Ecology of breeding sites and insecticide resistance of the potential malaria vectors in Mpumalanga Province, South Africa

By

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of MSc in Biology and Control of African Disease Vectors

Johannesburg, March 2013

DECLARATION

I, Manzane Frans Mbokazi, declare that this research report hereby prepared and submitted by me for the degree of MSc in Biology and Control of African Disease Vectors at the University of Witwatersrand, Johannesburg, is my own work and has not been submitted for any examination or degree in any other University or research institution.

Signature.....

On this.....28.....Day of.....MARCH.....2013

ABSTRACT

In spite of the reduction of morbidity and mortality rates reported recently, malaria remains a problematic disease in sub–Saharan Africa. An estimated 184 million people contracted the disease in 2010 with 611,000 deaths in the African region. Its transmission is sustained by the presence of highly efficient and anthropophilic *Anopheles* vector mosquitoes despite the continuous use of indoor residual spraying (IRS) and insecticide treated bed nets that have reduced the burden of malaria in some regions. The *Anopheles funestus* group and the *An. gambiae* complex contain the three most efficient vectors responsible for malaria transmission and are distributed across most of sub-Saharan Africa.

In Mpumalanga Province of South Africa, 85% of the malaria cases are imported and only 15% are acquired locally. This study aimed to review and update the entomological data on breeding sites of potential malaria vectors and to investigate the insecticide resistance status of these mosquitoes in Mpumalanga Province. The study was conducted in the Ehlanzeni district around the low and high risk areas of Nkomazi municipality. The study was carried out from October – December 2011.Selected breeding sites were monitored and all larvae sampled were reared to adults. One to five-day old female mosquitoes were used for insecticide susceptibility tests according to the WHO standard procedures. Female mosquitoes were morphologically identified and PCR was performed for members of the *An. gambiae* complex and the *An. funestus* group.

Twenty-five permanent mosquito breeding sites were mapped with 60% located in the high risk areas and 40% in the low risk areas. A total of 1200 anopheline mosquitoes were collected with 82.5% belonging to the *An. gambiae* complex and 27.5% belonging to the *An. funestus* group. 365 specimens were subjected to PCR for species-specific identification *Anopheles merus* and *An. rivulorum* were the most abundant species. Mosquitoes were subjected to the WHO insecticide resistance tests and were susceptible to all tested residual insecticides with an average of 99% mortality.

Additional mosquito collection methods, such as night-biting catches, carbon dioxide net traps and pit-trap collections need to be carried out to sample other proportions of the mosquito populations that may not have been sampled by larval collections.

DEDICATION

Through their unconditional love, I dedicate this research report to my parents, the late ZJ Mbokazi and NE Mbokazi, who encouraged me to further my studies. I also dedicate this work to my wife Phindile Lillian Mbokazi, who has always supported me during the difficult times of my research proposal development process and field data collection.

ACKNOWLEDGEMENTS

A special acknowledgement goes to Mr. MA Mabuza, Provincial Malaria Control Programme Manager, for his initial advice and support for the project. I am grateful to the Mpumalanga Provincial Department of Health for their financial support of the project. Likewise to my colleagues Gerdalize Kok and Daniel Williams, who instilled confidence and ideal in me in terms of mapping the identified mosquito breeding sites. In terms of field data collection, I would like to thank the entomology teams for their constant and correct GPS coordinates recording and Goodness Nkosi for the historical entomological data prepared and forwarded for the project.

I would like to thank Dr Riann Christian, Dr Belinda Spellings and Dr. Samuel B Vezenegho for their unconditional guidance in the molecular laboratory and for assistance with the PCR identification of my specimens. Most importantly, I would also like to thank my supervisor, Professor Maureen Coetzee, who initially guided and supported me in planning this research project.

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CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Malaria has been identified as a major public health problem in Africa. The disease is still having a great impact on morbidity and fatality rates as reported annually in sub–Saharan Africa (WHO, 2011). Out of the 46 malaria endemic African countries, about 184 million people were affected by the disease in 2010 with 611,000 deaths. The impact is more severe on children less than 5 years and pregnant women because of their low immune status compared to the other groups (WHO, 2011). Studies have shown that the malaria transmission cycle depends on the presence of populations of *Anopheles* vector mosquitoes that feed on humans.

The *An. gambaie* complex and the *An. funestus* group play a role in maintaining malaria transmission. Entomological studies have been conducted to draw a picture of its distribution in sub–Saharan Africa. These anopheline mosquitoes are closely related, and are difficult to differentiate morphologically. However, species molecular identification techniques such as polymerase chain reaction (PCR) assay have been developed to identify these anopheline mosquitoes to species-specific level (Scott *et al.,* 1993; Koekemoer *et al.,* 2002). In sub–Saharan Africa, *An. arabiensis*, *An. gambiae*

and *An. funestus* are the three species of *Anopheles* known as major vectors and responsible for the transmission (Gillies and DeMeillon, 1968; Gillies and Coetzee, 1987).

Indoor residual house spraying (IRS) with has been identified as the backbone of malaria vector control. This strategy has rapidly reduced disease burden, seasonal peaks and malaria epidemics in areas of seasonal or perennial transmission and also reduced intense malaria transmission. Most importantly, all the residual insecticides used for indoor residual house spraying belong to four main classes of insecticides. These main classes of residual insecticides include carbamate, organochlorine, synthetic pyrethroid and organophosphate. Dichlorodiphenyltrichloroethane (DDT 75% WP) and deltamethrin (K-Othrine 25%WG) are the most commonly used insecticides for indoor residual house spraying (WHO, 2011).

In spite of great achievements through the use of IRS for malaria vector control in Africa, drug and insecticide resistance are the major challenges for the malaria control programmes (WHO, 2011). The recent increase in the resistance to insecticides was due to the wide usage of a single class of insecticide for various vector control interventions. Out of the 45 countries around the world that reported resistance to insecticides, 27 of these are in sub Saharan Africa (WHO, 2011). Studies conducted on insecticide resistance in southern Africa showed that *An. funestus* was resistant to synthetic pyrethroid insecticides (Hargreaves *et al.,* 2000). Similar studies conducted in South Africa confirmed that *An. arabiensis* was resistant to both carbamate and synthetic pyrethroid insecticides (Hargreaves *et al.*, 2003).

1.2 THE MALARIA VECTORS IN SUB-SAHARAN AFRICA

Studies conducted in sub-Saharan Africa confirm that *Anopheles gambiae* and *Anopheles arabiensis* in the *An. gambiae* complex and *Anopheles funestus* in the *An. funestus* group are the most efficient malaria vectors (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Coetzee *et al*, 1993). These anopheline mosquitoes are distributed over most of the region and their distribution and abundance is dependent on seasonal parameters linked to rainfall (Gillies and Coetzee, 1987; Coetzee *et al*, 1993).

Transmission is influenced by the behaviour of the mosquitoes (Gillies and De Meillon, 1968). The vector mosquitoes are mainly anthropophilic (preferring to feed on humans) and endophilic (preferring to rest indoors), although *An. arabiensis* will readily feed on animals in the absence of humans (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). In addition, studies have shown that these vectors can vary in their ability to transmit malaria (White 1974; Gillies and Coetzee, 1987; Coetzee *et al*, 1993; Hunt *et al,* 1998). Each member of the *An. gambiae* complex and *An. funestus* group is discussed below.

1.2.1 The *Anopheles gambiae* complex

The *An. gambiae* complex currently consists of seven members that are further classified as vectors or non-vectors. The member species include *An. arabiensis*, *An. gambiae s.s.*, *An. merus*, *An. melas*, *An. quadriannulatus* species A, *An. quadriannulatus* species B and *An. bwambae* (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Hunt *et al.,* 1998). Of these, *An.*

gambiae and *An. arabiensis* are highly efficient vectors and responsible for malaria transmission in large parts of sub–Saharan Africa. *Anopheles merus*, *An. melas* and *An. bwambae* are considered to be minor vectors while *An. quadriannulatus* species A and B are non-vectors.

1.2.1.1 *Anopheles arabiensis*

Anopheles arabiensis is a fresh-water breeder and has both anthropophilic and zoophilic feeding patterns and will rest indoors or outdoors. This exophilic and endophilic behaviour presents a great challenge to vector control programmes that use residual insecticides sprayed inside houses (Gillies and Coetzee, 1987; Masendu *et al.,* 2005; Ntomwa *et al.,* 2006).

Anopheles arabiensis in most cases shares larval habitats with *An. gambiae* and *An. quadriannulatus*. Its widespread distribution depends on climatological factors such as rainfall, humidity and temperature (Lindsay *et al.,* 1998). Previous and current entomological studies have shown that the species is capable of surviving better during the dry period and at higher temperatures than *An. gambiae* (White 1974; Gillies and Coetzee, 1987; Coetzee *et al.,* 2000).

1.2.1.2 *Anopheles gambiae s.s.*

This fresh-water breeder is highly anthropophilic and endophilic in nature and thus a highly efficient malaria vector. *Anopheles gambiae* larvae can be found in various breeding sites ranging from permanent wells and irrigation channels to sunlit temporary pools. *An. gambiae* is classified into two molecular forms,

M and S, based on the sequence analysis of the intergenic spacer region (IGS) and the internal transcribed spacers (ITS) (Favia *et al.,* 2001; Gentile *et al.,* 2001; Della Torre *et al.*, 2002, 2005).

The M form is mainly distributed in Central and West Africa and adult females lay eggs in man-made breeding sites such as rice paddies and water canals used for agricultural purposes. In addition, this molecular form is acclimatized to survive the dry season. The S form occurs in all parts of sub–Saharan Africa and is abundant during the rainy season breeding in temporary sunlit pools and puddles (Della Torre *et al.,* 2005; Pinto *et al.,* 2007).

1.2.1.3 *Anopheles merus*

Anopheles merus is a salt-water breeder with anthropophilic and zoophilic behaviour. It is distributed in localized areas mainly along most parts of coastal East Africa but can also be collected in inland areas of South Africa, Mozambique, Zambia, Zimbabwe and Swaziland (Coetzee *et al.,* 1993; La Grange, 1995; Kloke, 1997; Masendu *et al.,* 2005). It is considered a minor malaria vector in localized areas in East Africa (Gillies and Coetzee, 1987). This species has also been incriminated as a vector of Bancroftian filariasis in coastal East Africa (Mosha and Petrarca, 1983). Similar studies conducted in the southern African region have also shown that this species is involved in malaria transmission. This was confirmed by the high sporozoite rates (11.6%) recorded in 1998 from Tanzania (Temu *et al.,* 1998). In comparison, studies conducted in Mozambique in 2007 showed an infection rate of 0.067%

while in 2009 the sporozoite rate had increased to 4.2% (Sharp *et al.*, 2007; Cuamba and Mendis, 2009).

1.2.1.4 *Anopheles melas*

Anopheles melas is best known as the West African salt-water breeder occurring along the coast from Senegal in the west to Angola in the south. Its distribution is associated with saline waters around tidal and mangrove swamp areas. It is considered to be a minor vector of malaria, usually occurring in sympatry with *An. gambiae* (Gillies and De Meillon, 1968; Diop *et al.,* 2002; Moreno *et al.,* 2004; Wondji *et al.,* 2005; Jawara *et al.,* 2008).

1.2.1.5 *Anopheles quadriannulatus*

Anopheles quadriannulatus is the third fresh-water breeder and a non-vector that prefers to feed on animals. In comparison with the distribution of *An. gambiae* and *An. arabiensis*, *An. quadriannulatus* is mainly found in three disconnected areas: Ethiopia, Zanzibar and south-eastern Africa (Gillies and Coetzee, 1987). This taxon is classified as two different species, namely *An. quadriannulatus* A *and An. quadriannulatus* B (Hunt *et al.,* 1998) based on sterility and chromosomal asynapsis. The species are distributed in different geographical areas with *An. quadriannulatus* A found in four southern African countries, South Africa, Swaziland, Mozambique and Zimbabwe and *An. quadriannulatus* B in Ethiopia (Hunt *et al.,* 1998). Neither species transmit malaria (Gillies and Coetzee, 1987; Hunt *et al.,* 1998).

1.2.1.6 *Anopheles bwambae*

Anopheles bwambae is found only in Uganda. It is identified as a minor and localized vector that in most cases breeds in pools around the mineral springs in the Semliki forest in Bwamba County. It has anthropophilic and zoophilic characteristics and has also been implicated as a possible transmitter of *Wuchereria bancrofti* (White, 1985). *An. bwambae* has been found susceptible to *Plasmodium falciparum* with a sporozoite rate of 0.7% (White, 1985).

1.2.2 The *Anopheles funestus* group

The *Anopheles funestus* group contains a major vector of malaria in sub–Saharan Africa (Gillies and De Meillon, 1968). The group consists of nine named members, namely *An. aruni*, *An. brucei*, *An. confusus*, *An. funestus*, *An. fuscivenosus*, *An. leesoni*, *An. parensis*, *An. rivulorum* and *An. vaneedeni* and two informally named species, *An. funestus*-like and *An. rivulorum*-like. The members are morphologically similar although some can be differentiated at specific stages of their immature development (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Based on phylogenetic analysis and molecular data, the *An. funestus* group has been classified into five subgroups (*An. aconitus, An. culicifacies, An. funestus*, *An. rivulorum* and *An. minimus* subgroups). Of these subgroups, only the *An. minimus*, *An. funestus* and *An. rivulorum* subgroups occur in sub–Saharan Africa (Harbach, 2004, Garros, 2005).

1.2.3 Reclassification of the *An. funestus* group

The Anopheles funestus group has been historically known to contain nine species (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). Genetic studies on close relatives of the *An. funestus* group from Asia resulted in reclassification of the group (Harbach, 2004).

1.2.3.1 *An. funestus* subgroup

This subgroup consists of six members, *An. funestus, An. funestus*-like, *An. parensis*, *An. aruni*, *An. vaneedeni* and *An. confusus.* Of these, *An. funestus* is the only important vector and is widespread over the greater part of sub-Saharan Africa. This species is known for its anthropophilic and endophilic behaviour and is amenable to control by indoor residual house spraying operations (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). All members of the *An. funestus* group prefer to use permanent, vegetated, clean water bodies as their breeding sites (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).The five remaining members of the subgroup are non–vectors and generally localized in distribution (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987; Spillings *et al.*, 2009).

1.2.3.2 *An. minimus* subgroup

The African members of this subgroup are *An. leesoni* and *An. fuscivenosus*. *An. leesoni* occurs in the savanna regions of East and West Africa and is exophilic and zoophilic in behaviour. The distribution of *An. fuscivenosus* is restricted to Zimbabwe and it also is known as an exophilic species (Gillies and De Meillon, 1968).

1.2.3.3 *An. rivulorum* subgroup

This subgroup consists of *An. brucei*, *An. rivulorum* and *An. rivulorum*–like. *An. rivulorum* is widespread in Africa while *An. brucei* is known only from Nigeria and *An. rivulorum*-like from Cameroun and Burkina Faso (Gillies and De Meillon, 1968; Cohuet *et al.,* 2003).*An. rivulorum* has been implicated as a malaria vector in Tanzania (Wilkes *et al.*, 1996).

1.3 DISTRIBUTION OF MALARIA VECTORS IN MPUMALANGA PROVINCE

Recent studies were conducted between 1995 and 2010 on the distribution and abundance of vector species in Mpumalanga Province, South Africa. Of the seven known members of the *An. gambiae* complex, only three (*An. arabiensis, An. quadriannulatus* and *An. merus)* have been collected in the past 13 years with *An. merus* occurring in large numbers at Martiens farm and Block C (Govere *et al.,* 2000).Similar studies conducted in the province at Tonga sub–district, on distribution and abundance of the *An. funestus* group, showed the presence of five species (*An. funestus, An. rivulorum, An. vaneedeni, An. parensis* and *An. leesoni*) (Ngomane *et al.,* 2007). During night–biting catches, *An. rivulorum* was identified as the predominant member of the *An. funestus* group, occurring in large numbers at Tonga Malaria Training centre (Ngomane *et al.,* 2007).

The occurrence of both *An. merus and An. rivulorum* in large numbers in the province is a concern (Govere *et al.,* 2000; Ngomane *et al.,* 2007).Similar studies in Mozambique have implicated *An. merus* as a malaria vector

(Cuamba and Mendis, 2009) and *An. rivulorum* has been shown to be a minor malaria vector in Tanzania (Wilkes *et al.,* 1996). In Mpumalanga, only *An. arabiensis* has been identified as the major vector responsible for transmitting malaria (Govere *et al.,* 2001).

1.4 MALARIA TRANSMISSION IN MPUMALANGA PROVINCE

Mpumalanga Province is one of the malaria endemic provinces of the Republic of South Africa. It is bordered by Mozambique in the east and Swaziland in the south. It is estimated that over 1.5 million people, 43% of the Province's population, live in the low-lying areas and are at risk of contracting malaria. The disease is endemic in Ehlanzeni district with the possibility of localized outbreaks even in non-malaria districts such as Gert Sibande and Nkalanga districts. Malaria transmission is unstable and seasonal (Mpumalanga Malaria Information System, data unpublished). Transmission is greatly influenced by climatic factors such as rainfall, temperature, and relative humidity. The malaria season usually starts after the first rains in October, reaches its peak in December and January, and then begins to wane in April/May. (Mpumalanga Malaria Information System, data unpublished).

In terms of economic development, the province is well situated strategically for new planned developments strengthening ties between Mozambique and Swaziland. These include the development of the "Maputo Corridor" aimed at promoting trade, industry and tourism mutually beneficial to Mozambique and Mpumalanga, and the Lubombo Spatial Development Initiative (LSDI) at least part aimed at reducing malaria disease burden in the three countries,

Mozambique, South Africa and Swaziland. (Govere *et al.,* 2001; Sharp *et al.*, 2007). Implementation of these economic developments has increased cross-border movements of both human beings and malaria vectors in the province. The commercial farming of bananas, mangoes, litchis and oranges is attracting large numbers of migrant and seasonal workers from Mozambique and Swaziland. Commonly, these workers come from hyperendemic malaria districts and many are parasite carriers (Mpumalanga Malaria Information System, data unpublished). In Mpumalanga, 85% of the malaria cases are imported (Fig. 1.1). All notified malaria cases are analyzed weekly and their original source of infections captured in the Malaria Information System database as part of surveillance activities.

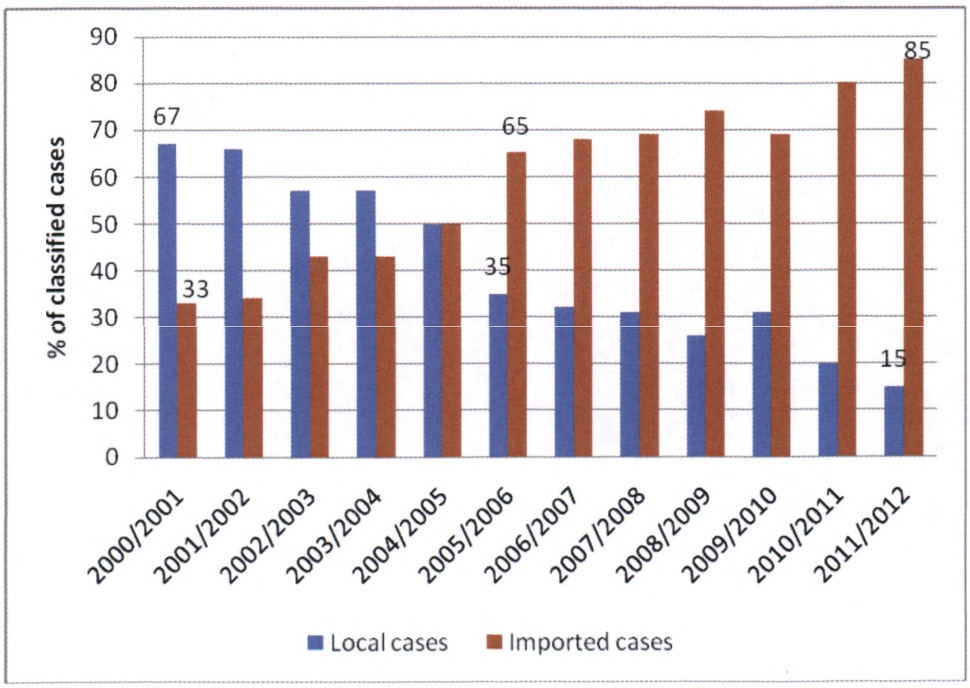

Figure 1.1: Distribution of local and imported cases from 2000/2001 to 2011/2012 malaria seasons

Of the five known *Plasmodium* species infecting humans (*P.vivax*, *P. ovale*, *P. falciparum*, *P. malariae* and *P. knowlesi*), *P. falciparum* accounts for 98% of the malaria cases in Mpumalanga while *P. malariae* and *P. ovale* account for 1% approximately each. From therapeutic efficacy studies on anti–malarial drugs conducted during previous malaria seasons, no records of *P. vivax* were reported in the province (Govere *et al.*, 1999; Mabuza *et al.,* 2001*;* Mabuza *et al.,* 2005). *Plasmodium knowlesi* has not yet been detected in sub-Saharan Africa and is known only from Southeast Asian countries (Singh *et al.,* 2004; Putaporntip *et al.*, 2009; Van den Eede *et al.*, 2009).

Figure 1.2 shows that more than half (58%) of the notified cases for the past twelve seasons have been contracted by males compared with 42% contracted by females. Health facilities are monitored weekly by plotting all notified cases to the health facility onto threshold charts for outbreak prediction. Most notified malaria cases are followed up by the surveillance teams through the use of routine epidemiological, special and mass surveys. Nurses and medical doctors are annually updated regarding changes in the malaria control strategies. Communities are also updated and informed about new developments in the programme. Indoor residual spraying is used as the main vector control strategy for adult mosquito control, accompanied by small scale larviciding for larval control (Mpumalanga Malaria Information System, data unpublished).

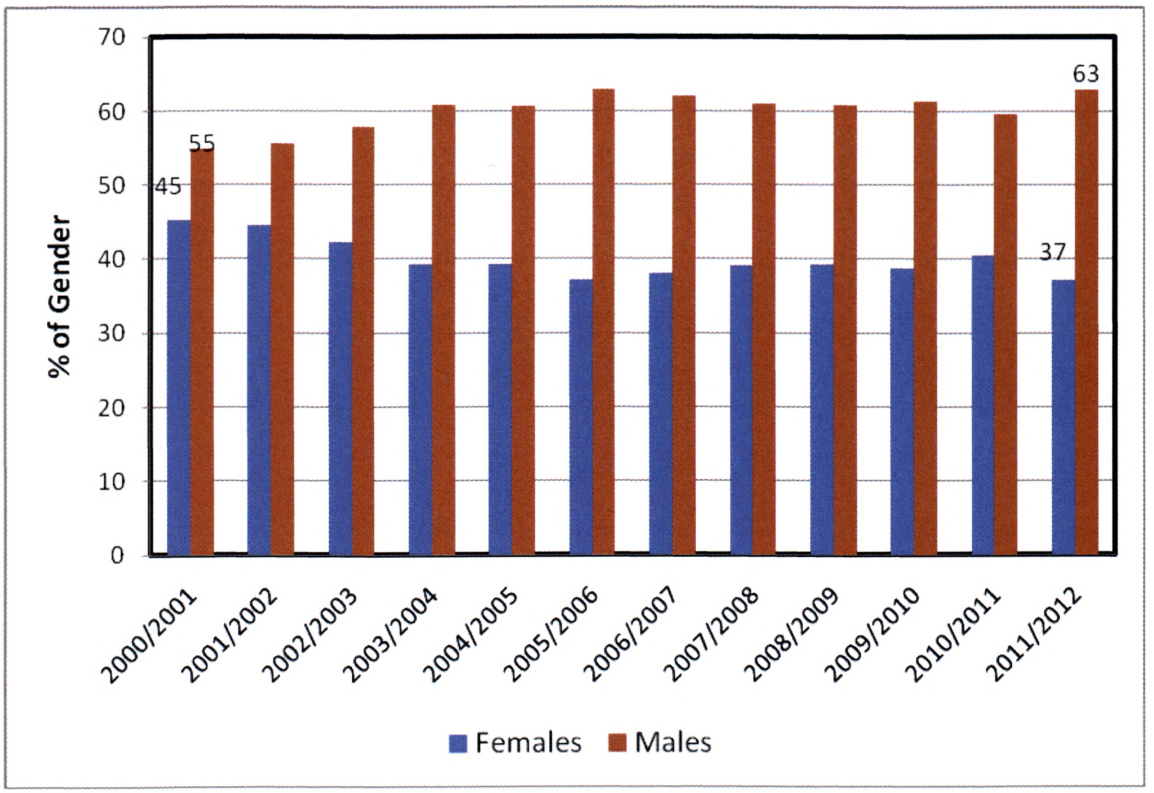

Figure 1.2: Distribution of malaria cases in relation to gender for the twelve Seasons

1.5 RESEARCH RATIONALE

More is known about the distribution of vector species and malaria parasites in Mpumalanga Province than in other malaria provinces of South Africa, but nevertheless there are numerous gaps in our knowledge. For example, the mapping of the potential breeding sites is not documented. Even the recent few studies conducted on the distribution of *Anopheles* mosquitoes (Govere *et al.,* 2000; Ngomane *et al.,* 2007), did not map the breeding sites of the potential malaria vectors. No studies have been conducted on the insecticide resistance status of potential malaria vectors in Mpumalanga Province. Mapping of breeding sites and monitoring insecticide resistance status will

provide potentially data important for planning and evaluating the provincial vector control programme.

1.6 STUDY OBJECTIVES

This study aimed to review and update the entomological data on breeding sites of potential malaria vectors and to investigate the insecticide resistance status of these mosquitoes in Mpumalanga Province.

Specific objectives were:

A. To Map the entomological historical data from 2005/2006 – 2009/2010 malaria seasons

B. To describe the current breeding sites

C. To identify new potential breeding sites and screen for the presence of malaria vector species

D. To determine species composition of *Anopheline* mosquitoes from the larval collections

E. To establish the insecticide susceptibility levels of potential vector species to the four classes of residual insecticides approved for indoor residual spraying, using WHO standard test kits

CHAPTER TWO

MATERIALS AND METHODS

2.1 STUDY AREA

The study was carried out in the high risk areas of Nkomazi municipality, Nkomazi – Mbombela sub–district, bordering on Mozambique to the east and Swaziland to the south (Fig. 2.1). These high risk areas included most of the rural settlements within Driekoppies, Figtree, Mgobodi, Naas and Tonga.

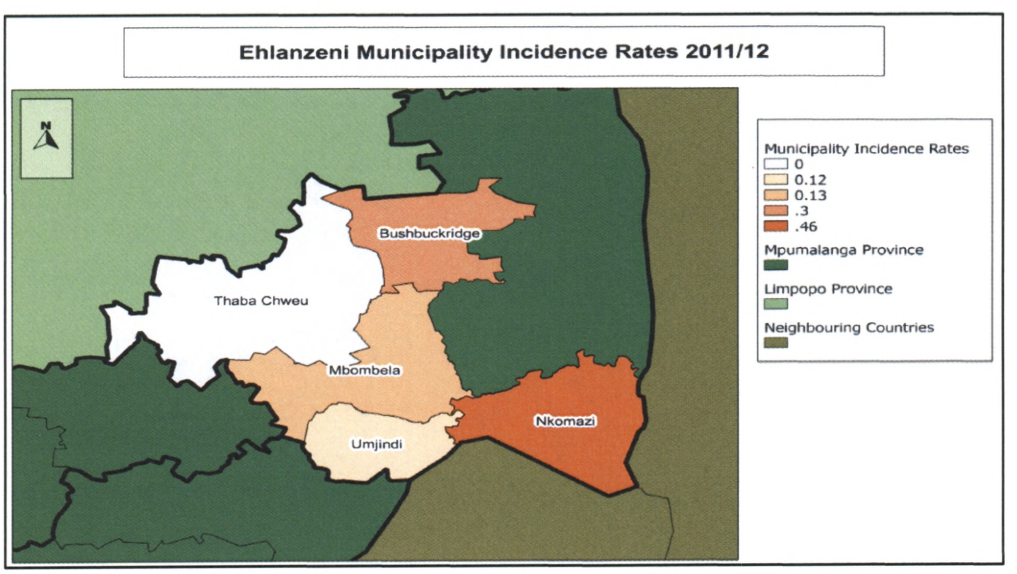

Figure 2.1: Map of Ehlanzeni showing the study area, Nkomazi municipality.

2.2 MAPPING HISTORICAL VECTOR SURVEILLANCE DATA

Of the 26 historical vector surveillance sentinel sites established over the years in the Nkomazi Municipality, 15 were chosen for this study based on >0.36 malaria case incidence rate. This is the classification that the

programme uses to identify "hotspots". Historical entomological data were retrieved from the provincial vector surveillance database dated for the 2005/2006 to 2009/2010 malaria seasons (5 years). Where map co-ordinates were not available for the sentinel sites, these localities were visited and GPS co-ordinates recorded. Excel spreadsheets were developed for data capturing. The completed data files were converted to dbf files (database files) and then imported into ArcView and visualized using the Nkomazi Municipality shape file.

2.3 SELECTED HISTORICAL AND NEW BREEDING SITES

On completion of the historical data review, a three month plan was developed to monitor the selected sentinel sites. Out of the 35 600 sq. km earmarked for programme monitoring, the project was allocated 47% (16732 sq. km) of the official area. Seven sites were chosen at random using the blue-red-bead method (Durrheim pers.comm.) as follows. Fifteen vector surveillance officers were requested to pick a marble, out of a bag containing 8 blue and 7 red marbles.

Those who picked the red marbles had their sentinel sites selected for the study. An additional ten new breeding sites were identified and their GPS co-ordinates recorded.

2.4 MOSQUITO COLLECTIONS

Larval sampling catches were used for monitoring the distribution patterns of the anopheline mosquitoes. The monitoring programme was instituted for three months, from October 2011 to December 2011. Field collection teams

visited each breeding site once a month. The collected larvae were transferred into labeled plastic cups and transported to the Driekoppies insectary for rearing to adults.

On arrival at the Driekoppies insectary, larvae were transferred into larval rearing bowls. Emerged adult mosquitoes from the wild-caught larvae were transferred to mosquito cages and provided with 10% sugar solution for five consecutive days. Emerged adult mosquitoes were morphologically identified according to the key of Gillies and Coetzee (1987) and recorded on the Excel spread sheets.

2.5 MORPHOLOGICAL IDENTIFICATION

Adult mosquitoes reared from larvae collected from the sentinel sites were killed by freezing, and then separated according to the sexes, females and males. Female mosquitoes were identified to species, using the *Anopheles* morphological identification key of Gillies and Coetzee (1987). Mosquitoes were individually placed in tubes containing silica gel desiccant and stored for molecular studies. These prepared specimens were recorded electronically in a separate Excel spreadsheet for both *funestus* group and *gambiae* complex. Specimens were assigned unique numbers with the purpose of differentiating each species.

2.6 MOLECULAR IDENTIFICATION

Prior to the molecular identification, *An. gambiae* complex and *An. funestus* colonies kept at the Vector Control Reference Unit insectary were prepared and used as reference positive controls. Then *An. gambiae* PCR (Scott *et al.,*

1993) was prepared and run followed by *An. funestus* PCR (Koekemoer *et al.*, 2002).

2.6.1 PCR identification of *Anopheles gambiae* complex

A total of 247 mosquitoes belonging to the *An. gambiae* complex were tested for species identification using the PCR method of Scott *et al.* (1993). In brief, the PCR reaction consisted of the following reagents: 1.25µl 10x reaction buffer (100m M Tris-HCL pH 8.3, 1mM KCl), 1.25µl 10x dNTP, 0.5µl $MgCl_2$ solution, 0.5µl Quad Primer, 1.0µl each of UN, AG, AR, ME and QD primers, 4.9µl deonised H2O and 0.1µl Taq. A volume of 12.5 µl of the Master Mix was aliquoted into each 0.2 µl PCR and DNA added. Negative controls consist of master mix and PCR tubes and content were centrifuged for 20 seconds at 16K rpm to collect reaction mixture. Reaction was subjected to PCR cycling conditions of 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, the samples were removed from PCR machine and stored in the freezer. Four microlitres of loading dye was added to the content of each tube and samples loaded in the well of the gel. Positive controls were loaded alphabetically (*An. arabiensis*, *An. gambiae*, *An. merus* and *An. quadriannulatus*) followed by negative controls to the end of the gel. Samples were loaded and gel electrophoresed at 100V/400mA for approximately 60 minutes. After electrophoresis the gel was placed into the GeneSnap cabinet (Vacutec G-Box from Syngene) and photographs take of the PCR products. Samples were scored by comparing the product size to

those of the positive controls and molecular marker size. An *An. gambiae* PCR file was developed and saved on the computer.

2.6.2 PCR identification of *Anopheles funestus* group

A total of 118 mosquitoes belonging to the *An. funestus* group were tested for species identification using the PCR method of Koekemoer *et al.* (2002). DNA was extracted using the method of Collins *et al.* (1990).

EDTA grinding buffer solution was prepared and 1 or 2 legs were crushed and ground in a 1.5ml microcentrifuge tube with pestles. Samples were homogenized and incubated at 70°C for 30 minutes on a heating block.

In brief, the PCR reaction consisted of the following reagents, 1.25µl 10x reaction buffer (100m M Tris-HCL pH 8.3, 1mM KCl), 1.25µl 10x dNTP, 0.75µl $MgCl_2$ solution, 1.0µl each of UV, *FUN*, *VAN*, *LEES*, *RIV* and *PAR* primers, 3.15µl deonised H2O and 0.1µl Rtaq.A volume of 12.5 µl of the Master Mix was aliquoted into each 0.2 µl PCR and DNA added. Negative controls consisted of master mix and PCR tubes and content were centrifuged for 20 seconds at 16K rpm to collect reaction mixture. Reaction was subjected to PCR cycling conditions of 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, the samples were removed from PCR machine and stored in the freezer. Four microlitres of loading dye was added to the content of each tube and samples loaded in the well of the gel. Positive controls were loaded according to the product size (*An. vaneedeni,*

An.funestus, An. rivulorum, An. parensis and *An. leesoni*) followed by samples with molecular marker at the end of the gel. Samples were loaded and gel electrophoresed at 100V/400mA for approximately 60 minutes. Gel was removed from the well and immersed into container containing pink dye for two hours. This process assists in binding the DNA from the electrophoresed gel then, placed into the GeneSnap cabinet (Vacutec G-Box from Syngene). Samples were scored by comparing the product size to those of the positive controls and molecular marker size. The gel was photographed and *An. funestus* PCR file was developed and saved on the computer.

2.7 WHO INSECTICIDE SUSCEPTIBILITY TESTS

Susceptibility tests were carried out on reared adult mosquitoes at room temperature of 28°C and relative humidity of 75% using the WHO standard procedures and test kits (World Health Organization, 1998). The anopheline mosquitoes collected from Block A sentinel site were subjected to the tests and then later identified into *An. merus* species. One to five day old females were selected and used for the experiments. A total of 352 female mosquitoes were separated into four batches and exposed to impregnated filter papers for 60 minutes.

Table 2.1: Insecticides used for the susceptibility tests.

Recommended Insecticide class	Insecticide name	Diagnostic dose
1. Carbamate	Bendiocarb	0.1%
2. Organochlorine	DDT	4%
3. Pyrethroid	Lambda-cyhalothrin	0.05%
4. Organophosphate	Malathion	5%

Control tests were prepared and loaded with 88 susceptible *An. merus* (MAF colony) mosquitoes from the Vector Control Reference Unit insectary at the NICD in Johannesburg. They were kept under the same conditions as the exposed group but without being exposed to the insecticide treated papers. At the end of the 60 min exposure, the mosquitoes were transferred into the holding tubes and fed with 10% sugar solution. Insecticide knockdown effects were recorded after 60 minutes post exposure and total mortality counted at the end of the 24 hours holding period. The control mortality rate required that Abbott's formula be used to correct the final mortality rate. The susceptibility test results were interpreted according to WHO (1998) where 98-100% mortality indicates full susceptibility, 80-97% mortality requires further investigation and <80% indicates resistance.

CHAPTER THREE

RESULTS

3.1 STUDY AREA

The map given in Figure 3.1 shows the collection localities of all sentinel sites used in this study. Of the 26 potential breeding sites mapped, 30.8% of the sites were classified as historical breeding sites, 26.9% of the breeding sites were selected and monitored from October 2011 to December 2011.The remaining 42.3% breeding sites were classified as new breeding sites (Fig. 3.1).

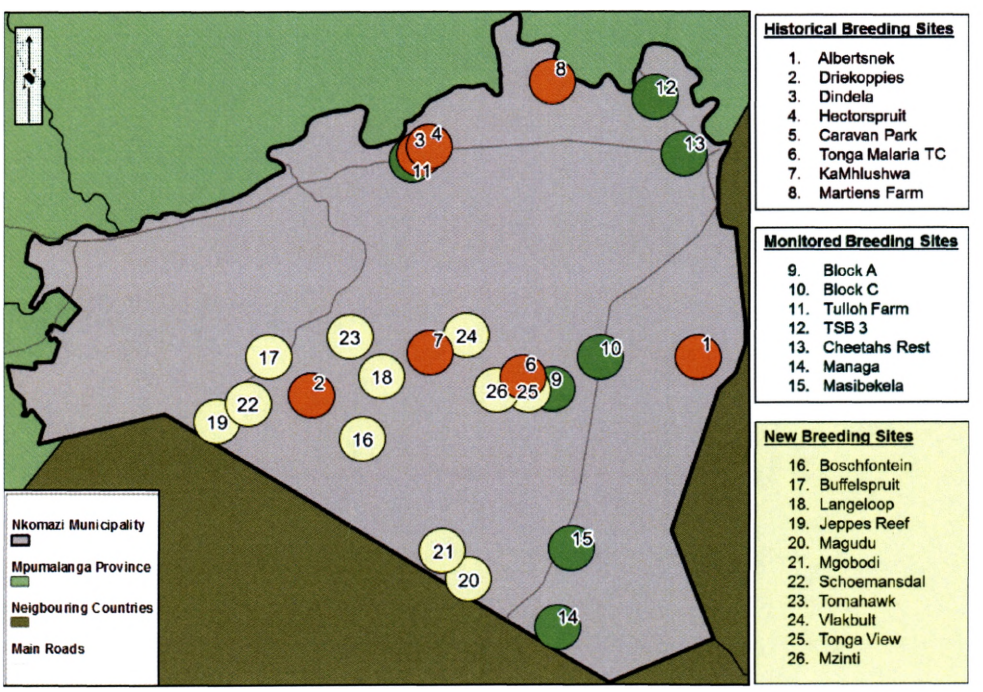

Figure 3.1: Map showing historical, monitored and new identified breeding sites at the study area.

3.2 COMPOSITION OF *ANOPHELINE* MOSQUITOES ANALYZED FROM THE HISTORICAL ENTOMOLOGICAL DATA

Of the 6259 *Anopheles* mosquitoes collected between 2005/2006 and 2009/2010 malaria seasons, 87% were members of the *An. gambiae* complex and the remaining 13% members of the *An. funestus* group (Table 3.1). Block A and Tonga Malaria Training Centre were identified as the most productive sentinel sites for these species. Tonga Malaria Training Centre accounts for 81.4% of the *An. funestus* group and Block A accounts for 54% of the *An. gambiae* complex sampled.

Table 3.1: Number of anopheline mosquitoes collected in Mpumalanga province from 2005/2006 – 2009/2010.

Locality	Latitude(-)	Longitude	*An. funestus* group	*An. gambiae* complex	Total
Albertsnek	25° 39' 34"	31° 57' 27"	12	5	17
Block A	25° 41' 35"	31° 48' 30"	5	2924	2929
Block C	25° 39' 38"	31° 51' 29"	5	504	509
Driekoppies	25° 41' 57"	31° 33' 51"	0	49	49
Dindela	25° 26' 51"	31° 40' 27"	11	43	54
Hectorspruit	25° 26' 24"	31° 40' 60"	22	407	429
Tulloh Farm	25° 27' 12"	31° 39' 58"	34	82	116
TSB 3	25° 23' 20"	31° 54' 48"	16	218	234
Cheetahs	25° 26' 50"	31° 56' 34"	17	0	17
Tonga Malaria TC	25° 40' 50"	31° 46' 45"	668	3	671
Mananga	25° 56' 26"	31° 48' 53"	26	229	255
Masibekela	25° 51' 33"	31° 49' 44"	2	431	433
KaMhlushwa	25° 39' 19"	31° 41' 07"	0	10	10
Martiens Farm	25° 22' 22"	31° 48' 33"	3	533	536
Total			821	5438	6259

Besides the above two mentioned sentinel sites, Block C and Martiens farm also produced good numbers of *An. gambiae* complex mosquitoes (Table 3.1). Of the 3130 anopheline mosquitoes subjected to species identification, 53.8% of specimens were identified PCR positive and the remaining 46.2% were unidentified. PCR results are presented in Table 3.2 showing that overall 44.3% of the specimens were *An. rivulorum* and 20.1% were identified as *An. merus* (Table 3.2).

Table 3.2: Species composition collected in the study area from 2005/2006 – 2009/2010.

Locality	*An. riv*	*An. van*	*An. par*	*An. lees*	*An. quad*	*An. mer*	*An. arab*	Total
Block A	0	0	0	0	226	250	0	476
Block C	2	0	0	0	0	20	1	23
Driekoppies	0	0	0	0	0	8	0	8
Dindela	1	5	0	0	0	0	0	6
Hectorspruit	12	22	2	2	0	0	0	38
Tulloh Farm	13	0	0	1	0	6	0	20
TSB 3	187	28	0	26	0	7	0	248
Tonga Malaria TC	521	158	42	30	0	0	0	751
Mananga	11	3	0	7	0	0	0	21
Masibekela	0	24	0	0	20	48	0	92
Martiens Farm	0	2	0	0	0	0	0	2
Total	747	242	44	66	246	339	1	1685

Key:

An. riv = An. rivulorum, An. van = An. vaneedeni, An. par = An. parensis, An. lees = An. leesoni, An. quad = An. quadriannulatus, An. mer = An. merus, An. arab = An. arabiensis

3.3 ANOPHELINE MOSQUITOES COLLECTED FROM THE MONITORED BREEDING SITES

The results presented in Table 3.3 confirmed that there were continuous distribution patterns of Anopheline mosquito species in the province. Of the 1047 specimens morphologically identified, 70% (737/1047) were the *An. gambiae* complex and the remaining 30% as the *An. funestus* group. The majority (52.7%) of specimens was collected from Masibekela and Block A with the *An. gambiae* complex being predominant at these sites.

Table 3.3: Distribution of anopheline mosquitoes collected at the seven monitored breeding sites from October – December 2011.

Locality	Latitude(-)	Longitude	*An. funestus* group	*An. gambiae* complex	Total
Block A	25° 41' 35"	31° 48' 30"	0	261	261
Block C	25° 39' 38"	31° 51' 29"	0	69	69
Tulloh Farm	25° 27' 12"	31° 39' 58"	9	56	65
TSB 3	25° 23' 20"	31° 54' 48"	66	57	123
Cheetahs	25° 26' 50"	31° 56' 34"	115	61	176
Mananga	25° 56' 26"	31° 48' 53"	45	17	62
Masibekela	25° 51' 33"	31° 49' 44"	75	216	291
Total			310	737	1047

38.1% of the total sample was identified by PCR as *An. rivulorum*, while 27.4% were identified as *An. merus* (Table 3.4). Of the 6 *An. arabiensis* collected during the survey, 4 were from Masibekela, 1 from Tulloh farm and 1 from Mananga. Of the seven monitored breeding sites, Cheetahs was noted as the most productive breeding site for *An. rivulorum* (Table 3.4).

Table 3.4: Species composition of mosquitoes at the seven monitored breeding sites from October - December 2011.

Locality	*An. riv*	*An. van*	*An. par*	*An. quad*	*An. mer*	*An. arab*	Total
Block A	0	0	0	1	28	0	29
Block C	0	0	0	4	26	0	30
Tulloh Farm	4	2	0	12	4	1	23
TSB 3	5	7	0	11	13	0	36
Cheetahs	73	2	5	0	0	0	80
Mananga	1	2	0	9	5	1	18
Masibekela	10	0	0	5	9	4	28
Total	93	13	5	42	85	6	244

Key:

An. riv = An. rivulorum, An. van = An. vaneedeni, An. par = An. parensis, An. quad = An. quadriannulatus, An. mer = An. merus, An. arab = An. arabiensis.

3.4 ANOPHELINE MOSQUITOES COLLECTED FROM NEW BREEDING SITES

A total of 153 mosquitoes were collected from the low malaria risk areas of which the Vlakbult breeding site was noted as being the most productive. Out of these 153, 87% (133/153) were *An. gambiae* complex and the remaining 13% *An. funestus* group. Of the 10 breeding sites identified, 90% were identified as *An. gambiae* complex productive sites, while 10% were identified as good *An. funestus* group sites (Table 3.5).

Table 3.5: Distribution of anopheline mosquitoes collected from the new breeding sites sampled in the low risk areas from October – December 2011.

Locality	Latitude(-)	Longitude	*An. funestus* group	*An. gambiae* complex	Total
Boschfontein	25° 44' 39"	31° 36' 58"	0	2	2
Magudu	25° 53' 28"	31° 43' 28"	0	1	1
Komatipoort Town	25° 26' 13"	31° 58' 02"	0	10	10
Mgobodi	25° 51' 40"	31° 41' 51"	1	9	10
Mangweni	25° 44' 34"	31° 48' 21"	0	15	15
Mzinti	25° 41' 31"	31° 43' 56"	0	25	25
Tomahawk	25° 38' 16"	31° 36' 12"	5	11	16
Sasol pump station	25° 27' 53"	31° 57' 59"	6	0	6
Vlakbult	25° 41' 41"	31° 47' 12"	0	40	40
Vukuzenzele	25° 51' 34"	31° 49' 44"	8	20	28
Total			20	133	153

Results of species composition presented in Table 3.6 show that *An. merus* and *An. arabiensis* were the most abundant species, with *An. merus* being more widespread having been found at all localities. Vlakbult was the only locality where *An. arabiensis* occurred in large numbers with other three sites yielding only two or three specimens each. *An. rivulorum* was the only member of the *An. funestus* group collected from the newly identified breeding sites (Table 3.6).

Table 3.6: Composition of species collected from the new breeding sites sampled in the low risk areas.

Stratification	Locality	*An. arab*	*An. mer*	*An. quad*	*An. riv*	Total
High risk areas	Komatipoort Town	0	3	1	0	4
	Mangweni	0	6	0	0	6
Low risk areas	Boschfontein	0	2	0	0	2
	Magudu	0	1	0	0	1
	Mgobodi	2	2	1	0	5
	Mzinti	2	8	0	0	10
	Tomahawk	3	5	1	7	16
	Vlakbult	23	2	0	0	25
	Total	30	29	3	7	69

Key:

An. arab = An. arabiensis, An. mer = An. merus, An. quad = An. quadriannulatus, An. riv = An. rivulorum.

The following maps show the distribution of the three species *An. arabiensis* (Fig. 3.2), *An. merus* (Fig. 3.3) and *An. rivulorum* (Fig. 3.4).

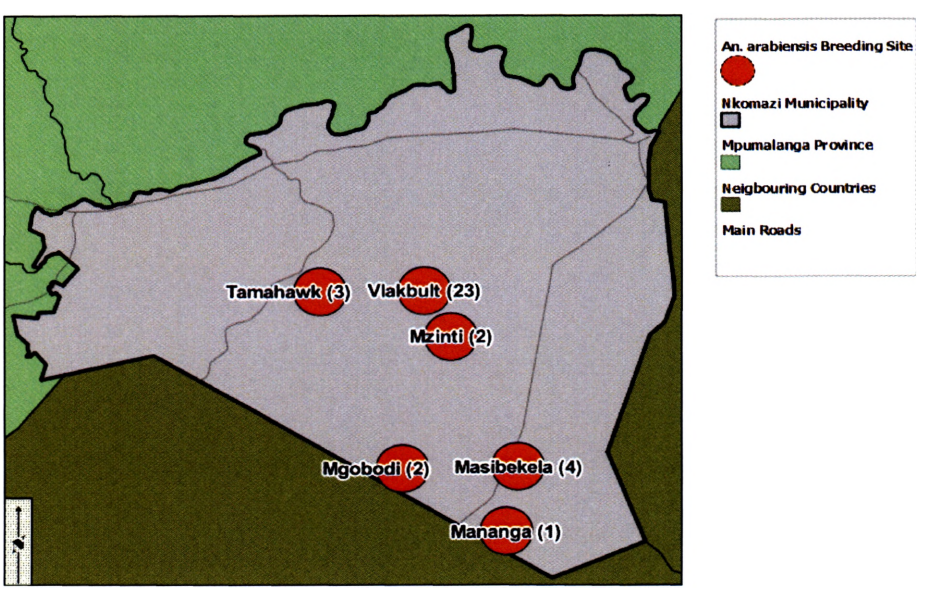

Figure 3.2: Distribution of the principal vector, *An. arabiensis,* in the study area, Nkomazi municipality.

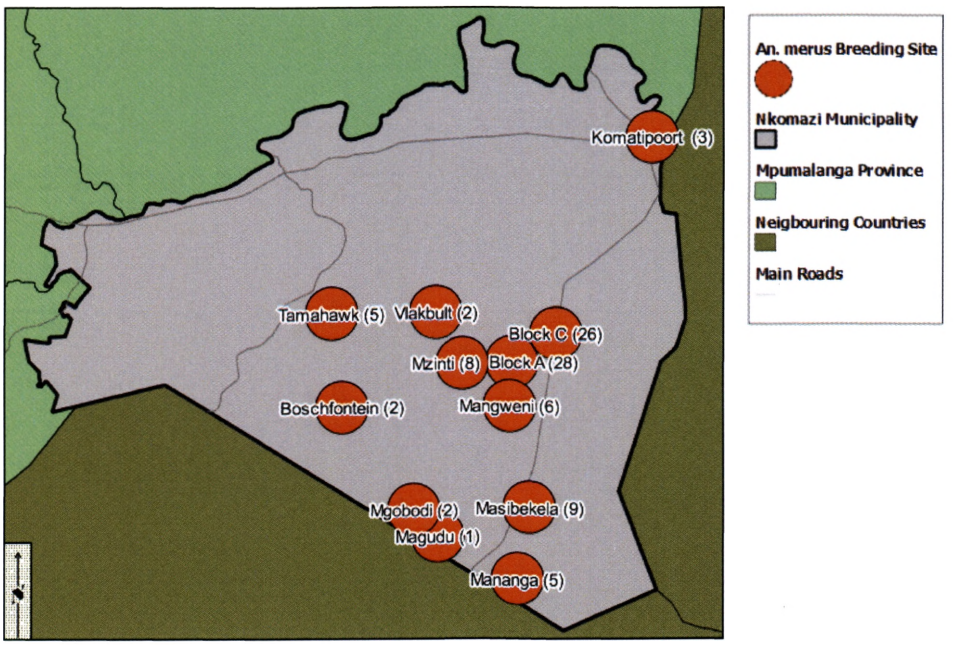

Figure 3.3: Distribution of *An. merus* in the study area, Nkomazi municipality

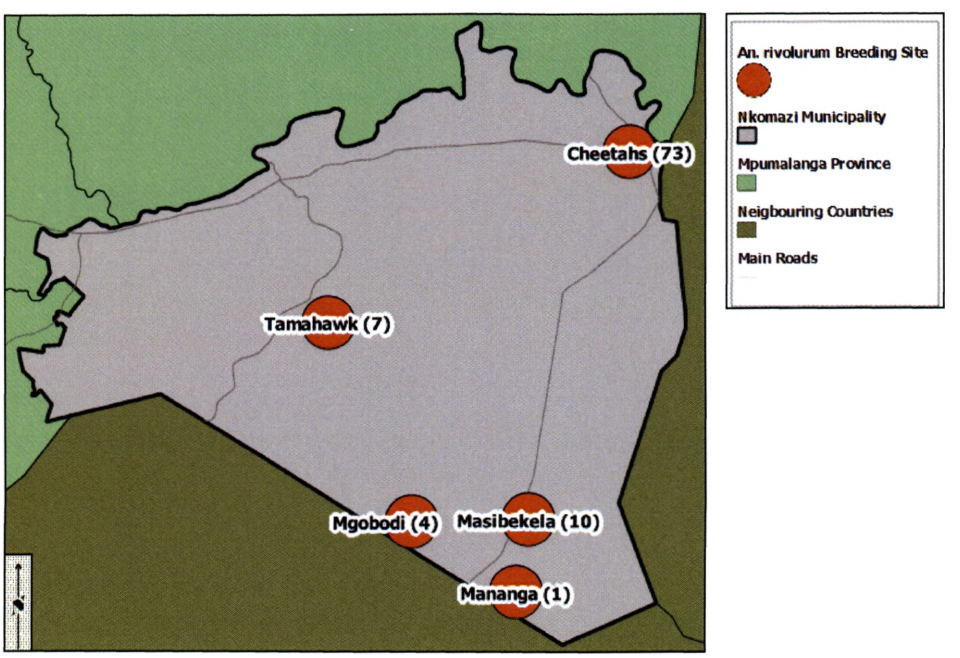

Figure 3.4: Distribution of *An. rivulorum* in the study area, Nkomazi municipality.

3.5 SPECIES IDENTIFICATION

In this present study, 365 mosquitoes were subjected to species identification using the Scott *et al.* (1993) method for the *An. gambiae* complex and Koekemoer *et al.* (2002) for the *An. funestus* group. Results of the PCR gels are presented in Figures 3.5 and 3.6.

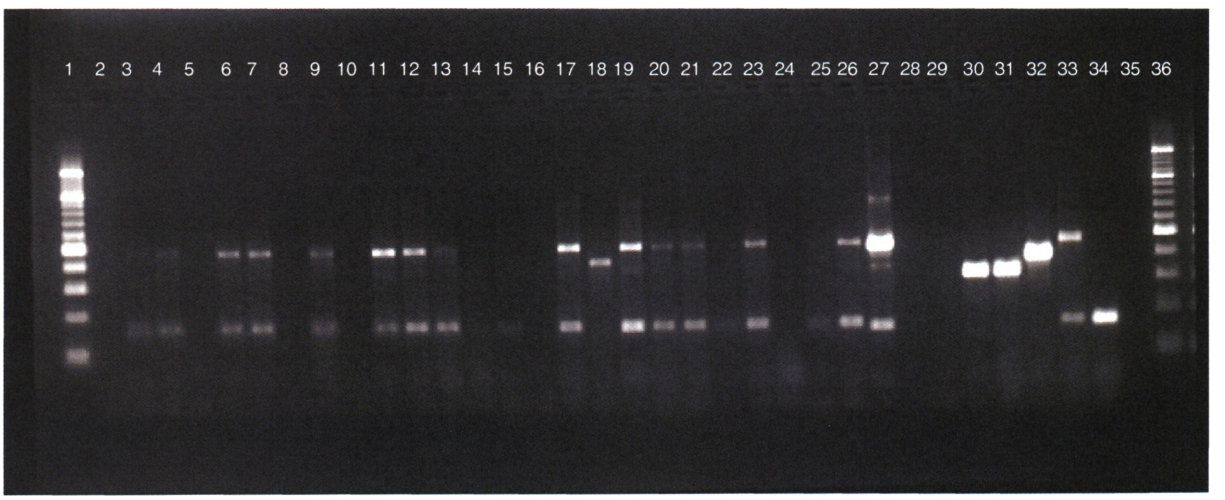

Figure 3.5: A 2.5% ethidium bromide stained agarose gel for the identification of the *An. gambiae* complex.
Lanes 1 & 36 represent molecular weight markers; lanes 2,5,8,10,14,16,24,28 & 29 represent No IDs; lanes 3,15,22 & 25 were identified as *An. quadriannulatus*; lanes 4,6,7,9,11–13,17,19–21,23,26 & 27 were identified as *An. merus*; lane 18 was identified as *An. gambiae s.s.* (this specimen was collected outside of the study area); and lane 30 identified as *An. arabiensis*. Lanes 31 – 34 were positive controls for *An arabiensis, An. gambiae, An. merus* and *An. quadriannulatus* while lane 35 was the PCR negative control.

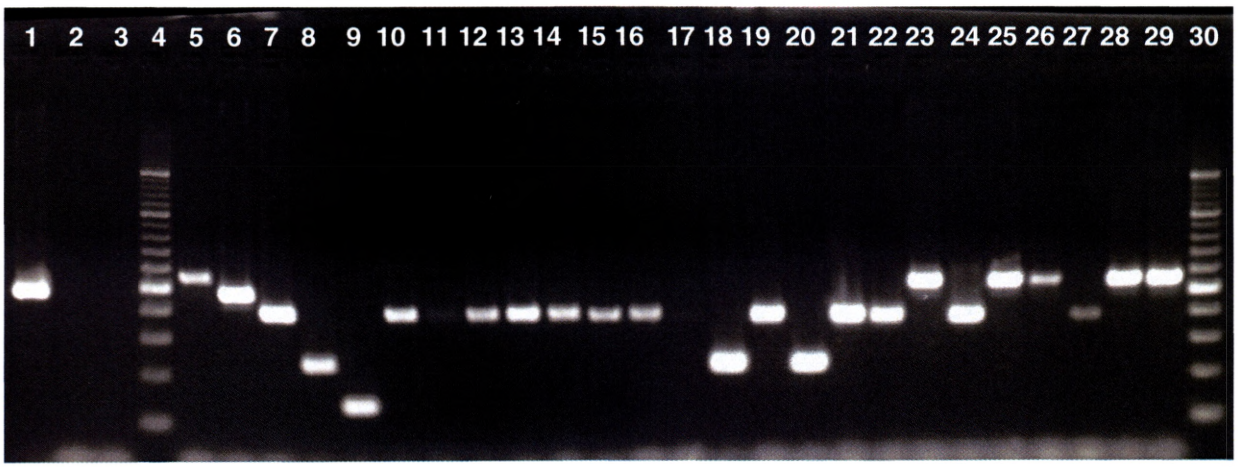

Figure 3.6: A 2.5% ethidium bromide stained agarose gel for the identification of the *An. funestus* group.

Lane 1 represents extracted DNA positive control, lanes 2 – 3 negative controls for extracted DNA and PCR, lanes 4 & 30 molecular weight markers, lanes 5 – 9 positive controls for *An. vaneedeni*, *An. funestus, An. rivulorum*, *An. parensis* & *An. leesoni*, lanes 10 – 17, 19, 21 -22, 24 & 27 samples were identified as *An. rivulorum*, lane 18 & 20 identified as *An. parensis*, lanes 23, 25 – 26 & 28 – 29 were identified as *An. vaneedeni*

Of the 365 specimens subjected to species identification, 85.8% (313/365) of the specimens were successfully identified with only 14.2% remaining unidentified. Of the 195 members of the *An. gambiae* complex successfully identified, *An. merus* represented 58.4% of the sample, *An. arabiensis* 18.5%, and *An. quadriannulatus* with 23.1%. A single individual of *An. gambiae s.s.* was identified from ..... Of the 118 *An. funestus* group, *An. rivulorum* represented 85%, *An. vaneedeni* 11 % and *An. parensis* 4% (Tables 3.3 and 3.4).

3.6 WHO INSECTICIDE SUSCEPTIBILITY TESTS

A total of 352 *An. merus* species collected at Block A sentinel sites were selected and tested according to the standard WHO operating procedure to confirm the levels of susceptibility to DDT (4%), bendiocarb (0.01%), lambda-cyhalothrin (0.05%) and malathion (5%). Of the 352 exposed *An. merus* samples, 99.4% were susceptible to the four classes of the recommended residual insecticides (Table 3.7). Even though the overall results showed high mortality, there was one survivor on DDT and one on lambda-cyhalothrin (Table 3.7).

Table 3.7: Susceptibility test results for four classes of insecticides

Insecticide	Diagnostic dose	No of mosquitoes tested	No of deaths in 24 hours	% Mortality
DDT	4%	91	90	98.9
Bendiocarb	0.1%	88	88	100
Lambdacyhalothrin	0.05%	93	92	98.9
Malathion	5%	80	80	100
Controls		88	13	14.7

CHAPTER FOUR

DISCUSSION

4.1 HISTORICAL RECORDS OF MOSQUITO SURVEYS IN MPUMALANGA PROVINCE

An entomological survey conducted between 1996 and 1999 provided information on the distribution of the *An. gambiae* complex and *An. funestus* group in the province. Of the 5084 anopheline mosquitoes collected, the *An. gambiae* complex accounted for 8.6% while 27.9% was the *An. funestus* group. 425 specimens were subjected to PCR identification of which 56.0% were identified as *An. merus*, 30.4% were *An. quadriannulatus* and 13.6% were *An. arabiensis (*Govere *et al.,* 2000, 2001).

A similar study on the distribution and abundance of the *An. funestus* group was conducted in the same study area from 1996 to 2005. Of the 4546 anopheline mosquitoes collected, 319 specimens were identified by PCR. *Anopheles funestus s.s.* accounted for 7.8%, *An. rivulorum* 60.2%, *An. vaneedeni* 10.7%, *An. parensis* 11.0% and *An. leesoni* 10.3% (Ngomane *et al.,* 2007). The historical data from 2005-2009 presented in Chapter Three (Table 3.1) shows that members of *An. gambiae* complex outnumbered the *An. funestus* group. These mosquito collections were achieved using various methods, including larval sampling, pit–traps, night-biting catches, window traps, cattle kraal collections, and indoor resting catches (Govere *et al.,* 2000,

2001; Ngomane *et.al*, 2007). This may account for the differences seen compared with the present study where only larval collections were done. Larvae of the *An. funestus* group are difficult to collect, preferring swampy, well-vegetated breeding sites, while the *An. gambiae* complex can be easily found in temporary pools at roadsides and in hoof prints around dams and pans.

An. funestus s.s. and *An. arabiensis* were both collected during the surveys of Govere *et al.* (2001) and Ngomane *et.al.* (2007) along with secondary and non-vectors (see Table 3.2). Comparing the collections of *An. funestus* between 1997 and 1998, a great reduction in the numbers was observed. Furthermore, collections of *An. arabiensis* were reduced by 13.5%. A possible explanation for this reduction in vector populations is the implementation of complete indoor residual house spraying for all localities in the high risk areas. It is also possible that low rainfall was responsible for the decreased numbers of vectors.

4.2 THE CURRENT SURVEY OF BREEDING SITES

Despite the present study having used only one collection method (larval catches), the species diversity was similar to that of previous surveys (Govere *et al.*, 2000, 2001; Ngomane *et al.*, 2007). Within the *An. gambiae* complex, *An. merus* remained the most abundant with almost 60% of the sample being identified as this species. This is comparable with the historical data where 56% were *An. merus*. The prevalence of the other two species showed that *An. quadriannulatus* had decreased (30% down to 24%) while the proportion

of *An. arabiensis* had increased (13% up to 18%). One specimen of *An. gambiae s.s.* was identified from outside of the study area. And this could have serious consequences for the malaria vector control programme. This major malaria vector has been recorded in South Africa on only very rare occasions (Smith *et al.*, 1977; Miles, 1979) but occurs extensively in neighboring Mozambique (Coetzee *et al.,* 1993, 2000). Increased entomological surveillance is recommended to ensure that this species is kept under control.

Within the *An. funestus* group, species identification results showed that *An. rivulorum* remained the most abundant member of the group, both historically and in the present survey. The present study found no specimens of *An. funestus* but since collections were carried out for only three months, early in the transmission season, this does not mean that this species is absent from Mpumalanga.

The recent incrimination of *An. merus* in Mozambique as a potential vector responsible for malaria transmission at Namacha district bordering on Mpumalanga is of great concern (Cuamba and Mendis, 2009). Although *An. merus* and *An. rivulorum* are known as secondary vectors in Tanzania (Wilkes *et al.,* 1996; Temu *et al.,* 1998), neither had been incriminated further south until 2009 when the Cuamba and Mendis paper was published. Given the extensive distribution of *An. merus* in Mpumalanga, regular screening of this species for parasite infections is recommended

When comparing the species composition recorded during the present study with the historical entomological data, significant changes were noted at three localities: Tulloh Farm, Mananga and Masibekela. This change was reflected in the collection of *An. arabiensis* that had not been collected at these sites before. Furthermore, this study showed that more *An. arabiensis* were being collected from low risk areas compared with the monitored breeding sites in the high risk areas. This extension of the distribution of *An. arabiensis* should also be monitored through entomological surveillance so that programmatic decisions can be evidence-based.

4.3 INSECTICIDE RESISTANCE STATUS OF THE *ANOPHELES GAMBIAE* COMPLEX

The insecticide susceptibility tests could only be carried out on *An. merus,* of which sufficient numbers were collected. The results confirmed that *An. merus* was susceptible to all the classes of insecticides approved by WHO for use in malaria indoor residual house spraying. Govere *et al.* (2001) carried out a similar study in Mpumalanga Province where the same results were produced. Even though the insecticide susceptibility results for *An. merus* are good, this does not mean that the *An. arabiensis* populations are also susceptible. The vector control programme, therefore, needs to consider the strategies to be used in managing the development of resistance to insecticides since indoor residual house spraying is the backbone of malaria vector control (WHO, 2011).

Insecticide resistance management strategies include rotation of insecticides, mosaic spraying, insecticide treated bed nets, integrated vector management and use of mixtures, although this latter option is not yet available from insecticide suppliers. Of the four strategies recommended for preserving the efficacy of insecticides, rotation of insecticides and mosaic spraying are identified as the most easy to implement (WHO, 2012). Resistance management strategies are already used in Mpumalanga whereby organophosphate insecticides are used for larviciding operations and organochlorines and pyrethroids are used for indoor residual house spraying, one in traditional houses and the other in cement houses. The house spraying results in a mosaic effect while the addition of larviciding with a different class of insecticides contributes to the integrated vector management strategy.

4.4 IMPLICATIONS FOR MALARIA VECTOR CONTROL IN MPUMALANGA PROVINCE

Presently, there is significant population movement and migration in and out of Mpumalanga which impacts on malaria transmission. Seasonal workers from Namacha district in Mozambique are recruited by commercial farming operations in the Nkomazi Municipality. Some of them are parasite carriers and can infect the local vector mosquitoes. Entomological surveillance and mapping of breeding sites is essential for the control programme, particularly for larval control. Finding *An. arabiensis* in what is considered to be the known low risk areas (e.g. Vlakbult which is the first record of *An. arabiensis* from this area) should trigger the entomological equivalent of a malaria case outbreak response and the IRS coverage adapted accordingly.

Anopheles arabiensis will feed on animals in the absence of humans or feed on humans both indoors and outdoors (Gillies and De Meillon, 1968). After taking a blood meal, it will rest indoors or outdoors, depending on the available microclimate or if the IRS insecticide has a repellent effect or not. Its exophilic/endophilic, exophagic/endophagic behaviour poses a great challenge to the efficiency and efficacy of vector control programmes that use residual insecticides (Gillies and Coetzee, 1987; Hargreaves *et al.,* 2003). If *An. arabiensis* maintains its exophilic behaviour, the desired impact of the indoor residual spraying will not be attained. Therefore, the programme needs to explore new strategies to supplement or support the main intervention of indoor residual spraying. The new strategies could include implementation of a winter larviciding programme and screening of houses to prevent mosquito entry.

4.5 CONCLUSION

The aim of the study was to review and update the entomological data on breeding sites of potential malaria vectors and to investigate the insecticide resistance status of these mosquitoes in the study area. Continuous identification and mapping of potential breeding sites from low and high risk areas will be useful for the malaria vector control programme, whereby more sentinel sites will be established. Coupled with this, the programme will be able to implement targeted indoor residual spraying according to the epidemiology of the disease and the distribution of potential vector mosquitoes.

Indoor residual spraying and intensive larviciding operations, implemented in areas where vectors are shown to be continuously breeding, will maintain effective control and reduce wasteful expenditure on implementing control in areas where there are no vectors present. The current residual insecticides used by the programme for indoor residual spraying are still effective according to the WHO susceptibility tests carried out here. However, the programme needs to keep in mind some of the strategies that are possible to delay the development of the resistance, including insecticide rotation and the use of mixtures when these become available.

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