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Salinity tolerance of the aquatic stages of *Anopheles funestus* group

By

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Malaria is a major public health disease across the globe, especially in Sub-Saharan Africa where 90% of deaths occur, causing high morbidity and mortality among children. Africa remains the most affected region with an estimated 247 million clinical cases amongst 3.3 billion people at risk with nearly 1 million deaths, despite advances in preventive and public health measures (WHO, 2008). In South Africa, about 10% of the total population lives in malaria risk areas in the three provinces of Mpumalanga, KwaZulu-Natal and Limpopo (Statistics South Africa, 2010). The communities living in the malaria endemic areas are all at risk due to unstable transmission. *Plasmodium falciparum,* the most dangerous human malaria parasite, accounts for 95% of the malaria cases in South Africa and it is associated with severe and fatal disease. *Anopheles arabiensis* Patton is an important malaria vector in South Africa together with *An. funestus* Giles (Annual malaria report, 2007).

1.2 Malaria situation in South Africa (past and present)

In the early part of the last century, malaria in South Africa was a serious health problem with a much wider distribution within the country than nowadays. Following more than 50 years of successful malaria vector control using indoor residual spraying with sustainable operational coverage of more than 85%, there has been a significant reduction in vector density leading to reduction in malaria cases and death (Annual malaria report, 2007). Malaria in South Africa is seasonal, unstable and now confined to the low altitude

areas of three provinces (KwaZulu-Natal, Limpopo and Mpumalanga). Malaria risk areas in fig 2.1 are situated along the borders of South Africa with Zimbabwe and Mozambique. Malaria in South Africa is primarily a cross-border movement problem. Very occasionally limited focal transmission occurs in the North West and Northern Cape Province along the Orange and Molopo rivers due to favorable breeding sites (Annual malaria report, 2007).

During the 2007 malaria season there were approximately 6,615 reported malaria cases with 60 deaths, a decrease of almost half the number of cases and deaths compared with 13,901 cases and 126 deaths in 2006. According to the unpublished Department of Health data, during the 2005/2006 and 2006/2007 malaria seasons the malaria burden was concentrated in the Limpopo and Mpumalanga provinces. While Limpopo province accounted for 43% and 44% of all malaria cases in each season respectively, Mpumalanga recorded 33% for both malaria seasons (Annual malaria report, 2007).

The annual malaria cases and deaths trends in South Africa from 1971 to 2007 are shown in figure 1.1. From 1971 to 1995 malaria cases and death has been very low. However, during 1999/2000 a major increase in transmission occurred, and a total of 61,934 cases and more than 400 deaths were reported. Many factors may have contributed to this. Two main factors were the evidence of pyrethroid resistance in *Anopheles funestus* (Hargreaves *et al.*, 2000) and the presence of parasite resistance to anti-malaria drugs, especially sulfadoxine-pyrimethamine (Maharaj *et al.*, 2005). Unusually heavy rains and influx of economic migrants from bordering countries carrying the malaria parasites were also contributing factors. Since then the number of cases and deaths have reduced drastically due to

reintroduction of dichloro-diphenyl-trichloroethane (DDT) in combination with pyrethroids in 2001, change in anti-malaria drug treatment and also cross-border collaboration with Swaziland and Mozambique known as the Lubombo Spatial Development Initiative (LSDI) (Annual malaria report, 2007).

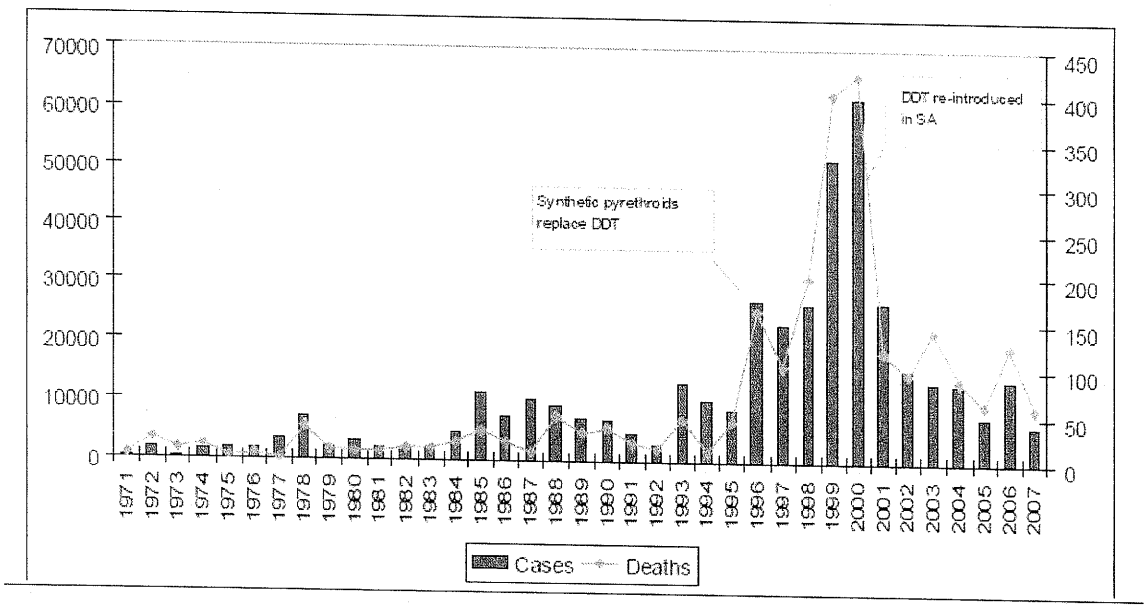

Fig 1.1 Annual notified malaria cases and deaths in South Africa, 1971- 2007 (Department of Health. Annual malaria report, 2007)

1.3 Malaria in Mpumalanga Province and current challenges

Mpumalanga Province is situated between KwaZulu-Natal Province in the south, Swaziland and Mozambique in the east, Limpopo Province in the north and Gauteng Province in the west. The highest malaria incidence in this province is along the Mozambique border. An estimated population of four million people reside in this province in predominantly rural areas (Fig 1.2) with repeated malaria outbreaks and epidemics (Coleman *et al.*, 2008). Despite the fewer number of reported cases in South Africa, both

Limpopo and Mpumalanga Provinces reported the highest number of cases during the past years (Annual malaria report, 2007).



Fig 1.2 A typical traditional hut in a rural area, Block A, Mpumalanga Province.

Malaria transmission in Mpumalanga Province still remains a challenge due to population migration from Mozambique for employment on farms (Durrheim *et al.*, 1999, Govere *et al.*, 2001), the suitable climatic conditions and breeding sites (Fig 1.3) in the area with the perennial Crocodile river (Fig 1.4) and tourists to Kruger National Park. Farming in the area has attracted a lot of workers from Mozambique and many are gametocyte carriers (Govere *et al.*, 2001). There are a variety of mosquito breeding sites in this province (Figs 1.3, 1.4). Targeting the mosquito breeding habitats is one of the interventions available for malaria vector control. Locating the larval habitats and understanding their distribution and factors affecting the larval abundance is an important component in planning the vector control measures. Larval control can help reduce the malaria burden even though most efforts are targeting the adults rather than the early stages.



A B C

Fig 1.3 Some potential breeding sites in Tonga (A and B) and Komatipoort (C) in Mpumalanga province.

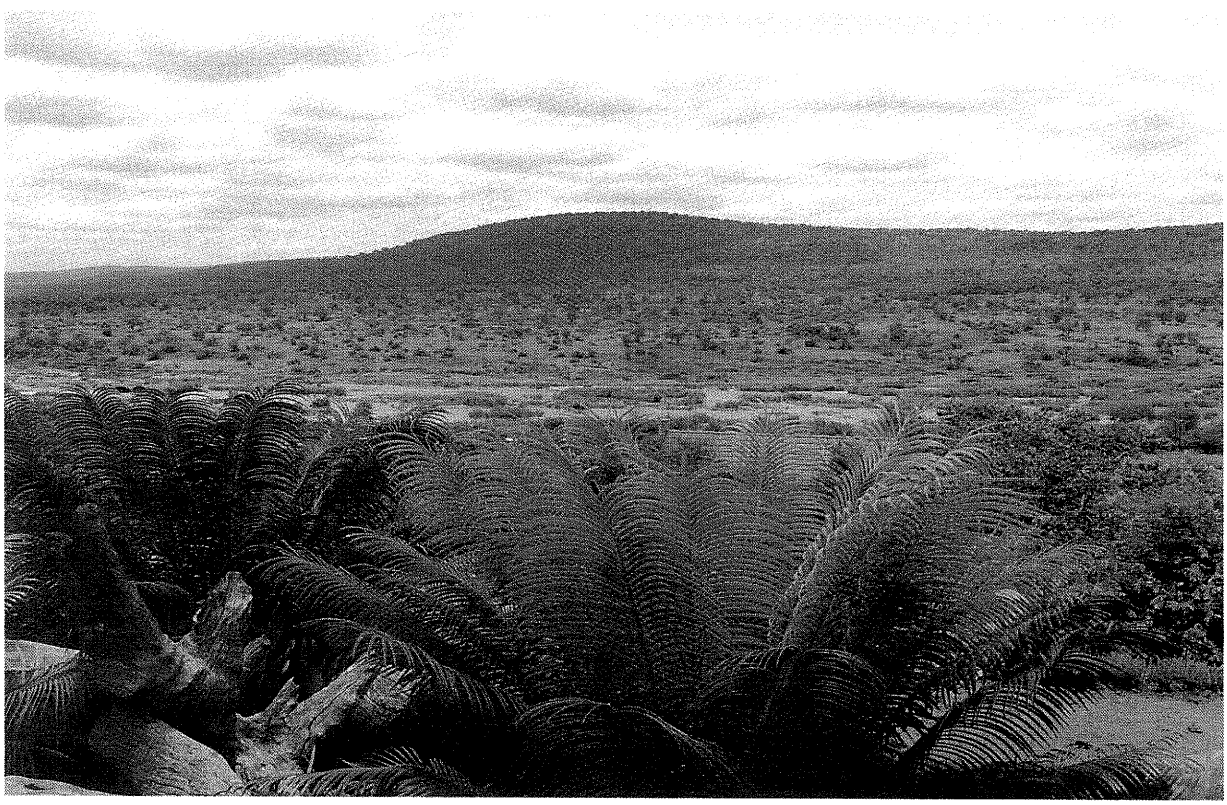

Fig 1.4 The perennial Crocodile River flows through Komatipoort.

According to the 2007 malaria report (Department of Health) the majority of cases in the province are amongst people returning from Mozambique (Fig 1.5). The highest malaria incidence was reported in the Ehlanzeni district, bordering Mozambique (Fig 1.6).

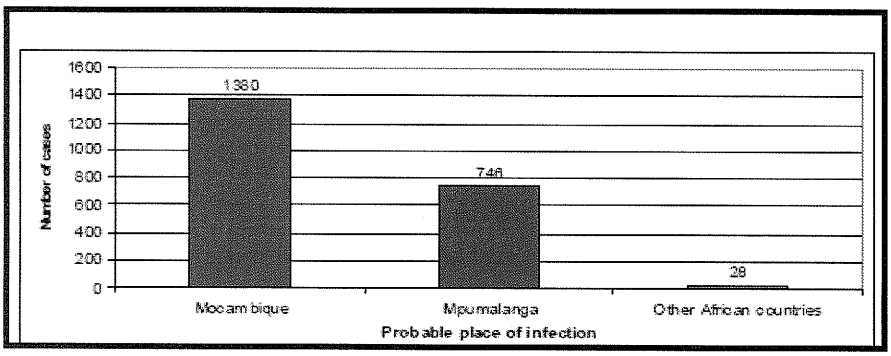

Fig 1.5 Reported malaria cases by probable place of infection (Mpumalanga) 2006/2007 malaria season (Annual Malaria Report, 2007)

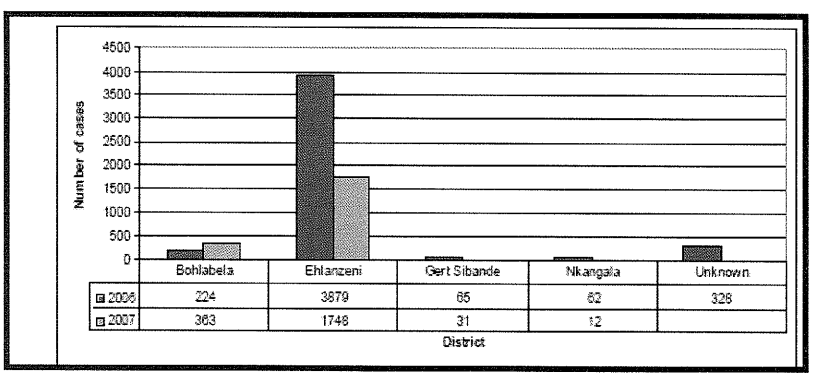

Fig 1.6 Reported malaria cases by district, Mpumalanga Province during 2006/2007 malaria season (Annual Malaria Report, 2007).

1.4 Malaria vectors in southern Africa

In the southern Africa region the two major malaria vector species *An. funestus* and *An, arabiensis* account for the bulk of malaria transmission. Although the two are usually found occupying the same geographical range *An. arabiensis* plays a dominant role in the drier or arid regions while *An. funestus* prefers humid and tropical regions. *Anopheles funestus* breeds mainly in vegetated permanent water bodies such as swamps and weedy streams. It

has been successfully controlled by indoor residual spraying due to its indoor feeding and resting habits (Gillies & De Meillon, 1968).

1.5 *Anopheles funestus* group

Historically, this group consists of nine Afrotropical species (*An. funestus* s.s, *An. rivulorum* Leeson, *An. leesoni* Evans, *An. vaneedeni* Gillies & Coetzee, *An. parensis* Gillies, *An. confusus* Evans & Leeson, *An. aruni* Sobti, *An. fuscivenosus* Leeson and *An. brucei* Service (Gillies & De Meillon, 1968, Gillies & Coetzee, 1987). Species of this group show great uniformity at the adult stage. Some can only be distinguished at specific immature stages in their development (Gillies & De Meillon, 1968). Members of the *An. funestus* group are small, dark mosquitoes with wing markings showing dark and light areas of costa and 1st vein, reduced pale spots on other wing veins and larvae with simple clypeal, mesopleural and saddle hairs and wide, sometimes deep, abdominal tergal plates (Gillies & De Meillon, 1968).

The *An. funestus* group has recently been re-classified based on phylogenetic analyses (Harbach, 2004). This group now consists of five subgroups and the African members belong to the three subgroups. Five species belong to the *An. funestus* subgroup: *An. funestus, An. vaneedeni, An. aruni, An. parensis* and *An. confusus.* The *An. rivulorum* subgroup consists of *An. rivulorum, An. brucei, An. rivulorum*-like (a new species from Cameroon) and *An. fuscivenosus. Anopheles leesoni* has been reclassified as belonging to the *An. minimus* subgroup (Harbach, 2004, Garros *et al.,* 2005). Recently a new member of the *An. funestus* group has been discovered based on cytogenetic and molecular analysis and this species was preliminary named *An. funestus*-like from Malawi (Spillings *et al.*,

2009).This species is morphologically similar to *An. funestus* and is found occupying the same geographical range in Malawi (Spillings *et al.*, 2009).

1.5.1 African members of the *An. funestus* group: their biology, vector status and distribution

An. funestus s.s

Anopheles funestus s.s is widely distributed and found in abundance in most Afrotropical regions occupying a variety of breeding sites. It is considered a major human malaria vector due to its high endophilic and anthropophilic behaviour. In some areas it has been eliminated by indoor residual spraying (Gillies & De Meillon, 1968, Gillies & Coetzee, 1987). Larvae prefer breeding in more or less permanent clear water bodies such as swamps, weedy streams, rivers, ponds, ditches especially where there is vegetation. The vegetation provides some shade which is an important ecological feature. In observations made there might be some interactions between larvae of *An. funestus* and other group members leading to competition for environmental resources (Gillies & De Meillon, 1968).

An. aruni

Anopheles aruni has been found only in Kaburi Kikombe in Zanzibar and is morphologically indistinguishable from *An. funestus* with only females differing due to having paler wings and more broad banded palps. Females bite humans outside at night, but little else is known about adult behaviour, vector status or biology of this species (Gillies & De Meillon, 1968).

An. brucei

This species is known only from one locality in northern Nigeria and the larval habitat has been noted as shady forest streams and partially dried riverbeds. There is limited information available on its biology (Gillies & De Meillon, 1968).

An. confusus

Anopheles confusus is localized in South Africa, Zimbabwe and Ethiopia and its larvae were recorded breeding in hippo footprints and slow moving streams. Its biology and vector status remains unclear even though occasionally it is found in houses (Gillies & De Meillon, 1968, Gillies & Coetzee, 1987).

An. fuscivenosus

The early stages of *An. fuscivenosus* are not known and its distribution is localized in Zimbabwe. The adult biology is not known but specimens were collected resting outdoors (Gillies & De Meillon, 1968).

An. leesoni

Anopheles leesoni is widely distributed in the savanna regions in West and East Africa. Adults resemble *An. funestus* but have quite distinct early stages especially the eggs that appear to be different from the other members of the group. Adults are thought to be zoophilic as they have been found indoors in cattle sheds (Gillies & De Meillon, 1968).

An. parensis

Anopheles parensis is an exophilic and zoophilic species found in Tanzania, Pemba Island, eastern African lowlands of Kenya and South Africa. It was first recognized in Tanzania after elimination of *An. funestus* by indoor residual spraying. Larvae are found in permanent swamps and ponds with vegetation. It is of no importance in malaria transmission (Gillies & De Meillon, 1968).

An. rivulorum

This is an exophilic and zoophilic mosquito which occasionally is found resting indoors. Although no salivary gland infections have been found in this species, it was caught biting humans outdoors in fairly large numbers in Tanzania (Gillies & Smith 1960). In contrast, Wilkes *et al.* (1996) found that *An. rivulorum* can in fact be a vector in Tanzania by detecting five sporozoite-positive specimens in 1022 samples examined. Larval habitats are characterized by gently flowing water with vegetation, along the margins of open water bodies and irrigation channels especially in eastern Africa. It is found in eastern, southern Africa and West African savannas (Gillies & De Meillon, 1968).

An. vaneedeni

This is an outdoor human biting species. During daytime quite a large number of females are found resting in pit shelters (De Meillon *et al.,* 1977). In laboratory tests the species showed full susceptibility to *P. falciparum* malaria but its involvement in malaria transmission still remains uncertain (De Meillon *et al.,* 1977). Larval habitats are not different from *An. funestus* (Gillies & Coetzee, 1987).

1.6 Colonization of *Anopheles funestus*

Anopheles funestus is quite difficult to colonize in the laboratory. This was proven by Service & Oguamah (1958) where they attempted to colonize this species and only succeeded after three years. The first successful colony was established from egg batches of wild caught females after both guinea pig and human blood feeding. However, after a few months the colony died out due to diminishing egg production. A year later the same authors tried again to establish another colony from wild caught gravid females given

guinea pig blood meals only and this was more successful. Eggs were laid on filter paper immersed inside water and larvae were reared in tap water with floating water weed *Elodea canadensis* and fed with pure dried yeast. Adults (males) were given a sugar solution. Some egg batches were sent to London to establish another colony but this was never maintained (Service, 2010).

Hunt *et al.* (2005), in the Vector Control Reference Unit in Johannesburg, established the first long-term *An. funestus* colony (FUMOZ) from material collected in southern Mozambique. This colony has been in existence since 2000 and is highly stable in the insectary.

1.7 Physiology of salinity tolerance in mosquito larvae

In order for mosquitoes to complete their development, the early life stages require aquatic environments and they have adapted to diverse aquatic habitats ranging from freshwater rain pools to brackish, salt marshes etc. Mosquito larvae, like any aquatic organisms, live in environments which can fluctuate in salinity due to environmental factors such as rainfall and evaporation. Rainfall causes the habitat to become rapidly dilute while evaporation can cause increasing levels of salinity (Smith *et al.,* 2008). Most of the mosquito larvae breed preferable in freshwater tolerating a certain salinity range, while others are found in brackish and saline environments (Bradley, 1987).

Wigglesworth (1933a,b) noted that larvae of certain mosquitoes can live both in high salinity water as well as freshwater, while others are easily killed by salt water. These

differences can occur within the same species due to physiological adaptations. Mosquito larvae, just like any organisms that live in harsh environments, can develop specialized organs to adapt to such conditions. The rectum shown in figure 1.7 below is one of the important organs responsible for the ion regulation in mosquito larvae. In saline tolerant culicine species, the posterior rectum secretes hyper-osmotic urine in saline habitats (Smith *et al.,* 2008). In their study, Bradley & Phillips (1977) showed that *Aedes taeniorhynchus* larvae can withstand high salinity (200% seawater) during the developmental period by using the rectum for ion excretion. In addition, many mosquito species show the anal papillae as the site of salt and water uptake (Coetzee & Le Sueur, 1988, Bradley, 1987, Garrett & Bradley, 1984, Wigglesworth, 1933a).

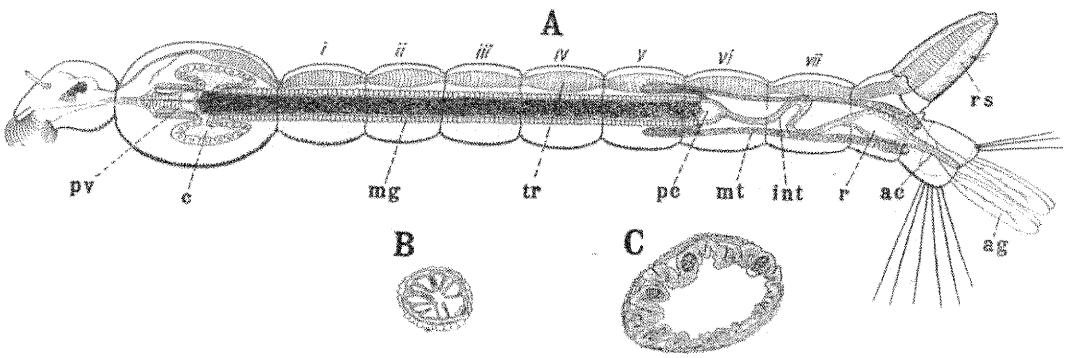

Fig.1.7 Anatomy of a mosquito larva (semi-diagrammatic). A: *ac,* anal canal; *ag,* anal gills; *c,* caeca; *int,* intestine(hind-gut); *mg,* mid-gut; *mt,* Malpighian tubes; *pc,* pyloric chamber (hind-gut); *pv,* proventriculus;*r,* rectum; *rt,* respiratory siphon; *tr,* one of the main tracheal trunks. The figures i, ii, etc., indicate the respective abdominal segments. B and C show cross sections of the hind-gut, B through the intestine *(int),* C through the rectum (r) (Wigglesworth, 1933a).

Patrick & Bradley (2000) examined the response of two species of *Culex* mosquito larvae to seawater: *Culex quinquefasciatus,* a freshwater breeder and *Culex tarsalis,* a saline tolerant breeder. They noted that the mosquito larvae of the osmo-conforming *Cx. tarsalis*

accumulated high levels of proline and trehalose (organic osmolytes) in the haemolymph in response to increased environmental salinity.

1.8 Salinity tolerance test method

Ribbands (1944) used a simple salinity test to distinguish between the larvae of *An. gambiae* and *An. melas* that was later modified by Muirhead Thomson (1951). Larvae of the two species show a clear difference in reaction to salinity and this simple test was used to identify the progeny of wild caught females of the salt water breeding species from freshwater breeding species. Laboratory reared larvae of *An. gambiae* died within 48 hours in 50% sea water while *An. melas* larvae survived in more than 100% sea water.

The method of distinguishing larvae using the salinity tolerance test has proved to be practical for separating *An. merus* from freshwater members of the complex (Mosha & Mutero 1982, Coetzee & Le Sueur, 1988, Paterson 1964). Such a remarkable physiological difference can be used to determine the proportion of either species in mixed batches of larvae. Coetzee & Le Sueur (1988) used the salinity test method to identify *An. merus* and confirmed species identification by enzyme electrophoresis. Therefore this simple and less expensive method can be used to separate saltwater breeding species from freshwater breeding species in areas where resources are limited.

1.9 Research rationale

There is little information on the biology of the early stages of the *An. funestus* group and the factors affecting larval survival are poorly studied. There is some information by Jepson *et al.*, (1947) on *An. funestus* salinity tolerance on Mauritius. In their study they found that

An. funestus larvae can survive in pools with a maximum salinity of 0.73% NaCl (25% sea water) but regularly occur in pools with salinities of 0.2% NaCl (7% seawater). In their laboratory trials they noted full development of the adults in salinities ranging from 0-0.3% NaCl (0-10% seawater). It is recognized that *An. funestus* shows little salinity tolerance in laboratory findings and even in natural breeding sites. While *An. funestus* is a freshwater breeder, rearing other members of this group in distilled water in the laboratory has proved to be problematic (Vector Control Reference Unit, unpublished data).

Understanding these variations in salinity tolerance can provide some vital information on the biology and ecology of members of the *An. funestus* group. Furthermore, this study provides information on factors that can influence larval abundance and distribution (Fillinger *et al.*, 2004).

1.9.1 Aim and objectives of the project

This study aimed to determine the effects of different saline concentrations on survival rates of members of the *An. funestus* group by exploring the salinity ranges tolerated and favored by the members of the group.

Specific Objectives of the Project

1. To establish a baseline salinity tolerance range using the laboratory reared *An. funestus* colony (FUMOZ).

2. To identify wild caught *An. funestus* group using the morphological keys and to conduct Polymerase Chain Reaction (PCR) assays to identify species within the *An. funestus* group.
3. To rear and maintain the wild caught *An. funestus* group egg batches in the insectary in different concentrations of saltwater.

CHAPTER TWO

MATERIALS AND METHOD

2.1 Mosquito colony

Anopheles funestus colony (FUMOZ)

According to Hunt *et al.* (2005) wild female *Anopheles funestus* group were collected inside houses in southern Mozambique in 2000. Females were set up for egg laying in the VCRU insectary at the National Institute for Communicable Diseases (NICD) in Johannesburg. Wild caught females were further identified to species level using the PCR-single strand conformation polymorphism method developed by Koekemoer *et al.* (1999). All mosquitoes identified as *An. funestus* were pooled and the FUMOZ colony established. This colony was used as a reference to establish a baseline salinity tolerance range for the *An. funestus* group.

2.2 Establishing the baseline salinity tolerance using FUMOZ

Approximately 50-60 eggs obtained from individual FUMOZ females were reared in different seawater concentrations using commercial sea salt (Cerebos® iodated sea salt). The following salinities were selected after preliminary studies exposing the FUMOZ eggs to different salt concentrations (0%-50% saltwater). Both 40% and 50% seawater concentrations were too high for eggs hatching. The salinity concentrations used were 0% (distilled water), 5% (0.145% NaCl), 10% (0.29% NaCl), 15% (0.435% NaCl), 20% (0.58% NaCl), 25% (0.725% NaCl) and 30% (0.88% NaCl) seawater (31.7g/litre of NaCl is

equivalent to 100% seawater). For each family five replicates per concentration were tested. To ensure that the salt concentration was not affected by evaporation all the rearing bowls were covered with a plastic sheet throughout the experiments. These were maintained in the insectary at 25 ± 2°C, relative humidity of ± 80% and photoperiod LD 12:12. The larvae were fed with a mixture of dog biscuits and brewer's yeast ground to fine powder. The different immature stages were counted and recorded daily until they reached the adult stage. The survival rates from the baseline trial determined the salinity tolerance ranges for the project on other members of the *An. funestus* group.

2.3 Study area

Samples were collected in Mpumalanga Province from different sites: Cheetahs, TSB3 and Caravan Park are all situated within Komatipoort (S 25°26.540', E 31°56.547'); Albetsnek (S 25°39.862', E 31°57.589'); Martiens farm (S 25°41.435', E 31°44.158'); Block A (S 25°42.077', E 31°48.552'); G & L Fourie baby buffalo farm (S 25°25.766', E 31°57.785') and Tonga (S 25°40.050', E 31°46.543'). All these localities are in the Nkomazi municipality. Komatipoort, Martiens farm and G&L baby buffalo farm are about 10km away from each other while Tonga and Block A are about 50 km from Komatipoort. There is intensive commercial sugarcane and banana farming in the province with the majority of workers coming from Mozambique. The majority of mosquito samples were collected in Komatipoort (Fig 2.1) which is situated on the Mozambique/South Africa border.

Wild mosquitoes were collected during the first week of February 2010 and some samples were collected by the entomology team in the Mpumalanga Province for two weeks during

February 2010 and sent to the Vector Control Reference Unit (VCRU) insectary at the National Institute for Communicable Disease (NICD) in Johannesburg.

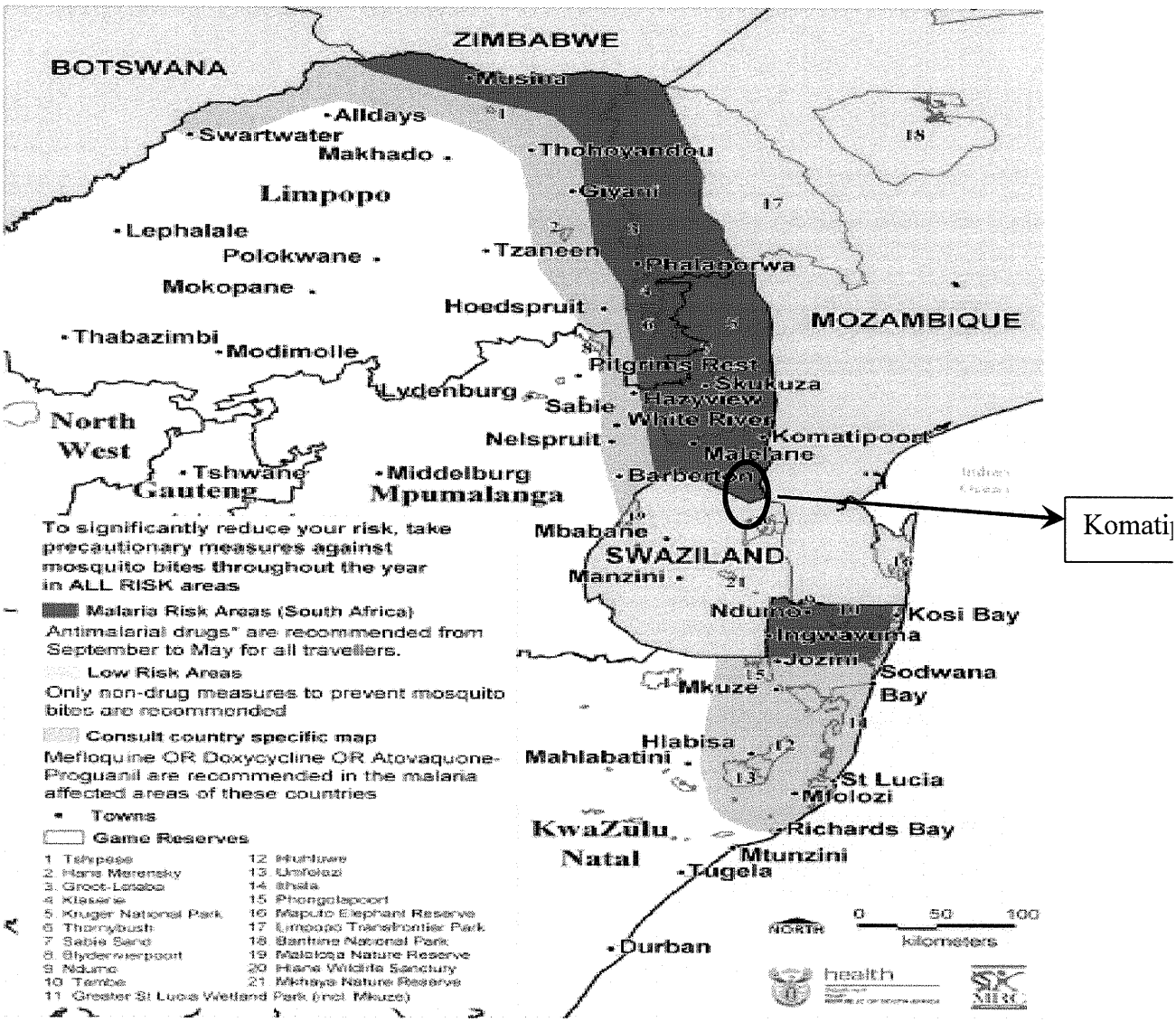

Fig 2.1 Map showing malarious areas in South Africa. Study area circled.

2.4 Wild mosquito collections

The Mpumalanga Malaria Control Programme's entomology team kindly provided mosquitoes collected by them during their routine entomological surveillance activities. These mosquitoes were collected using standard techniques in malaria vector control, i.e. indoor house searches, window exit traps and human landing catches (Service, 1976). In addition to these collections, the following trapping techniques were employed specifically for this project:

2.4.1 Carbon dioxide–baited net traps

Carbon dioxide strongly attracts mosquitoes to their host (Gillies, 1980, Snow, 1970). In this study, carbon dioxide in the form of dry ice was used as a bait to attract mosquitoes into the nets traps. The dry ice was placed in a polystyrene cool box inside a net trap with the sides tucked up as shown in Fig 2.2. Regular collections were done during the night at 30 minutes intervals from 1900hrs to 0000hrs and early in the morning at 0600hrs. All mosquitoes were collected using an aspirator and torch and were identified during capturing or on a later occasion. Collected mosquitoes were placed in paper cups and provided with cotton wool soaked in 10% sugar solution. This method was only used for two nights during this study.

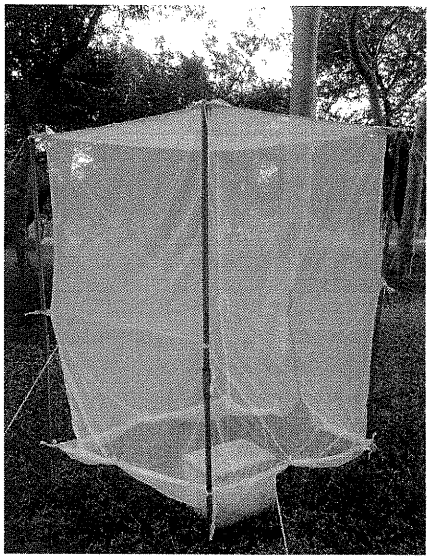

Fig 2.2. Net trap with sides tucked up containing a cooler box filled with dry ice at the Komatipoort shunters / caravan park.

2.4.2 Pit traps / Natural shelters

Large pits (1.30m x 1.30m x 1.30m) with horizontal shafts were dug in shaded areas under trees or bushy areas as shown in Figure 2.3. Also, adult mosquitoes were searched for in natural shelters such as crevices and holes near breeding sites along riverbanks (Fig 2.4). There was a problem with flooding of some pits in Block A. Regular mosquito collections were done every morning around 0630 and 0930 using an aspirator and a torch. Every morning all the pit traps were thoroughly searched and mosquitoes were collected into prepared paper cups and identified later.

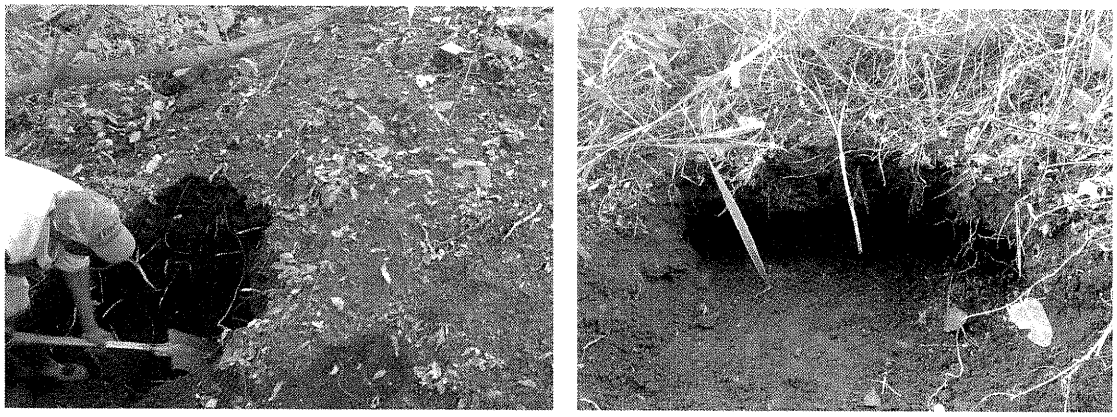

Fig 2.3 Construction of pit traps in caravan park



Fig 2.4 Searching for mosquitoes in their natural shelters along riverbanks

2.5 Morphological identification

All the wild caught adult mosquitoes were identified using the keys of Gillies & Coetzee (1987) while the eggs of *An. funestus* group members were morphologically identified using illustrations (Gillies & De Meillon, 1968).

2.6 Rearing of wild caught mosquitoes

All wild caught female mosquitoes were kept in glass vials in the VCRU insectary until they oviposited (Fig 2.5). Unfed females were offered guinea pig blood meals daily. All

egg batches from identified *An. funestus* group species were reared in different salinity concentrations.

Eggs from each batch identified as a member of the *An. funestus* group were divided amongst the different salt concentrations (see section 2.2 above) depending on how many eggs were oviposited. To ensure that the salt concentration was not affected by evaporation all the rearing bowls were covered with a plastic sheet throughout the experiment. All these were maintained in the insectary under standard conditions (25 ± 2°C, relative humidity of ± 80% and photoperiod LD 12:12). The larvae were fed with a mixture of dog biscuits and brewer's yeast ground to fine powder. Daily counting of larvae and pupae was done until they all reached the adult stage.



Fig 2.5 Glass vials where wild caught female mosquitoes were kept until they oviposited.

2.7 Species identification

All morphologically identified mosquitoes belonging to the *An. funestus* group were further identified by Polymerase Chain Reaction (PCR) assay to determine the species present (Koekemoer *et al.*, 2002). Extraction of DNA was done using the method of Collins *et al.* (1987).

An EDTA grinding buffer solution was made up of 1600µl 1M NaCl, 1.095g of Sucrose, 2400µl 0.5M EDTA, 1000µl of 10% SDS, 2ml 1M Tris-Cl (pH 8.6) and ±13 ml deionised water to make a total volume of 20ml. Wild mosquito abdomens and a known mosquito from the FUMOZ colony (positive control) were placed in individual 1.5ml microcentrifuge tubes and finely ground with a pestle in 100µl grinding buffer. When all the mosquitoes were homogenized they were incubated at 70°C for 30 minutes. A negative control was included using the same procedure being carried out but with no mosquito included. After incubation 14µl 8M KAc was added to all the samples and mixed gently by tapping with the fingers.

Samples were incubated on ice for 30minutes and then centrifuged for 15minutes at 15K.rpm. Supernatant were transferred into newly labeled microcentrifuge tubes bearing the same numbers as the original tube. The old microcentrifuge tubes were discarded. Then 200µl 100% ice cold ethanol (-20°C) was added and mixed by inverting the tube. The samples were kept overnight at - 20°C freezer.

The following day samples were taken out of the freezer and centrifuged for 15minutes at 15K.rpm. The 100% ethanol was removed and discarded in all samples without disturbing the pellet. Then 100µl of ice cold (-20°C) 70% ethanol was added to all samples ensuring that the pellet was not washed off. The samples were centrifuge for 30minutes at 15K.rpm and the 70% ethanol discarded. The pellet was allowed to air dry overnight and re-suspended in 100µl of 1x TE buffer. Samples were stored in a freezer for further use.

The PCR reagents for the Master Mix were 1.25µl of 10x buffer (500mM KCl, 100mM Tris-HCl pH 8.3), 1,25µl of 10x dNTP's (200 µM of each dNTP, 0.75µl 1.5mM MgCl, 1.0µl of 3.3 pmol/ primer each of UV,FUN,VAN,LEES,RIV,PAR, 3.15µl deionised water and 0.1µl of 0.5 units RTaq to give a final volume of 12.5µl per sample to be tested. All microcentrifuge were labeled corresponding to the extracted DNA wild samples and the five positive and negative controls. Then 12.5µl of master mix was aliquoted into all the tubes and 1µl of DNA was added to their respective labeled microcentrifuge including the controls. The microcentrifuge were then centrifuged for a few minutes to ensure that all the reagents mixed well. The samples were then placed into the thermocycler.

Amplification conditions were as follows: Initial denaturing at 94°C for 2 minutes, followed by a further 40 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 50°C for 30 seconds and extension at 72°C for 40 seconds and final autoextension at 72°C for 10 minutes. Four microlitres of ficol dye was added to all the samples and the controls after amplification and mixed thoroughly. Five microlitres of molecular marker was placed in the first well of the 2.5% TAE agarose gel to identify the

species with the base pair ladder, followed by 12µl of each of the positive controls of *An. vaneedeni, An. funestus, An. rivulorum, An. parensis* and *An. leesoni* and negative control in the other wells respectively. The amplified samples to be identified were loaded into the wells after the controls in their respective order.

Visualization and amplified product identity was done by electrophoresis on a 2.5% agarose gel, stained with ethidium bromide. The agarose gels were visualized under ultra violet light and the samples were identified by comparing the amplicons to the controls and the molecular weight marker ladder. A picture of each gel was taken and stored on the computer database.

2.8 Data Analysis

Data were statistically analyzed on Microsoft Office Excel 2003 and using Statistix 7. Survival rates were calculated for the different stages in different salt concentrations for each of the species. Percentage mean survival with standard error was calculated for the different concentrations. Analysis of variance (P values) for the different salt concentrations was calculated using Statistix 7 for the two species.

CHAPTER THREE

RESULTS

3.1 Baseline salinity tolerance using FUMOZ

A total of 1016 first instar larvae that hatched from eggs in different salt concentrations were reared through to adults.

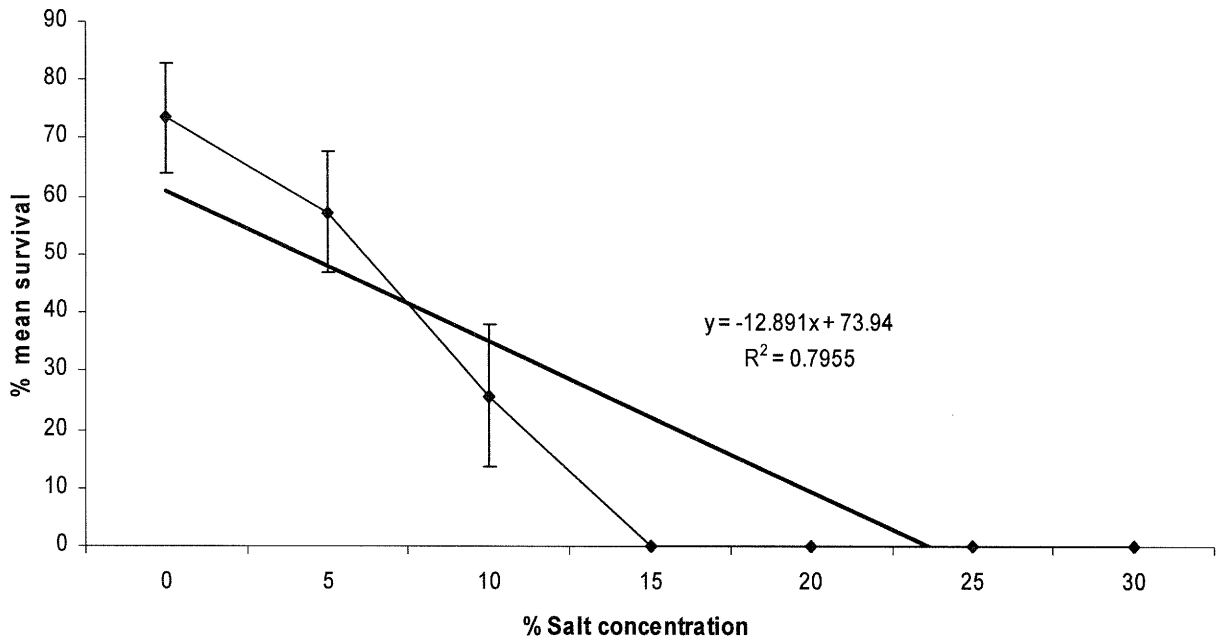

Fig 3.1 Percentage mean survival rates (± SE) of first instar larvae to adults (FUMOZ colony) exposed to the different seawater concentrations (0-30%). The regression line formula with slope (y=-12.891x+73.94, r2=0.7955), P value = 0.007.

The results in figure 3.1 show that FUMOZ decreased in levels of tolerance to saline water up to 10% but not including 15% saltwater. Increasing the salt concentration had less first instar larvae surviving to adult stage than those reared in distilled water. None of the first instar larvae survived to the adult stage when exposed to 15% seawater concentration and above. As a result, the salinity concentration range used for the other members of the *An. funestus* group collected in Mpumalanga was from 0%-15% seawater concentration.

Members of the *An. funestus* group species in South Africa are limited in numbers. Small egg batches (764 eggs) and eggs that were not viable contributed to only four concentrations being selected for this project. This low oviposition rates might be due to laboratory conditions not being ideal for such species compared with their natural environments.

3.2 Salinity tolerance of members of the *Anopheles funestus* group

3.2.1 Wild caught mosquito identification

A total of 315 adults were collected at the different sites in Mpumalanga Province using the different collection methods and identified morphologically using keys (Gillies & Coetzee, 1987, Gillies & De Meillon, 1968). Polymerase chain reaction assay was carried on those identified as *An. funestus* group (Koekemoer *et al.,* 2002). *Anopheles funestus* group were dominant (n = 244, 77.5%) followed by the *An. marshallii* group (n = 29, 9.2%), *An. gambiae* complex (n = 24, 7.6%), *An. coustani* group (n = 12, 3.8%) and *An. pretoriensis* (n = 6, 1.9%) (Table 3.1).

Table 3.1. Summary of identified *Anopheles* mosquito species collected from Mpumalanga Province in January/February 2010.

Collection method	***An. rivulorum***	***An. leesoni***	***An. vaneedeni***	***An. merus***	***An. quadriannulatus***	***An. coustani* group**	***An. marshallii* group**	***An. pretoriensis***
Indoor search (men's bathroom)	4	-	-	-	-	-	-	-
Window traps	-	-	-	-	-	-	1	-
Pit traps	58	-	2	13	5	-	-	6
Night bite catches	146	14	-		3	2	17	-
Carbon-dioxide baited nets	15	5	-		3	10	11	-
Total	**223 (70.8 %)**	**19 (6.0%)**	**2 (0.6%)**	**13 (4.1%)**	**11 (3.5%)**	**12 (3.8%)**	**29 (9.2%)**	**6 (1.9 %)**

The most successful collection method, where the bulk of the *An. funestus* specimens were collected, was through night biting catches (n = 160/244, 65.6%). This was followed by pit traps (n = 60/244, 24.6%), carbon dioxide baited net traps (n = 20/244, 8.2%) and indoor searches (n = 4/244, 1.6%), while window traps were considered unsuccessful in all cases.

Polymerase chain reaction assays were carried out on those specimens belonging to the *An. funestus* group (Fig 3.2). The results showed that *An. rivulorum* (n = 223/244, 91.4%) was the dominant species in that area followed by *An. leesoni* (n = 19/244, 7.8%) and finally *An. vaneedeni* (n = 2/244, 0.8%).

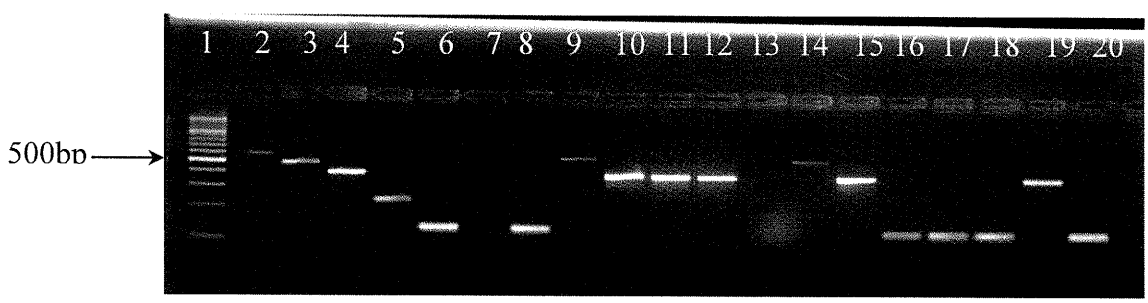

Fig 3.2: PCR identification of the members of the *Anopheles funestus* group from Mpumalanga Province. Lane 1: Molecular weight marker. Lane2: *An. vaneedeni* positive control. Lane 3: *An. funestus* positive control. Lane 4: *An. rivulorum* positive control. Lane 5: *An. parensis* positive control. Lane 6: *An. leesoni* positive control. Lane 7: negative control. Lane 8,16,17,18 & 20: *An. leesoni.* Lane 9&14: *An. vaneedeni.* Lane 10, 11, 12, 15&19: *An. rivulorum.*

3.2.2 Effect of salinity on hatch rate

A total of 1883 eggs of FUMOZ strain and 764 eggs for *An. rivulorum* were reared on different concentrations of salt water at the start of this experiment. The number of eggs used for each concentration was: FUMOZ (0%= 274, 5%= 292, 10%= 268, 15%= 305, 20% = 273, 25%= 267, and 30%= 202) and *An. rivulorum* (0%=226, 5%= 200, 10%= 201 and 15%= 137).

The results in Fig 3.3 showed a significant negative trend in *An. funestus* (FUMOZ) while *An. rivulorum* showed no significant trend in hatch rate exposed to different salt concentrations. The graph clearly shows that the hatch rate for *An. funestus* decreased with increasing salt concentration while for *An. rivulorum* the hatch rate was more or less similar in response to increased salinity.

Analysis of variance (P=0.0009) indicated that there was statistically significant trend in hatch rate amongst the salt concentration for *An. funestus*, while there was no significant trend in hatch rate between the salt concentrations for *An. rivulorum* (P= 0.748). FUMOZ showed a substantially higher hatch rate than *An. rivulorum* in all the concentrations.

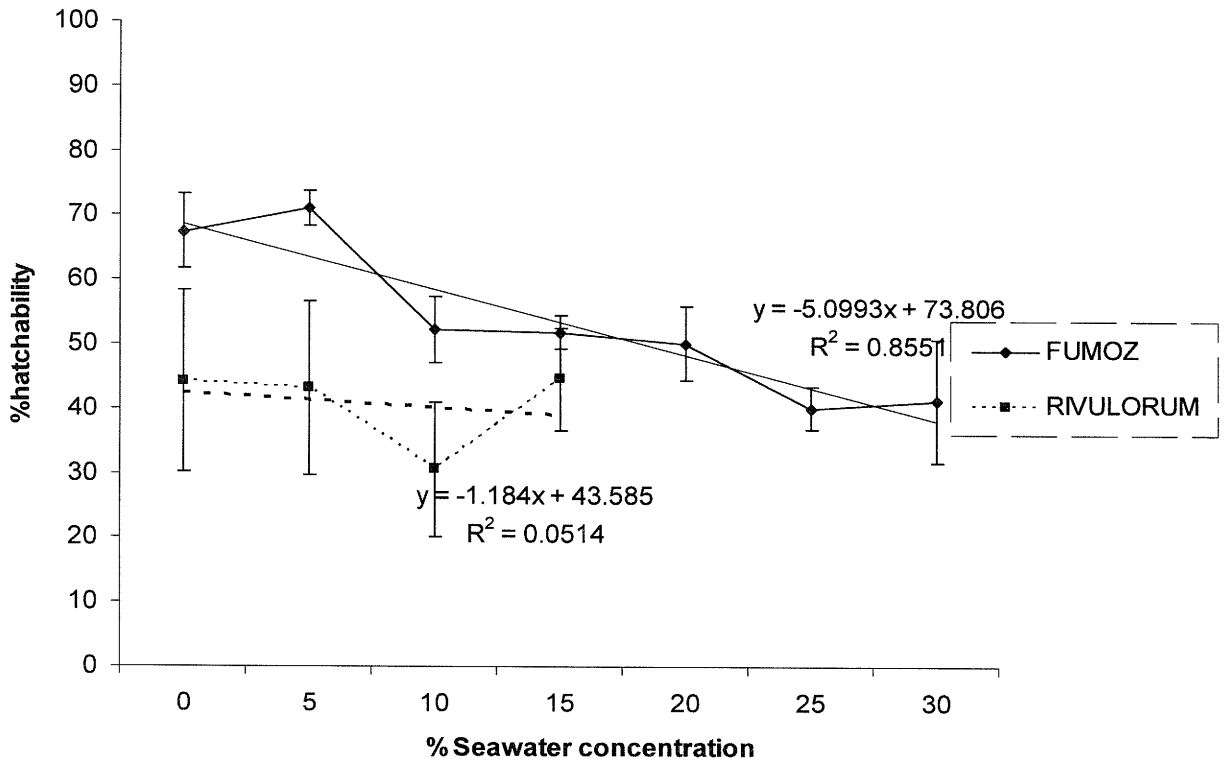

Fig 3.3 Percentage mean hatch rate of the two species *An. funestus* and *An. rivulorum* (± SE) for each salt concentration. The hatch rate was calculated by dividing the number of eggs hatched into first instar larvae by the total number of eggs exposed in each salt concentration. Analysis of variance for *An. funestus* (P= 0.0009), *An. rivulorum* (P=0.748) for all the salt concentrations.

The eggs kept in lower salt concentrations hatched more quickly than those in higher concentration for *An. funestus*. Concentrations of 0%, 5% and 10% had most of the eggs hatched by day 3 after oviposition. In higher concentrations (15% - 30%) eggs hatched 3 to 9 days after oviposition, although fewer eggs hatched on days 6-9. More eggs hatched on day 4 for *An. rivulorum* in all salt concentrations. *Anopheles funestus* hatched more quickly than *An.*

rivulorum. Only one *An. leesoni* female laid eggs and when exposed to all the different salt concentrations showed that they survive well in both 10% and 15% seawater.

3.2.3 Effect of salinity on larvae

All the first instar larvae that hatched from eggs in different salt concentration were observed until they reached the adult stage for the two species with mortality being recorded daily.

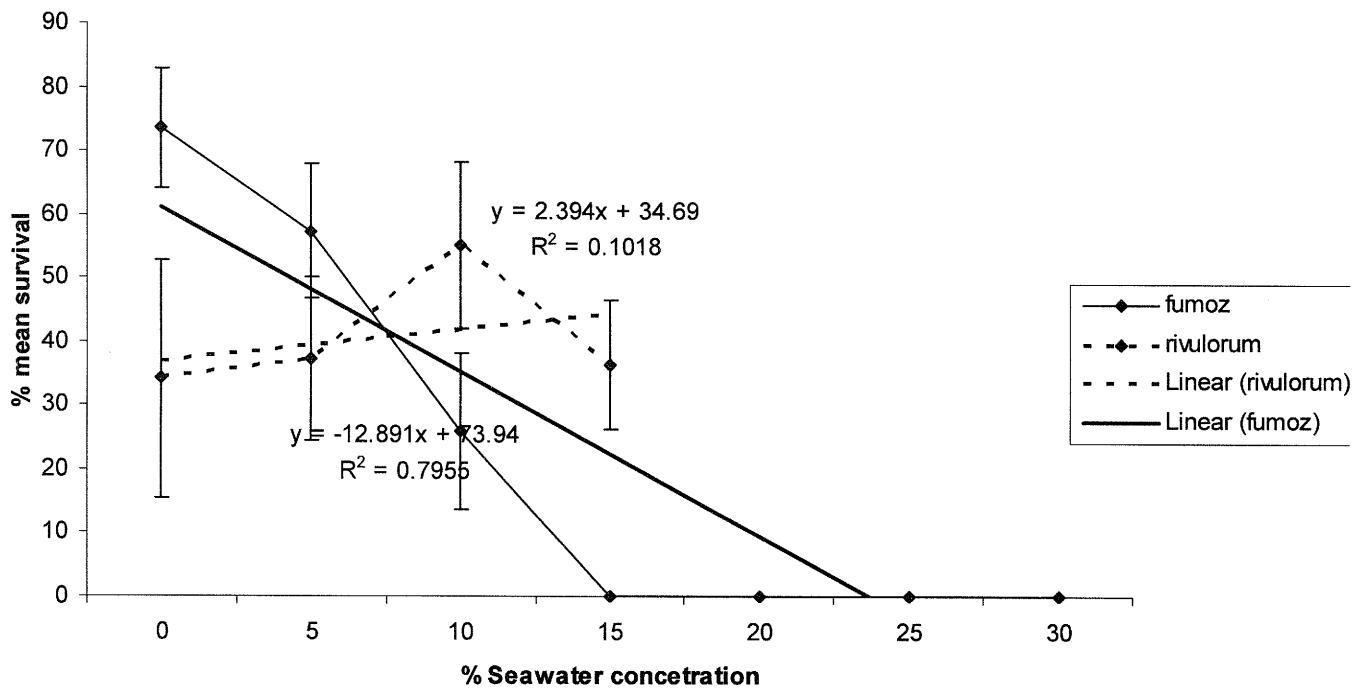

Fig 3.4 Percentage mean survival rates (± SE) of first instar to adults of *An. funestus* (FUMOZ) and *An. rivulorum* exposed to the different seawater concentrations. Survival rates were calculated by dividing the total number of adults emerged from the total number of first instars for each concentration for the two species. Analysis of variance for FUMOZ (P=0.0002) and *An. rivulorum* (P=0.909) for all the salt concentrations.

The percentages of first instar larvae that survived to the adult stage in different seawater concentrations are shown in Fig 3.4. The two species showed some differences in their response to increasing salinity. The *An. funestus* larvae surviving to the adult stage decreased progressively with increased salinity. The full development from egg to adult for *An. funestus* was observed in 10% saltwater (0.3% NaCl) and lower concentrations. *Anopheles funestus* showed a better survival rate in freshwater than in any of the saline concentrations. *Anopheles rivulorum* showed no significant trend (P=0.6809) with increased salinity and maximum adult emergence occurred at 10% (0.3% NaCl) seawater.

3.2.3.1 Effect of salinity on early immature larval stages (1st and 2nd instar)

Survival rates were recorded for all the first instar larvae that developed through to the 3rd larval stage (early immature larval stages) exposed to the different salt concentrations for the two species. The data were analyzed by using the two terms "early larval stages" (1st and 2nd instars) and "late larval stages" (3rd and 4th instars).

Fig 3.5 clearly shows a significant downward trend for *An. funestus* with increasing salinity concentration in the early immature larval stages. *Anopheles rivulorum* has no significant trend with increasing salt concentrations. However, at the 5% salt concentration the survival rates for the early immature larval stages of the two species were more or less the same.

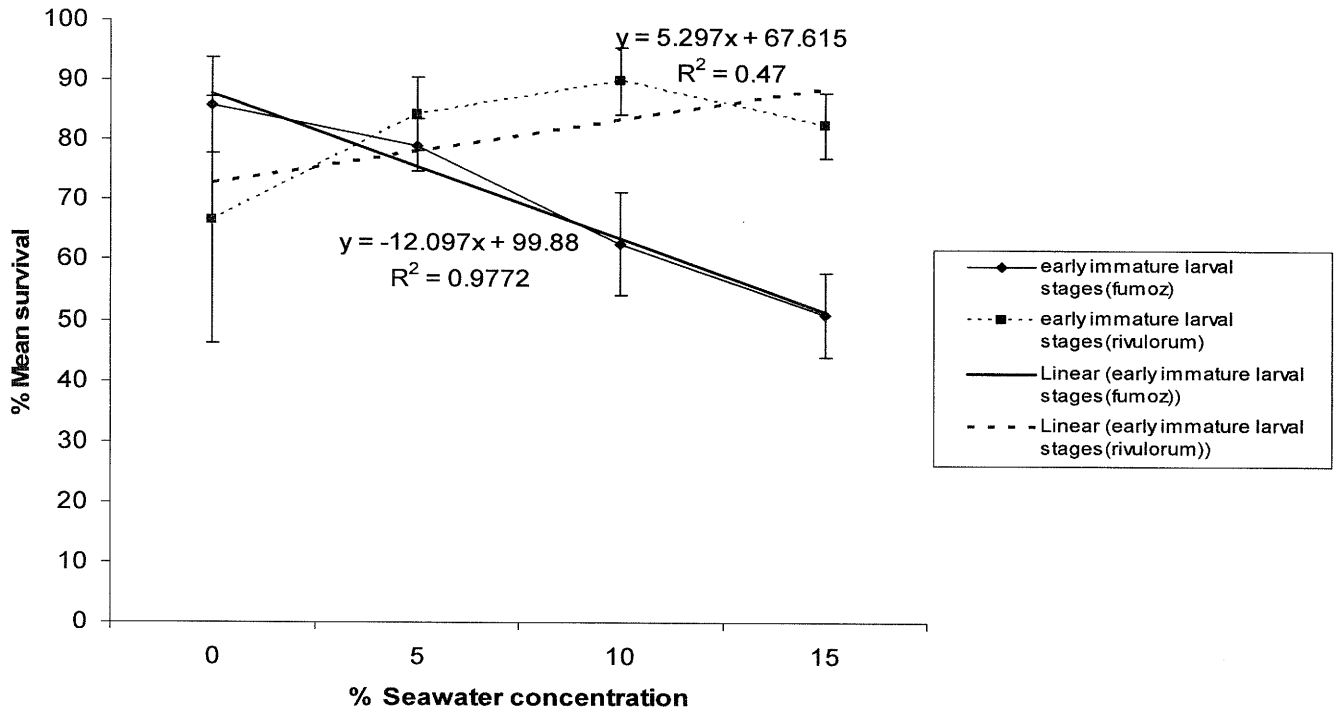

Fig 3.5 Percentage mean survival rates (± SE) of early immature larval stages of *An. funestus* (FUMOZ) and *An. rivulorum* exposed to the different seawater concentration. Survival rates were calculated by dividing the total number of all the 1st instar that survived to 3rd stage larval instar by the total number of first instar larvae for each concentration for the two species. Analysis of variance for *An. funestus* (P=0.02) and *An. rivulorum* (P=0.774).

3.2.3.2 Effect of salinity on late larval stages (3rd and 4th instar)

Survival rates were recorded for all the 3rdinstar larvae that developed through to the pupal stage (late larval stages) exposed to the different salt concentrations for the two species.

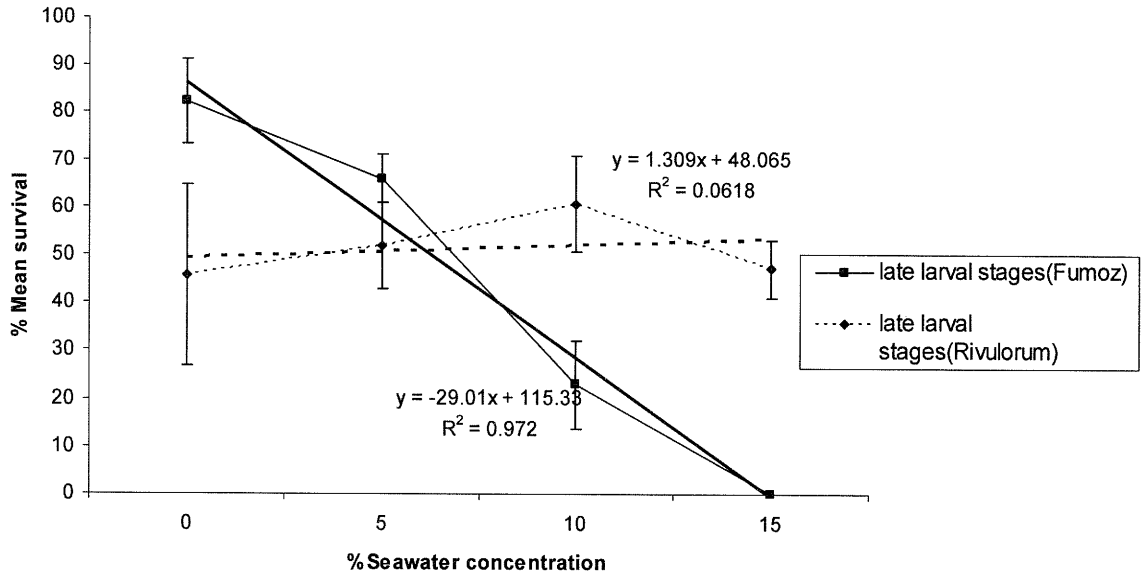

FIG 3.6 Percentage mean survival rates (± SE) of late larval stages of *An. funestus* (FUMOZ) and *An. rivulorum* exposed to the different seawater concentration. Survival rates was calculated by dividing the total number of all the 4th instar larva that survived to pupal stage by the total number of 3rd stage larval instar for each concentration for the two species. Analysis of variance *An. funestus* (P=0.001) and *An. rivulorum* (P=0.774).

The regression line data on Fig 3.6 above shows clear significant trends in late larval stages of *An. funestus*, increasing salt concentration decreases the larval survival to the pupal stage. This was observed in salt concentrations of 15% and above where the larvae died before reaching the pupal stage. There appears to be no significant trend in late larval stage for *An. rivulorum* at least up to 15% seawater, but higher concentrations were not tested.

3.2.4. Effect of salinity on pupal eclosion

The number of pupa surviving to the adult stage in this study was determined for each salt concentration.

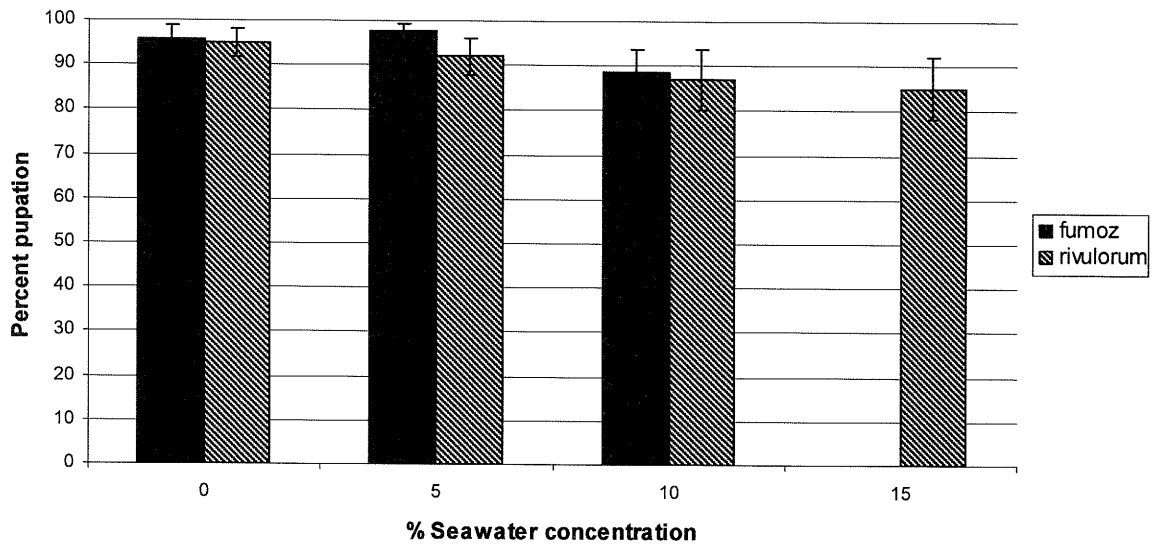

Fig 3.7 Percentage of pupae surviving to the adult stage of *An. funestus* (FUMOZ) and *An. rivulorum* (± SE) reared in different seawater concentrations. The percentage was calculated by dividing the adults emerged by the total number of pupae. Analysis of variance for two species exposed to different salt concentrations (P=0.477).

The graph in Fig 3.7 indicates that an increase in salt concentration does not significantly reduce the adult emergence once pupation has occurred. Pupal eclosion in both *An. funestus* and *An. rivulorum* was above 80% in all concentrations except the 15% concentration where the late larval stage did not survive to the pupal stage in *An. funestus*. Statistically, there is no significant difference in pupal eclosion in 0, 5 and 10% salt concentrations for both species.

CHAPTER FOUR

DISCUSSION

4.1 Baseline salinity tolerance using FUMOZ

Although a similar study on the effect of salinity on mosquito larvae was conducted on *An. funestus* on Mauritius by Jepson *et al.* (1947), these data were collected over 60 years ago and are not comparable with current data due to population variation over time. In the present study, a baseline salinity tolerance range was established using the FUMOZ strain from Mozambique, which borders the study area, Mpumalanga Province, and which was considered to be more appropriate than using the data from Mauritius. However, there was no difference in response to salinity between the two populations from the study conducted in Mauritius and laboratory colonized *An. funestus* from Mozambique.

4.2 Salinity tolerance of members of the *Anopheles funestus* group

4.2.1 Wild caught mosquitoes

The majority of the mosquitoes were collected at the three sites in Komatipoort. Irrigation schemes used for extensive commercial (sugarcane and banana) farming in the area provide abundant mosquito breeding sites. Data from the routine entomological surveys in Mpumalanga Province (Ngomane *et al.*, 2007) indicate that five out of the nine members of the *An. funestus* group are found in this region, namely *An. funestus s.s., An. rivulorum, An. vaneedeni, An. leesoni* and *An. parensis*. The dominant species within the *An. funestus* group found in this area is *An. rivulorum* with the other members (*An. funestus s.s, An. vaneedeni, An. leesoni* and *An.*

parensis) being present in low densities (Ngomane *et al.,* 2007). *Anopheles rivulorum* is the second most abundant and widespread Afrotropical species in the *An. funestus* group (Gillies & De Meillon, 1968, Gillies & Coetzee 1987). The present study confirmed the Ngomane *et al.* (2007) data.

The majority of mosquitoes were collected using night biting catches. Out of 23 window traps placed in the different localities only one mosquito was collected belonging to the *An. marshallii* group at Martien's farm. Exit window traps were considered a failure because local householders did not allow the traps to be set in the houses where they slept due to concerns for their safety with regard to criminals. Most of the window traps were therefore placed in unoccupied huts/houses and it is not surprising that no mosquitoes were collected by this method. Carbon dioxide baited net traps were second to human biting catches but both were affected by weather conditions - rain or wind adversely affects these methods.

Factors such as production of small egg batches and some females laying eggs that were not viable, contributed to the selection of the salinity range that was used for exposing wild material. A possible explanation for the production of small egg batches might be due to laboratory conditions not being ideal for such species compared with their natural environment and also not being adapted to guinea pig blood meals.

4.2.2 Effect of salinity on hatch rate

Mosquito eggs are found in a variety of different habitats such as rain pools, animal hooves, salt marshes, tree holes and other man-made containers. There is little information regarding the

study of the biology and ecology of mosquito eggs (Service, 1976). Female mosquito ovipostion site selection plays an important role in determining the larval survival (Bentley & Day 1989, Clements 1963, Roberts 1996). Some authors report contradictory laboratory findings on female oviposition site choice for determining egg eclosion and larval survival (Pappas & Pappas, 1983, Osborn *et al.,* 2006). Presumably female mosquitoes use sensory organs on their tarsi and palps to test salinities in nature. In this study, all wild females were placed in tubes containing distilled water for egg-laying and it is not known if fewer females would have laid eggs had they been given a choice of a range of salinities.

The effect of salt concentration on the hatch rate has not been studied for members of the *An. funestus* group. Although *An. leesoni* and *An. vaneedeni* were collected from the study area, females of these two species either laid eggs that were not viable or did not take blood meals. *Anopheles funestus* egg hatching was adversely affected by increasing salinity compared with *An. rivulorum* which showed a more or less similar hatch rate for all salt concentrations. These results are similar to those reported by *Bailey et al.* (1981) on *An. albimanus* Wiedemann from El Salvador, a freshwater breeding species that can tolerate some degree of salinity, but with the hatch rate decreasing with increasing salinity.

Comparison of time of hatch showed that more eggs hatched on day 3 for *An. funestus* with late hatching occurring in higher salt concentrations. Time of hatch for *An. rivulorum* showed more eggs hatched on day 4. High salinity in larval habitats might influence egg hatching for freshwater breeding species. In this study *An. funestus* hatch rate was drastically affected by increased salinity.

4.2.3 Effect of salinity on larval stages

Anopheles mosquito larvae are found in many different types of habitats. It is quite difficult to find larvae of members of the *Anopheles funestus* group due to their preference for swampy, vegetated habitats. Understanding the larval biology and ecology is crucial although most of the malaria control programmes target the adult stage.

This study showed that *An. funestus* larvae are less tolerant to salinity than *An. rivulorum*. An increase in salt concentration adversely affected the larvae surviving to the adult stage. These results are similar to Jepson *et al.* (1947) on Mauritius who noted that the full development to adult stage took place in 10% seawater and below. They noted that even in nature the larvae of this species were found regularly in no more than 7% seawater (0.2% of NaCl). It never occurred in brackish water.

Anopheles rivulorum shows a better tolerance to salinity. This species survived well in a variety of salt concentrations throughout the aquatic stages and presumably would survive well in a variety of natural breeding sites. Interestingly, while the hatch rate for *An. rivulorum* in 10% seawater concentration was lower than other concentrations, more adults emerged from this concentration. It would have been interesting to find the larvae of these species in nature and determine the salinity of these water bodies.

Further work could be carried out on the effect of salinity on the anal papillae of the fourth instar larvae of *An. rivulorum.* Coetzee and Le Sueur (1988) studied the effect of salinity on anal

papillae of *An. merus* larvae when raised in both fresh and saline water. They noted that when reared in freshwater the anal papillae increased in length to twice that of the larvae reared in saline water. It was not possible to carry out these observations in the present study due to lack of time.

Salinity tolerance of larvae has been used to distinguish saltwater from freshwater member species, particularly of the *An. gambiae* complex (Ribbands, 1944, Muirhead Thomson 1951, Sweeney 1987). This simple method might be used to aid identification in the field where resources can be limited.

4.2.3.1 Effect of salinity on early immature stages and late larval stages

The early immature larval stages for *An. funestus* were drastically affected by increased salt concentrations leading to fewer of them reaching the late larval stages. Increasing salt concentration for *An. rivulorum* did not affect the early immature larval stages. More late stage larvae were observed with increased salt concentrations for *An. rivulorum*. Osborn *et al.*, (2006) in their work on *Anopheles aquasalis* Curry, a salt tolerant species, found that larval mortality occurred mostly in the early immature larval stages (first and second instar).

There might be some physiological differences between the early and late larval stages due to their uptake of salts and water. The anal papillae of the late larval stages (third and fourth instar) might be well developed to deal with saline environments. Salt appears to have inhibitory effects on growth for early immature larva by reducing the food supply, while the growth of mature

larvae appears to be hastened in saline water (Graham, 1910). Larval growth in higher salt concentrations was delayed for *An. funestus* although no clear conclusion could be drawn in this study. For 202 eggs exposed in the highest seawater concentration (30%) for *An. funestus* 83 (41%) hatched into first instar larvae but died immediately within 24-48hrs after hatching (personal observation).

4.2.4 Effect of salinity on pupal eclosion

This study showed that increasing salt concentration did not affect the chances of adults emerging once pupation occurred. More than 80% of the pupa emerged as adults for both species. Since pupae do not drink or eat, their survival in a variety of habitats raises questions on how they deal with such environments since they lack the mechanisms used by the larvae (Bradley, 1987).

Sheplay and Bradley (1982) concluded that pupae are extremely impermeable and thus may survive in high concentrations of magnesium sulphate in 50% seawater while all the larvae were killed. In comparing the larvae and pupae Christopher (1960) reported that the larva cuticle is thin and flexible while that of the pupa is thick, highly sclerotized and covered by a waxy substance which makes it impermeable. Therefore it is evident that pupal osmoregulation depends more on passive resistance of ion uptake (Bradley, 1987).

CHAPTER FIVE

CONCLUSION

Members of the *Anopheles funestus* group are found in Afrotropical regions occupying a variety of habitats. Although this group consists of vectors and non-vectors of malaria, it is very important to understand the factors that affect the survival of their aquatic stages. This study focused on the salinity tolerance of the early stages for this group.

Due to the limited numbers of this group collected in Mpumalanga, only two species, *An. rivulorum* and *An. funestus* (FUMOZ strain) were used for this study.

The baseline salinity tolerance showed that there was a similar response in salinity for *An. funestus* from the Mozambique colony used in this study and work done in Mauritius. *Anopheles rivulorum* was the dominant species in the different collecting sites in Mpumalanga. Other members of the group were found in low densities. The objectives of this study were achieved with interesting results.

Egg hatching for *An. funestus* was adversely affected by increasing salt concentrations compared with *An. rivulorum* which was not affected. Similar patterns were also observed in larval survival for the two species. Increased salinity adversely affected the larvae of *An. funestus* while for *An. rivulorum* the larvae survived well in all concentration. This study showed that increased salinity does not affect pupal eclosion for both species.

Based on these results it is evident that *Anopheles funestus* is a freshwater breeding species since it showed little tolerance to salinity in all its aquatic stages. In comparison, *An. rivulorum* survived better in saline water in the early stages. Further investigations need to be done for the other species of the group, and also exposing *An. rivulorum* to higher seawater concentrations above 15%. The effect of salinity on anal papillae for *An. rivulorum* should be studied since it showed better tolerance to salinity. A better understanding of variations in salinity tolerance will provide some vital information on biology, ecology, planning for malaria control and rearing the other members of the group.

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