Dr. R Maharaj, MRC Durban, Regional LSDI Co-ordinator, personal communication), and the reintroduction of lambdacyhalothrin for IRS (NMCP, IRS programme manager at Manhica health post, personal communication). Added to this is the reported further cessation of DDT for IRS in this area (NMCP, IRS programme manager at Manhica health post, personal communication). The only insecticide to be used in this area now is lambdacyhalothrin, to which *An. funestus*, the principle vector of malaria in this area, is highly resistant and *An. arabiensis* exhibits low levels of resistance, at this time. The continued use of lambdacyhalothrin to which *An. funestus* is already highly resistant will result in even higher levels of resistance in this vector and also possibly in the *An. gambiae* complex. Complete resistance to this insecticide is highly likely with its continued use in this area, which may result in increased malaria transmission and morbidity and mortality amongst the surrounding local community outside of Maragra. This will also impact on Maragra in increased numbers of malaria cases at their clinic, as local labour is employed by the estate for cane cutting and work in the Mill and present to the clinic for diagnosis and treatment, which will have a financial cost.

Control of *An. arabiensis* is problematic due to its life habits of endophily and exophily and anthropophagism and zoophagism. Whilst ITN's may appear to be a strategy that can be employed to protect the human population in this area, the level of pyrethroid resistance in this vector and the high levels of resistance to the same insecticide in *An. funestus* should preclude this insecticide for IRS vector control.

Which also begs the question as to what other insecticide can be used for ITN's since only pyrethroids are approved by WHO for this purpose at present?

In northern KZN proximal to the Mozambican border from 1998 to 2000 there was a huge surge in malaria cases over this period, due to the change from DDT to pyrethroids and the reappearance of *An. funestus* from across the Mozambique border which was resistant to pyrethroids (Hargreaves *et al*., 2000 Brooke *et al*., 2001). The change back again to DDT (in traditional housing) and to deltamethrin (for western housing), resulted in a dramatic reduction in transmission and cases of malaria and an equally dramatic reduction in the *An. funestus* population in this area. Consequently, DDT resistance has now been demonstrated in *An. arabiensis* and *An. quadriannulatus* in the KZN area (Hargreaves *et al*, 2000, 2003), for the first time since the use of DDT was first introduced in 1946 (Maharaj *et al*., 2005). DDT resistance has now also recently been demonstrated in *An. merus* in the KZN area (K. Hargreaves, personal communication). Prior to the change from DDT to synthetic pyrethroids no resistance to DDT was detected in this vector in entomological monitoring. What has prompted this change of resistance status is not certain, but it has been suggested that the change to pyrethroids and then back to DDT again may have been the trigger (K. Hargreaves, personal communication).

This situation could develop in the Manhica district if the same phenomenon or trigger results in *An. arabiensis* and *An. funestus* becoming resistant to DDT when and if it is used for IRS again. 'The change to DDT in this area was economically and scientifically driven, informed by accurate monitoring and evaluation of the local vector insecticide resistance profile' (Coleman *et al.*, 2008). The need for scientific

entomological monitoring and evidence based control decisions in this area is critical for informed programme management.

4.3 Vector status of *Anopheles funestus* and *An. arabiensis* and parasite density.

Plasmodium falciparum parasites in the malaria vectors detected by ELISA, demonstrated very high levels of infection in *An. funestus* (6.02%). This study confirms its predominance as the principle vector of malaria and also indicates the hyperendemicity of malaria in the human population in this area at the time of this study. Mayor *et al.* (2007) report almost half of the adults from Manhica town were positive for *P. falciparum* during the dry season, and that rates may be even higher in the wet season. Mabunda *et al.* (2008) reported human malaria parasite prevalence of 58.9% of which 52.4% were due to *P. falciparum*, the burden being in the northern areas and also the coastal areas, where Maragra is situated. The high sporozoite rate of 6.02% in the *An. funestus* population assayed for malaria parasites in this study correlates well with this level of endemicity and places it as a vector of the highest priority for control. The one *An. gambiae* complex that showed positive for *Plasmodium falciparum* could not be identified to specific species as this particular sample did not amplify on PCR, but gives a rate of 3% for this complex. As this investigation and collection of mosquitoes was done from January to March, in the season of high malaria transmission in this part of Mozambique, the results for *An. funestus* however correlate well with this high transmission period.

4. 4 *Anopheles funestus* and *An. gambiae* complex populations in and out of Maragra vector control area.

Mosquito collections carried out in sprayed areas, revealed that the insecticide sprayed houses contained no resting anopheline mosquitoes despite intensive searches under and behind furniture and beds. This is confirmation that the IRS programme at Maragra is killing and/or repelling malaria mosquito vectors and effecting control measures which are protecting those that fall under this control programme. This is despite large numbers of vectors and intense malaria transmission outside the area of control as evidenced by the sporozoite rates in these vectors.

Although anophelines were captured resting in western type dwellings in the unsprayed areas, the numbers captured in this manner were considerably less than in the traditional dwellings. This may be due to greater affluence of the occupants of the western type dwellings and the use of mosquito coil repellents, which we found to be used in many of these dwellings.

An interesting observation made during these collections was the number of mosquitoes found resting inside unsprayed houses where young children were sleeping at night. Virtually without exception the vector mosquito numbers were significantly higher, often in the region of 15–30+, in rooms with children < 5 years of age, than in rooms where older adults slept, where only 1 – 5 vectors may have been captured at best. The same increase in the numbers of mosquitoes was found where adults and children slept together in the same room.

Lacroix *et al.* (2005) reported on the greater attraction of mosquitoes by children infected with *Plasmodium falciparum* gametocytes than those infected by natural means with asexual parasites (non-infective) and those uninfected. The children

infected with gametocytes attracted twice as many mosquitoes as the others in the control group. Once the gametocytes had been cleared with anti-malarials the attractiveness of these children to mosquitoes reduced to the same level as the control groups. This would appear to correlate with the findings of this study, of greater numbers of mosquitoes in rooms where young children were sleeping than in rooms where only adults slept. This may be due to the lower levels of gametocytaemia in older adults, rendering them less attractive to vector mosquitoes. In a retrospective study of malaria in children < 10 years in the Manhica district over a two year period, children < 2 years accounted for 60% of all the malaria cases in over 8000 patients with 19% of all paediatric deaths due to malaria (Bassat *et al*., 2008). A study carried out in this regard by Saute *et al.* (2003) confirmed that children < 10 years suffered the most with the highest incidence of malaria, especially those from 6 months to < 4years.

This high percentage of infected children may be due to the attractiveness of mosquitoes to children, especially those harbouring gametocytes, as demonstrated by Lacroix. In another study carried out in Mozambique it was found that parasite infection and density peaked in the second year of life and decreased with increasing age (Mayor *et al*., 2007). It may be useful to correlate such studies with vector densities in the different age groups. Further study needs to be done on this aspect of malaria transmission, as it has implications for the control of malaria vectors in this area and may be an area for vector control programme managers to focus control activities.

During this study period an unplanned and impromptu once-off night man-net catch was carried out by the author from 19h00 to 23h00 in the unsprayed area, about 3 metres from a traditional house in which young children slept, and in which *An. funestus* and *An. gambiae* complex had been collected the previous few days and cleared that morning. The author and another entomologist acting as bait (both on malaria prophylaxis), sat inside a netted tent with the bottom 6 inches of the tent raised off the ground, and the entrance open to allow access for circulating mosquitoes. No anopheline malaria vectors entered the tent to rest or feed. However, the next morning this same room, near to which the man net catch took place, in which previous collections had been very successful, was searched to see if there were vectors again resting in the room. The room again contained many *An. funestus*, bloodfed and resting inside. It would appear from this observation that *An. funestus* may predilect to the interior of more sheltered permanent structures and prefers to feed indoors rather than outdoors.

Knockdown collections were more successful than the exit window traps with more *An. funestus* being collected than *An. gambiae* complex. The low numbers of anophelines collected correlates with the collections done by Mendis *et al.* (2000) and Aranda *et al.* (2005) in which indoor spray catch (knockdown collection) mosquito collection numbers were also lower than resting indoor collections in houses in the Matola and Manhica areas. However, despite the low numbers collected in these studies, they correlate with the greater proportion of *An. funestus* to *An. gambiae* complex that were collected in indoor resting catches in this study, and with the high rate of *P. falciparum* infection confirm its status as the predominant vector of malaria in this area.

Fig. 4.1.a Typical local community housing (note lack of windows)

A problem encountered with local reed and corrugated roofed houses (Fig. 4.1a) which was the majority housing in the unsprayed areas around Maragra, was the lack of windows in the majority of these structures, hence choice of site selection for this type of dwelling for trap installation was very limited, restricting the full capability of this trapping technique. Those homes that did have windows (Fig. 4.1b) were not always the most suitably sited in proximity to potential breeding sites, and in occupancy with young children, which was the premium site desired. Hence a larger proportion of traps were installed in western style housing rather than traditional reed housing. The occupants of these houses are at a higher socio-economic level than those of the reed houses and were more prone to using insect repellent coils than those in reed houses, which adversely affected collections from these houses.

Fig. 4.1.b Typical western type housing at Maragra

4.5 Implication of the sugar cane industry on malaria vector production, malaria transmission and insecticide resistance at Maragra sugar estate

Previous work carried out on *P. falciparum* infection in *An. funestus* and *An. arabiensis* was carried out by Mendis *et al*. (2000) in Matola, a suburb of Maputo, by dissection of the salivary glands of vector mosquitoes and identification of sporozoites by microscopy. The rate was calculated as a percentage of infectivity relative to those mosquitoes dissected and positive, to those negative for sporozoites. In this study Mendis reported plasmodium rates of 2.42 ± 1.24% in *An. funestus* and 1.11 ± 1.25% in *An. arabiensis* over the period November 1994 to April 1996. Aranda *et al.* (2005) surveyed the vector populations in the Manhica area from October 1997 to September 1998 and reports on an estimated *Plasmodium* rate of 1.2%. These rates are significantly lower than those reported in this study, which was carried out during the mid to latter part of the rainy season.

From the results of this study it would appear that the sugar cane farming industry in malarious areas does have significant implications, particularly on malaria vector production and malaria transmission in those areas where it is situated. Maragra started cane farming in 1998 with extensive rejuvenation of the cane fields and irrigation systems and employment of local labour to assist in this programme of activities. This would have changed the local ecology at the time and provided more opportunity for further invasion of vectors into these areas as more and more permanent breeding sites were established, and a greater introduction and a greater concentration of malaria parasites introduced into this area from the local labour force employed.

In 1998 *Anopheles funestus* represented 72.3% of the vector population with a sporozoite rate of 1.2% in the Manhica area (Aranda *et al*., 2005), which is very close to Maragra sugar estate. A large section of the cane fields and irrigation systems lie immediately below Manhica town on the NKomati floodplain where Aranda carried out his study. This study 11 years later in the same site on the same vector and the parasite infection rate of this vector, has found that *An. funestus* now represents 90.8% of the vector population in this area with a *Plasmodium falciparum* rate of 6.02%. This is an increase of 18.5% increase in the *An. funestus* predominance and a 4.8% increase in the *P. falciparum* sporozoite rate of this vector over this period.

As Mabunda (2008) points out in his study, the greatest concentration of malaria is in the northern and coastal districts of Mozambique, and this is where the sugar industry in Mozambique is primarily situated. Not all of his studies were conducted around sugar estates and in areas where the vector *An. funestus* predominates, but with the

expansion of the sugar industry in the coastal and major river systems in Mozambique the potential for increased vector production and malaria transmission is extremely high, if not almost certain.

In comparison tests carried out comparing levels of infection by this method and by ELISA, it was demonstrated that with the ELISA method, the count could be 1.5 to 2 times higher than assessed by the microscopy method. The dissection technique ensures greater accuracy in estimating the burden of malaria in an area of vector and human populations, but is not always practical in the field situation and with time and resource constraints. Although there is controversy with regard to the accuracy of ELISA infection rates on anterior portions of malaria vector mosquitoes in relation to rates by dissection of salivary glands (Beier *et al*., 1990), the ELISA technique is still useful for measuring transmission intensity and changes over time. This study gives some validity to this argument as the difference of the *P. falciparum* rates over the 11 years demonstrates the validity of this method as a field tool to monitor the parasite rate increase or decrease.

4.6 Conclusion

The incidence of insecticide resistance is an increasingly critical problem for vector control managers in Africa. Without the necessary knowledge of which vectors are resistant to particular insecticides in a particular area, the implementation of a vector control programme is meaningless and an expensive waste of manpower, finance and other resources (Mouatcho *et al*., 2007). If implemented without this knowledge it may create a bigger problem than that originally defined or intended, by increasing the levels and intensity of vector resistance to the insecticide in use with increased

transmission of malaria parasites to the human population. Coupled with this is the necessity of ensuring the programme is managed and implemented on the planned basis - that the designated areas planned for IRS operations are sprayed as and when planned, and that the IRS is carried out correctly in accordance with WHO guidelines (World Health Organization, 1997, 2006).

The vector control programme around the outskirts of Maragra appears to have such management problems. Planned spray programmes are not always adhered to, with villages and housing being left out in the spray schedule, and the technique of IRS is in many instances not at an acceptable standard (personal observation). The spray programme this year has also been implemented in July, with the malaria transmission season starting only after the rains in November. This means that the insecticides will have lost their efficacy before the end of the transmission season in May 2010. The NMCP has changed from bendiocarb for IRS to lambdacyhalothrin for this year's spray round in this area, an insecticide to which, this and other studies have confirmed, *An. funestus* is highly resistant (Brooke *et al*., 2001, Casimiro *et al*., 2006 a, b). These factors will lead to rapidly increased levels of resistance in this vector to this insecticide and may increase levels of malaria transmission and cases as in South Africa in 1999 - 2000.

A further complication to insecticide resistance in malaria vectors is the leakage from NMCP storage and uncontrolled sale of carbamate and DDT insecticide on the black market (personal observations; Presidents Malaria Initiative: Malaria Operational Plan – FY 09) which will in time, in unskilled hands, impact further on the insecticide resistance status of *An. funestus* and *An. arabiensis*.

Maragra sugar estate's vector control programme is surrounded by the NMCP and these actions may have dire negative consequences for its malaria vector control programme, particularly the community outside of the Maragra vector control programme. Accountability, vigilance and new strategies of vector control are of paramount and urgent importance under such conditions. Malaria has increased in Mozambique in 2001 to 2006 (World Health Organization, 2008)and so have sugar estates. The increase in vector production, malaria transmission and malaria cases is a serious problem in Mozambique, and sugar estates and NMCP's need to be accountable to the communities in which they operate in their roles and responsibilities in the control of malaria vectors and malaria disease. Both have major implications in the control of malaria vectors and disease, or the converse of increasing numbers of insecticide resistant malaria vectors and an increasing malaria burden and concommitant human and economic loss to the country.

Malaria parasites and malaria mosquito vectors appear at times to be advancing as rapidly as the new drugs and insecticides are developed and brought into play. As fast as therapeutic drugs are discovered and distributed, the parasite begins to develop immunity to these drugs. The same scenario is being played out with mosquitoes and insecticides, with insecticide resistance management playing an increasingly important role in malaria vector control programmes utilising IRS. New insecticides and new drugs and vaccines are being sought in partnership with private sector industry in an effort to control malaria parasites (Hemingway *et al.*, 2006). New and innovative methods of vector control are being and need to be sought, in an effort to curb and control the vectors of this disease.

The burden of responsibility to ensure programme success rests on the malaria vector control programme manager in the field, who must make operational decisions for the control of malaria in his area, to achieve the best outcome against the vector with the resources allocated to that task. As with any battle commander, the more intelligence he has about his target, the better he can plan and optimise for the best outcome. In this regard it is essential that he at the very least, knows what species of vectors he is dealing with in his area of operations, and to what extent they are resistant or susceptible to insecticides. To this end he will need as much entomological intelligence as he can muster and he will need the support of a medical entomologist trained in malaria entomology to achieve this.

The entomological and disease monitoring of such IRS programmes is increasing in importance, and requires an increase in entomologists and technical expertise to proactively assist and advise programme managers on the best courses of action, to evaluate implemented measures and manage the resistance profile for control of the vectors and disease transmission (Beier *et al*., 2008).

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