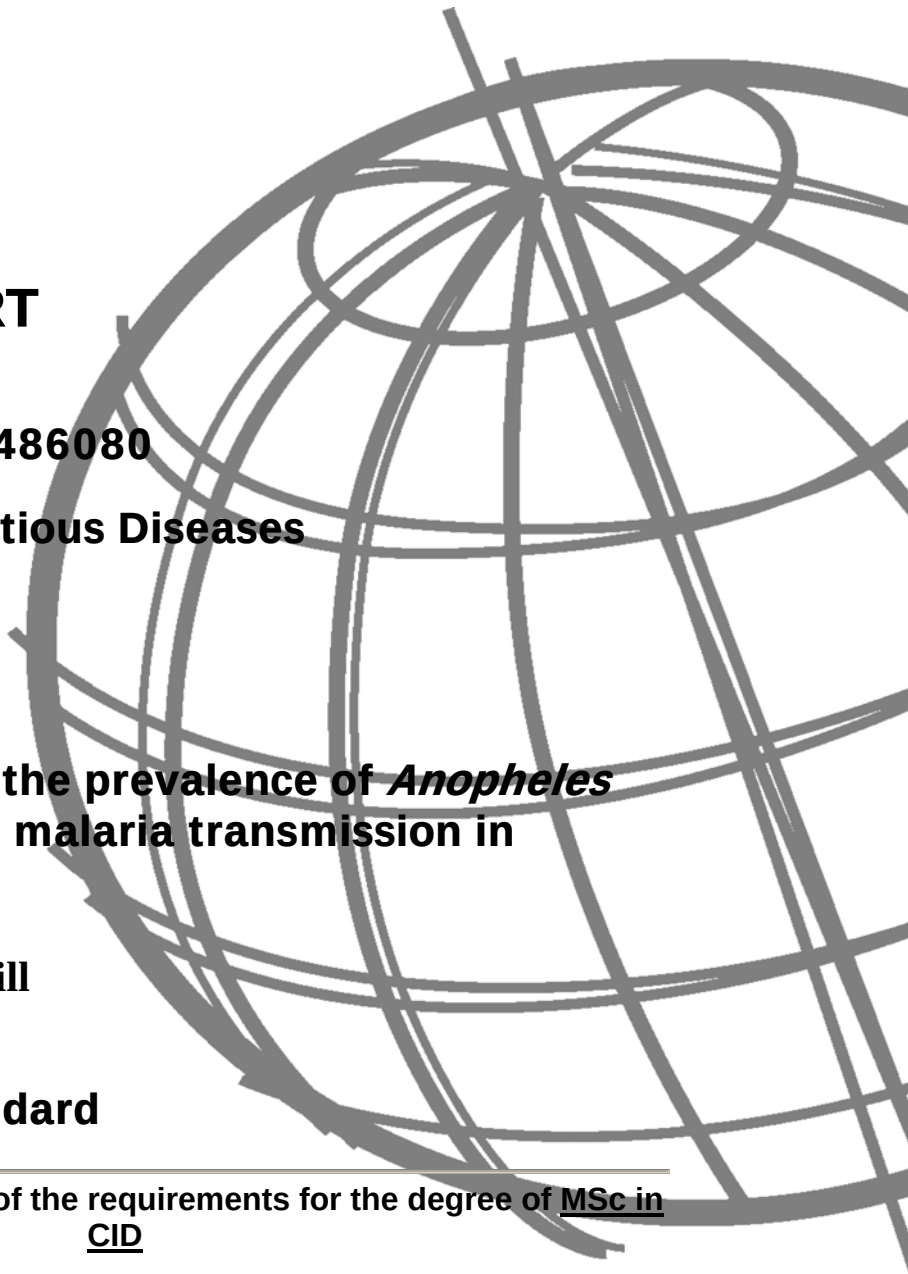


PROJECT REPORT

Candidate Number: 486080

MSc Control of Infectious Diseases

Title: A snap shot of the prevalence of *Anopheles merus* and its role in malaria transmission in Pemba

Supervisor: Dr. Nigel Hill
Word Count: 9,948

Project Length: Standard

Submitted in part fulfilment of the requirements for the degree of MSc in CID

For Academic Year 2007-2008

ACKNOWLEDGEMENTS

I am profoundly grateful to Dr. Sinead Fitzpatrick for her assistance, steadfast support and enthusiasm. I truly could not have than this without you. I will also like to extend my thanks to Dr. Quentin Bickle, Dr. Nigel Hill, Ms Shahida Begum, Dr. Bakar Khatib and the ZMCP. Lastly to the late Prof. Curtis for inspiring me and making this project available to me.

ABSTRACT

Background

Malaria is a serious public health problem in Pemba. It is holoendemic and thus affects mostly children and pregnant women. Following intense vector control activities in recent years, there has been a notable decline in malaria cases, presumably due to reduced populations of *An. gambiae* s.s. However, it was noted by Wastling (2007) that some areas in Pemba were supporting large populations of *An. merus*, a vector which the ZMCP recorded as absent in 2005 (ZMCP, 2007). It is probable that changes to vector population previously observed following vector control activities of the 1960's may be taking place. To ensure the continued effectiveness of the Pemban malaria vector control campaign, it is prudent to determine the relative proportion of *An. merus* (and other members of *An. gambiae* s.l.) in Pemba; to document where the larvae and adults are found and consider its potential impact in the transmission of *Plasmodium* and the implications for clinical malaria in the region.

Methods

Larvae were sampled from a diverse range of water habitats (41 sites) and ecological features of each larval site were recorded in order to investigate their relationship to species distribution and relative abundance. CDC light traps were used to collect host seeking mosquitoes from households and a goat shed neighbouring suspected *An. merus* breeding sites. A PCR assay was used to identify the specimens collected.

Results

Ribosomal DNA (rDNA) analysis was performed on a total of 216 larvae from 19 populations. 120 larvae were positively assigned to 3 species: *An. merus* (50%), *An. arabiensis* (5%) and *An. quadrannulatus* (1%). Whilst 96 larvae (44%) remained unidentified. Of the 5 mosquitoes collected 2 were identified as *An. merus* and 3 were unidentified. *An. merus* dominated in brackish-waters, showing great plasticity in its choice of larval habitat. However its relative abundance decreased at high altitudes.

Conclusion

The species composition of the *An. gambiae* complex appears to be undergoing the same changes as observed during the spraying campaigns of the 1950s and 60s. The occurrence of endophilic members of the *An. gambiae* complex have reduced significantly whilst the exophilic members; (mostly) *An. merus*, *An. arabiensis* and *An. quadriannulatus* thrive. However the role of this species in malaria transmission cannot be commented upon due to limited sampling results.

Abbreviations

ACT	Artemisinin Combination Therapy
AFM	Africa fighting malaria
ANC	Antenatal Care
AQ	Amodiaquine
AS	Artesunate
COX1	Cytochrome Oxidase 1
CSA	Circumsporozoite antigen
DDT	Dichloro-diphenyl-trichlorethane
DNA	Deoxyribonucleic Acid
DOT	Directly Observed Therapy
GOZ	Government of Zanzibar
GPS	Global Positioning System
HBI	Human blood index
IPT	Intermittent preventive treatment
IRS	Indoor residual spraying
ITN	Insecticide treated net
LLIN	Long lasting insecticide treated net
LSHTM	London School of Hygiene and Tropical Medicine
PCR	Polymerase Chain Reaction
RBM	Roll Back Malaria
rDNA	ribosomal DNA
RDT	Rapid diagnostic test
USAID	United States Agency for International Development
WHO	World Health Organisation
ZMCP	Zanzibar Malaria Control Program

CONTENTS PAGE

1. INTRODUCTION

1.1. Zanzibar

The Zanzibar archipelago comprises two large islands; Unguja and Pemba, and several smaller islands, all of which are located off the north-eastern coast of the Tanzania mainland. The Revolutionary Government of Zanzibar was founded in 1963, following independence from Britain. Later, its union with The United Republic of Tanganyika led to the formation of The United Republic of Tanzania.

1.2. Malaria in Zanzibar

Malaria has been a serious public health problem in Zanzibar for the past 50 years. The disease is characterised by perennial stable transmission with children under five and pregnant women, who represent 20% and 4% of the total population respectively being most affected. It is estimated that the former group experiences 4-6 episodes of febrile illness (an indicator of malaria) per annum. Overall about 100% of the population inhabits areas at risk of malaria (RBM, 2008; ZMCP, 2007; AFM, 2008).

The main determinants of malaria in Zanzibar include the favourable climatic conditions for the parasite and vectors, socio-cultural factors and poverty.

Plasmodium falciparum is the main malaria species accounting for 97% of all malaria infections (RBM, 2008). *P. malariae* and *P. ovale* are also found in small numbers in Pemba.

In recent years the malaria burden has been significantly reduced. Indeed the disease is said to be under control for the first time since 1968. One aim of the present study

was to review the current status of the malaria control programme records during a visit to the Zanzibar Malaria Control Programme (ZMCP). This data was obtained from ZMCP reports and more recent unreported data.

1.3. Review of malaria control records

Presently the strategy adopted by the ZMCP falls into two categories:

1) Case management involving the use of Artemisinin Combination Therapy (ACT) (amodiaquine (AQ) and artesunate (AS)) as firstline treatment and quinine for treating severe malaria and malaria in 1st trimester of pregnancy (with AS and AQ being used in 2nd and 3rd trimester).

2) Prevention comprising of vector control activities (insecticide treated nets (ITNs), long lasting insecticide treated nets (LLINs), re-treatment of untreated nets and indoor residual spraying (IRS)**;** early diagnosis and prompt treatment; intermittent preventative treatment (IPT) with sulphadoxine-pyrimethamine; surveillance and epidemic preparedness.

Case Management

Zanzibar adopted ACT as its 1st line drug following the development of chloroquine resistance (up to 60% treatment failure). ACTs are supplemented by supportive drugs such as diazepam and glucose. Treatment is supplied through all public health facilities and administered free of charge to patients with confirmed malaria.

An ancillary to this policy change is the recent adoption of microscopic diagnosis and rapid diagnostic tests (RDTs). RDTs first piloted in 2005 are now administered to anyone under five suspected of having malaria. Additionally the following strategies

have been adopted to improve case management: DOT system strengthening in all facilities, mixing of antimalaria medicine at health facility in order to improve proper dose, malaria based home care and equitable distribution of resources.

The current adopted strategies have lead to improvement in malaria case management (please see graphical representation below)

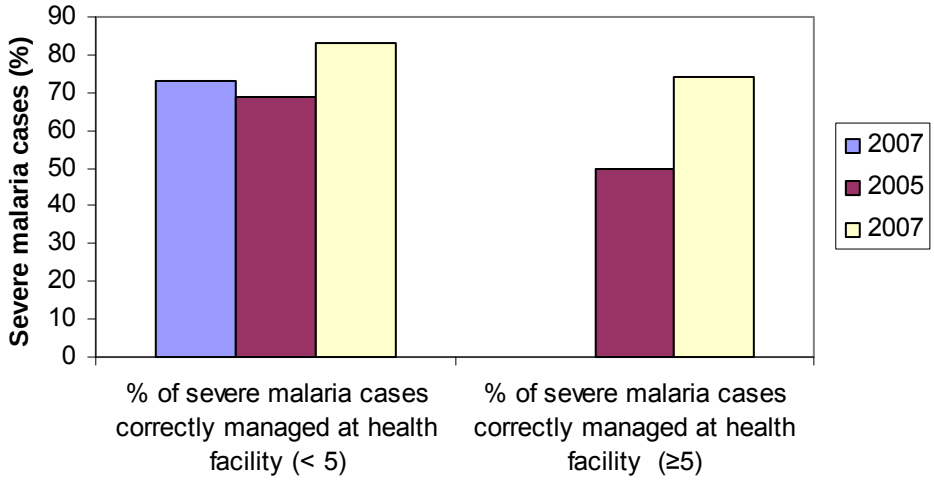
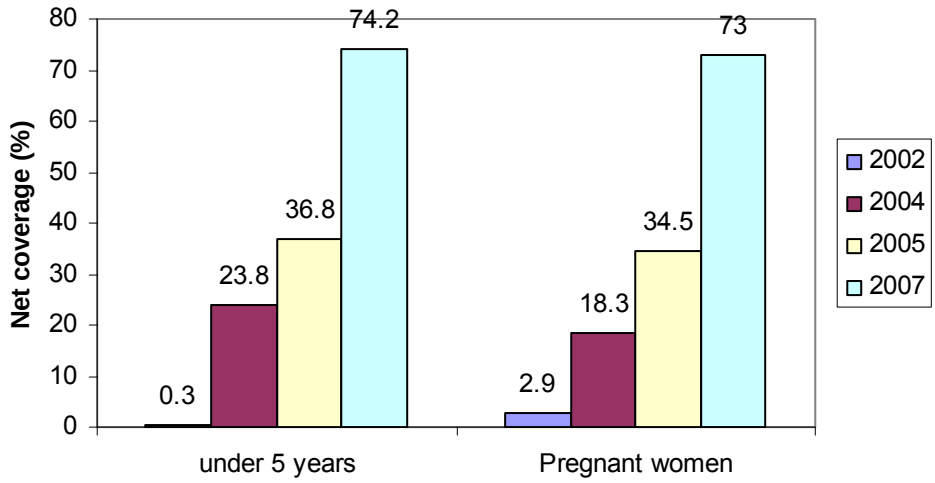


Figure 1. In Patient Department management of severe malaria cases in children < 5 and ≥5 in 2002, 2005 and 2007**.**

Also progress has been made in the scaling up of ITNs and IRS (see fig 2 and 3 and section 1.3 for detailed description of vector control programme in Zanzibar)



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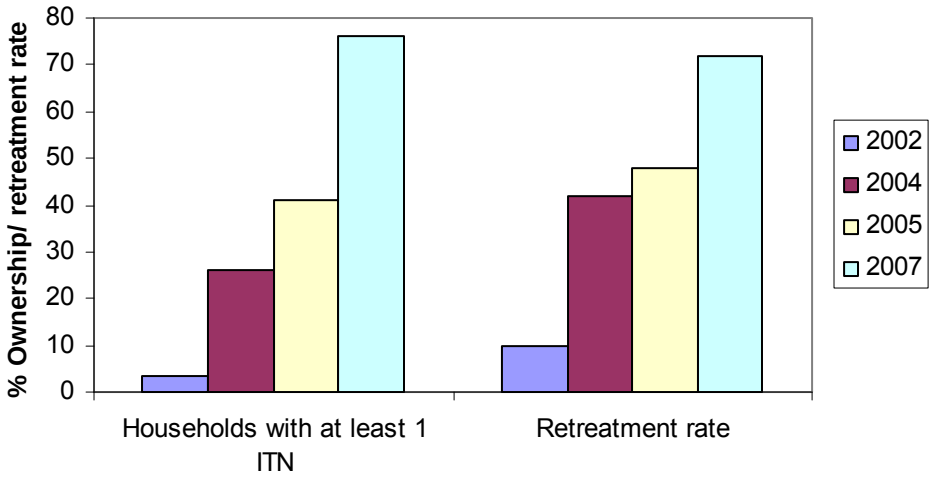


2b)

Figure 2: **2a.** Net coverage in children <5 and pregnant women who sleep under ITNs/LLINs; **2b**. LLINs/ITNs household ownership and conventional net re-treatment rate

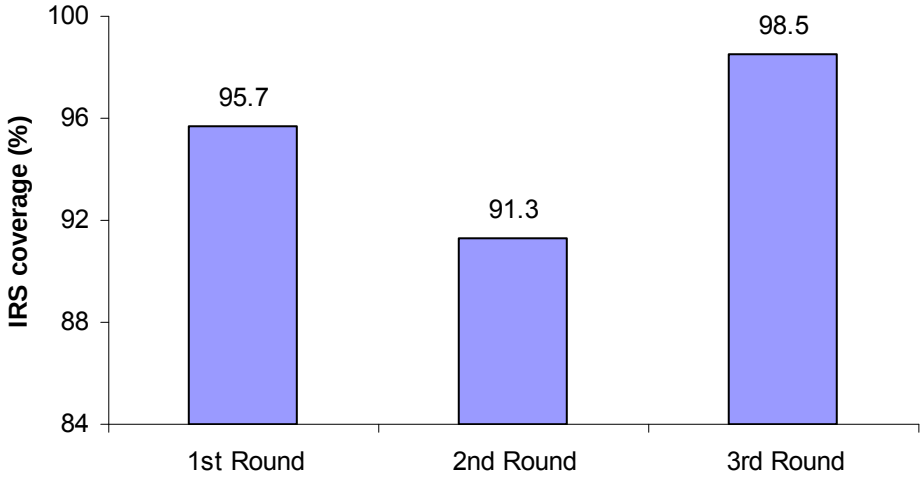


Figure 3: IRS coverage between 2006 and 2007

The introduction of Artemisinin Combination Treatment and the general improvements in case management coupled with the intensification of vector control activities have lead to great successes in malaria control (see fig. 4). In 2003 the ZMCP documented malaria rates (probable and parasitologically confirmed) of 351 per 1000 which decreased to 42 malaria cases per 1000 in 2007. Furthermore, prevalence surveys of malaria parasitaemia conducted between 2003 to 2007, convey reductions in parasitaemia of up to 90% (ZMCP, 2007).

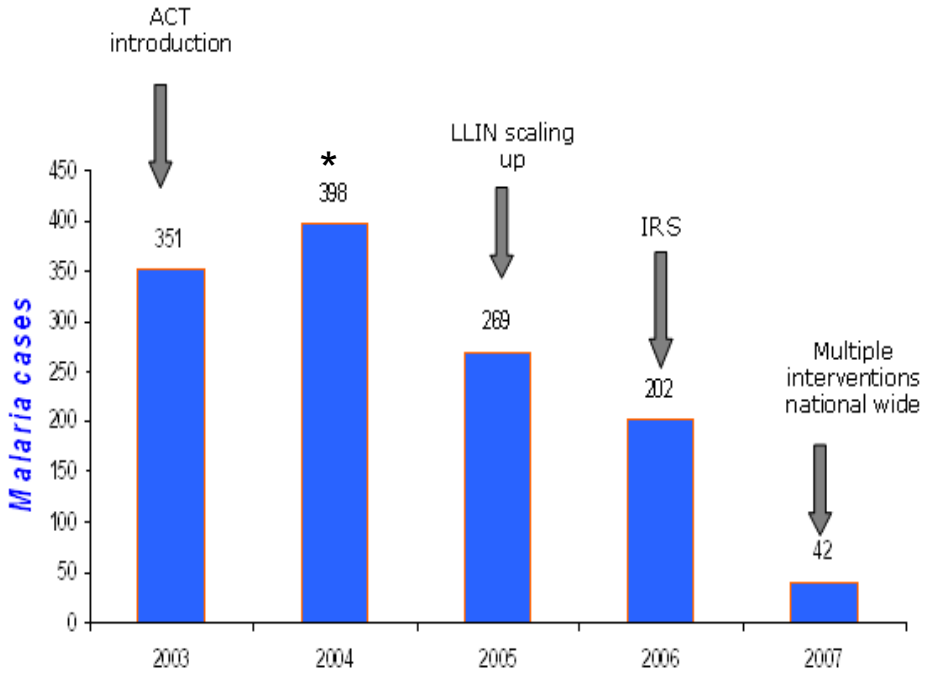


Figure 4. Out Patient Department Malaria Probable Cases (confirmed and unconfirmed) per 1000 of the population taken from RBM, 2008.

* No intervention introduced in 2004

However this new found success brings new challenges for the ZMCP. This is due to the shift from holo-endemicity to hypo-endemicity. The lowered exposure to *P. falciparum* resulting from this means that natural immunity acquired in early childhood and latter years is likely to decrease, making future generations more susceptible to malaria epidemics. Consequently there is great debate now on how best to maintain control, improve surveillance to prevent future epidemics and make certain that holoendemic malaria does not resurface in Zanzibar as it did in the 1970s.

1.4. A history of vector control and vector populations in Zanzibar

Historically, *Anopheles gambiae* sensu lato (s.l.) and *Anopheles funestus* have been the primary malaria vectors on Unguja and Pemba. Documentation of the vectors dates as far back as the 1930s. McCarthy (1941) during 1934 and 1937 found both species regularly resting indoors with high sporozoite rates. Equally Iyengar (1962) in 1958 discovered substantial populations of *An. gambiae* s.1. resting indoors and large numbers of its saltwater variant in coastal areas. To date, four of the seven sibling species of the *An.* gambiae complex have been recorded in Zanzibar: *An. gambiae* s.s., *An. arabiensis, An. quadriannulatus* and *An. merus.*

Vector control in Zanzibar spans three decades. In 1958, as a part of the World Health Organisation campaign for the global eradication of malaria (in 1957) indoor residual spraying was implemented in Zanzibar. Four rounds of dieldrin were administered between 1958 and 1961, followed by biannual spraying until 1968. The programme produced remarkable results in the incidence of malaria; overall parasite rates fell to 1.6% in Pemba and it was observed by Iyengar (1962) that *An. gambiae* s.l. became undetectable in houses and fresh water breeding sites. Conversely, the Pemban mangrove swamps were supporting large populations of the salt water breeding highly exophilic species *An. merus.* Between 1964 and 1967 the very endophilic *An. gambiae* s.s was not observed in both islands (Iyengar, 1962; Odetoyinbo & Davidson, 1968). It seems that not only did the spraying campaign affect the density of *Anopheles* but also altered the structure of the *An. gambiae* species complex such that the highly exophilic and zoophilic *An. merus* became dominant on Pemba and the less endophilic *An. arabiensis* became prevalent in Zanzibar. In Zanzibar *An. arabiensis* was accredited with the remaining transmission although behaviouristic resistance meant

that it was more exophilic and zoophilic (Odetoyinbo and Davidson, 1968). Additionally Curtis and Mnzava (2000) report that *An. funestus* which is entirely endophilic became barely detectable. In 1968 when the spraying programme was halted, malaria morbidity trends increased rapidly thus holoendemic levels returned (Curtis and Mnzava, 2000) and an ensuing reversal in species composition was observed in Unguja with *An. gambiae* s.s once more becoming dominant (Mnzava and Kilama, 1986). It is logical to believe that the same scenario occurred on Pemba.

Indoor residual spraying was restarted in the early eighties as a joint initiative between the United States Agency for International Development (USAID) and the Government of Zanzibar (GOZ). DDT and Malathion were used in Pemba as well as a more reliable supply of antimalarial drugs. The project ended in 1989 due to logistical problems, infrequent supplies of insecticide and drug resistance. Then onwards, malaria control activities were limited until 1997 when Zanzibar became one of the first countries to put into action the Accelerated Malaria Control Programme; a WHO initiative. In 2000, Zanzibar as a part of the United Republic of Tanzania became a signatory to the Abuja Declaration which is a pledge to make ITNs and LLINs universally accessible to the Zanzibari population. ITNs were formerly introduced in Zanzibar in 2002 and they have been provided at a reduced price to the public since 2004. Also since 2005 free LLINs have been distributed to vulnerable groups including pregnant women and children under five through antenatal care (ANC) facilities. As of 2007 the price of LLINs for vulnerable groups has been subsidized with a voucher scheme. But the continued use of conventional untreated nets remains high whilst re-treatment rate remains inconsistent in some areas consequently there

are free mass re-treatment campaigns to address this (RBM, 2008; ZMCP, 2007; AFM, 2008).

ITN coverage has increased significantly from 0.3% and 2.9% in 2002 to 74.2% and 73% in 2007 in under fives and pregnant women respectively (ZMCP, 2007). The ZMCP has set a target of 90% ITN or LLIN coverage in pregnant women and under fives by 2010 (ZMCP, 2007).

Recently the GOZ launched a comprehensive IRS program to complement the existing ITN and treatment efforts. As of 2006 there have been three rounds of spraying in Pemba using lamdacyhalothrin, the pyrethroid insecticide used in treating ITNs. Over 90% of households have been reached in the campaign (ZMCP, 2006; AFM; 2008).

Prior to the IRS activities, an entomological survey was conducted to obtain baseline data to assist in measuring the impact on malaria transmission in Zanzibar. In Pemba the following species were recorded; *An. gambiae* s.l., *An. funestus* and *An. coustani* with *An. gambiae* s.s and *An. arabiensis* predominating. Preliminary results in the post-spraying period showed a significant fall in man-vector contact; from 4.33 and 4.50 to 0.54 and 0.20 bites per person per night in Unguja and Pemba respectively (X^2 = 15.64, P<0.0001) (ZMCP, 2006). However, further attempts to collect entomological data following the above surveys has been impossible, consequently the ZMCP has not been able to monitor vector response to insecticide selection pressure and changes in behaviour. As of 2007, entomological monitoring activities have resumed but information on composition of the current vector species and

behaviour is still lacking. However, Wastling (2007) that some areas in Pemba were supporting large populations of *An. merus*, a vector which the ZMCP recorded as absent in 2005 (ZMCP, 2007). It is probable that changes to vector population previously observed following the vector control activities of the 1950's and 60's may be taking place.

To ensure the continued effectiveness of the Pemban malaria vector control campaign, it is prudent to determine the relative proportion of *An. merus* (and other members of *An. gambiae* s.l.) in Pemba; to document where the larvae and adults are found and consider *An. merus'* impact on the transmission of *Plasmodium* and the implications for clinical malaria in the region. It is proposed to do this in the present project.

1.5. The *Anopheles gambiae* complex: biology, distribution and mating relationships

Among the Afrotropical mosquitoes, the *Anopheles gambiae* species complex (Diptera, Culicidae) includes some of the most efficient vectors of human malaria (Bass *et al.,* 2008). Four of the seven sibling species are freshwater species: *An. gambiae sensu stricto*, *An. arabiensis, An. quadriannulatus* species A and B; one a mineralwater species: *An. bwambe*; and two are saltwater associated species: *An. merus* and *An. melas* (White, 1974; Hunt *et al.*, 1998; Coetzee, 2004; Besansky *et al.,* 2006).

Up to four members of the complex may be sympatric in some regions, and at least two occur in most malaria endemic zones (Coluzzi *et* al., 1979; Gale & Crampton, 1987; Scott *et al.*, 1993; Favia *et al.*, 1997; Bass *et al.,* 2007). The most

anthropophilic members of the complex are *An. gambiae* s.s and *An. arabiensis* and they are the most widespread in distribution. However, their proportions within a given area is a function of climatic factors with *An. gambiae* s.s predominating in high humidities and *An. arabiensis* showing a propensity to increase in arid zones (White, 1974; Lindsay *et al.,*1998). Also *An. arabiensis* has the ability and tendency to lean towards exophilic, exophagic and zoophilic behaviours a feature stemming from its numerous chromosomal inversion polymorphism which endows it with immense ecophenotypic plasticity (White, 1974; Tirados, 2006). These behaviours are also commonly observed in *An. quadriannulatus* species A which is more restricted in its distribution, occurring mainly in Zanzibar and southern Africa (White 1974; Pates *et al.,*; 2001; Fettene *et al.,* 2002). Similarly the markedly zoophilic and exophilic *An. merus* is confined to East Africa where it occurs in coastal areas and often mingles with *An. gambiae* s.s and *An. arabiensis* (Gale & Crampton, 1987; White, 1974). Its occurrence inland has also been well documented (Gillies & Coetzee, 1987). *An. bwambae* only occurs in the geothermal springs located within the Semuliki National Park of Bwamba County, Uganda (Charalambous *et al.,* 1999). However Besansky *et al.,* (2006) noted that the species may be more prevalent in eastern Africa than presently realized. *An. bwambae* behaves exophagically with many non-human hosts (Coluzzi *et al.*, 1979).

Generally the freshwater breeders are quite similar in their choice of larval ecology whereas the saltwater forms show great plasticity. Overall though shallow open sun-lit, slow flowing, clear water is the most preferred larval habitat. Such pools include: borrow-pits, drains, brick-pits, car-tracks, hoof-prints; pools resulting from: overflowing rivers, rainwater collecting in natural depressions and so on. Human

activities (directly or indirectly) contribute a great deal to the formation of these sites. The partial drainage of permanent or seasonal swamps for agriculture has been shown to provide plentiful breeding-sites. Flooded or partly flooded rice fields constitute prolific breeding grounds for freshwater *An. gambiae* species. Other water habitats include: irrigation channels, edges of swamps, stream edges and so on (Gillies & De Meillon, 1968; Coluzzi *et al.*, 1979; Gillies and Coetzee, 1987; Shililu *et al.*, 2003).

An. merus breeds not only in freshwater but brackish water although the latter is most favoured (Gillies & De Mellion, 1968; Mosha & Mutero, 1982, Tsy *et al.*, 2003). It commonly occurs around the belts of *Avicennia* mangroves and *Paspalum* sedge, brackish lagoons, ponds and swamps. Such waters are often dark with a high level of organic pollution (Muirhead-Thompson, 1951a; White, 1974). In Pemba it has been found in pools and puddles which were flooded at high spring tides and subsequently diluted by rainfall or seepage from land (Iyengar, 1962).

Altitude often dictates the abundance of *An. gambiae* s.l.. Shililu *et al.,* (2003) noted that the densities of *Anopheles* larvae (including *An. arabiensis*) was higher at high altitude zones where fairly high precipitation leads to the formation of many stream puddles. Kulkarni *et al.,* (2006) however noted the opposite with *An. arabiens*is density decreasing at high altitudes. They attributed this variation to topography and climatic factors (rainfall and temperature).

Male progeny of interspecific crosses between members of the complex produce sterile male hybrids whilst females are fertile. However in certain crosses few or no females are produced thus making the reproductive impediment near complete

(Gillies & De Mellion, 1968; White, 1974). Crosses do occur in both adult and rather less often larval forms (Davidson, 1964; Gillies & De Mellion, 1968; White, 1974; Coluzzi *et al*, 1979).

1.6. Disease transmission capabilities of *An. merus* and 3 freshwater species of the *An. gambiae* complex.

Generally the consensus on *An. merus* is that it is not of primary medical importance as *An. gambiae* s.s and *An. arabiensis.* This is due in part to its preference for domestic animals. Where animals are present it would feed solely on them, showing a partiality for cattle. In Pemba where animals are tied near swamps all night, *An. merus* is exclusively non-domestic. Iyengar (1962 ; 1972 (cited in White,1974)) recorded from bloodmeal analyses of outdoor-resting *An. merus* females a Human Blood Index (HBI) of 1.7% with bovids being the foremost food source. When the preferred host is absent *An. merus* readily bites man both indoors and outdoors. However sporozoite rates in the species remains lower than *An. gambiae* s.s and *An. arabiensis* (Gillies & DeMellion, 1968; White, 1974).

Despite the large population of *An. merus* in Pemba following vector control activities of the 1950's, the parasite rate documented was less than 2% thus cementing *An. merus* as an inefficient vector of malaria in Pemba (Odetoyinbo & Davidson, 1968). Others like Bushrod ((1981) in Tanzania) and Mosha and Petrarca ((1983) in Kenya) have however argued contrarily to this view. Recently Temu *et al* (1998) found that *An. merus* played an important role in malaria transmission along coastal Tanzania where the occurrence of both *An. merus* and *An. gambiae* s.s was identical and their circumsporozoite antigen (CSA) rates were comparable (11.6% and 12.5%

respectively) and higher than those of *An. arabiensis* (7.7%) and *An. funestus* (4.6%). Equally in Madagascar, Tsy *et al.,* (2003) recorded for the very first time evidence of *An. merus's* role in malaria transmission. The species is now on the list of malaria vectors in Madagascar. Overall though observations of the abundance and sporozoite rates in *An. merus* is inadequate to fully appreciate its role in the transmission of malaria (Temu *et al.,* 1998).

The medical importance of *An. arabiensis* and *An. gambiae* s.s has been well documented. However even within this highly efficient duo there is some disparity with the predominantly endophilic and anthropophagic *An. gambiae* s.s being a more efficient vector. HBI in this species may reach 100% when man is the only available host. Its only when *An. gambiae* s.s is absent that *An. arabiensis* dominates fully (White, 1974). Within this species there seems to be an east-west behavioural cline with *An. arabiensis* feeding more readily on humans (HBI 80-100%) indoors in West Africa whilst feeding mainly on cattle, outdoors in East Africa (White, 1974; Fontenille *et al.*, 1997; Tirados *et al.*, 2006). In Pemba (2005) HBIs of 100% was recorded in *An. gambiae* s.l. with *An. gambiae* s.s. predominating at 95% and *An. arabiensis* at 4%. No bloodmeals from domestic animals was recorded (ZMCP, 2007). Furthermore in East Africa parasite infection rates of 4.37 ± 0.71 and 0.32 ± 0.16% has been documented in *An. gambiae* s.s and *An. arabiensis* respectively (White *et al.*, 1972; Tirados *et al.*, 2006).

An. quadriannulatus species A, historically has been considered a non-malaria vector as it is entirely zoophilic and this is the case in Pemba (White, 1974; Pates *et al.*, 2001; ZMCP, 2007). Although Pates *et al.,* (2001) obtained results that demonstrated antropophagic behaviour previously not seen in this species.

1.7. Identifying members of the *Anopheles gambiae complex*

Members of *the An.* gambiae complex are isomorphic and so cannot be reliably differentiated using morphological characteristics. They vary substantially in their behaviour, ecology and vectorial capacity and respond dissimilarly to control measures thus posing a great challenge for malaria control programmes. Identification of species within a given area therefore remains elemental for any epidemiology study and control programme (Scott *et al.,* 1993; Coetzee *et al.*, 2000; Bass *et al.*, 2007).

In addition to the mating incompatibilities shown through cross mating techniques other methods that have been used in identifying species of singular specimen within the *An gambiae* complex includes allozyme analysis, polytene chromosome banding patterns, high performance liquid chromatography of cuticular hydrocarbons and hybridization with DNA probes (Gale & Crampton, 1987; Scott *et al.,* 1993). Salt water tolerance is also a proven basis for separating freshwater from saltwater types (Iyengar, 1962). However a limitation of all these methods is their impracticability when it comes to their comprehensive use in epidemiological studies and vector control programmes. Currently a better alternative involves the use of a standard polymerase chain reaction (PCR) assay (Fanello *et al.,* 2002; Walker *et al.,* 2007) such as the one developed by Scott *et al.,* (1993) for the identification of five of the most widespread species (i.e. excluding *An. bwambae* and *An. quadriannulatus* B). This method uses diagnostic primers which attach to species-specific nucleotide sequences in the ribosomal DNA (rDNA) intergenic spacer region. Together with a universal primer that binds to a homologous region of all five species, the assay produces DNA fragments that differ sufficiently in size to allow unambiguous differentiation by gel electrophoresis. Selection of rDNA for this purpose is optimal

because it occurs in hundreds of tandem arrays so only a small part of an individual can provide enough template for PCR amplification. Also it contains highly conserved, evolutionarily labile regions which vary between very closely related species thus enabling the identification of appropriate sequences for designing both universal and species-specific primers. Lastly intraspecies variation is generally less in rDNA sequences than single copy loci. The assay can be used to identify species irrespective of the life stage. Segments of mosquitoes may be added directly to the reaction mixture although extracted DNA offers more sensitivity (*Scott et* al., 1993). The ZMCP in a recent entomological survey (in 2005) on Pemba and Unguja used this method to identify 76 species from 5 sentinel sites: 72 of the species identified were *An. gambiae* s.s ; 3 were *An. arabiensis* and 1 *An. quadriannulatus* (ZMCP, 2007).

2. AIMS AND OBJECTIVES

2.1. Aims

To review data recorded by the ZMCP and assess the impact of recent vector control activities on malaria control in Pemba. To conduct a preliminary survey of the distribution and relative prevalence of *An. merus* compared to other members of the *An. gambiae* complex and to assess the role of *An. merus* in the transmission of malaria in Pemba.

2.2. Specific objectives:

1. To obtain data during placement in Pemba from records kept by the ZMCP (see review achieved in section 1.3) and assess the impact of IRS and ITNs on malaria control in Pemba.

2. To determine the species composition of *An. gambiae* s.l. immatures in >50 different freshwater and brackishwater sites and adults from 10 households and livestock sheds.

3. To relate larval species distribution and relative abundance to larval habitat salinity, using correlations of tested salinity in natural habitats to establish whether *An. merus* is occupying new less saline niches and if there is an overlap in suitable habitats for *An. merus* and other members of the *An. gambiae* complex.

4. To utilise the above information to explore what impact *An. merus* may be having on *Plasmodium* transmission and the implications for clinical malaria and future vector control activities of the ZMCP.

MATERIALS AND METHODS

3. Study Area

This study was carried out in Pemba an island of the Zanzibar archipelago. It is located between 5° – 6° S and 39° - 40° E and has a land area of about 984 km^2. Pemba has pronounced hills and valleys and and its climate is tropical and humid. Humidity ranges from 50 to 80% and average maximum temperature during the hot season of December to March is 30° C. This decreases to about 20° C during the cool season. Pemba is characterised by 2 distinct rainy seasons; heavy rains (Masika) of March and May and the shorter rainy period (Vuli) from October to December. It is during Masika that *An. gambiae* s.l. is most abundant (ZMCP, 2007; ZMCP, 2007). Administratively Pemba is separated into 4 districts, Michenweni, Wete, Chake Chake and Mkoani. Each of these districts is further separated into constituencies and Shehias.

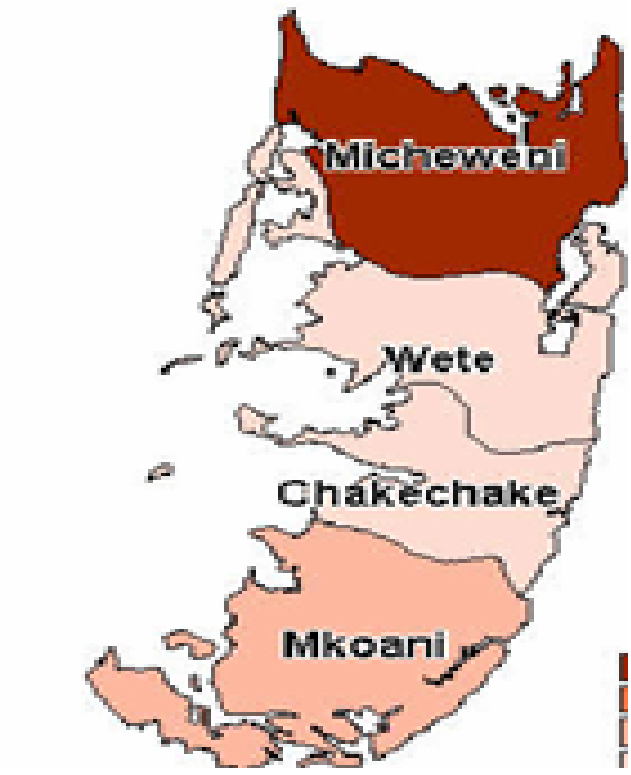


Figure 5. Map of Pemba

3.1. Sampling

3.1.1. Larvae

Sampling of *An. gambiae* s.l. immatures was conducted between June 2008 and July 2008. A total of 41 sites from the 4 districts of Pemba were investigated (see appendix 1 and 2). Sites were primarily distributed on the east coast of Pemba, along a north to south transect (with some scattering on the west). Sampling sites were chosen using the following criteria: 1) accessibility 2) suitability of sites for *Anopheles* breeding (as attested by the knowledge and experiences of the ZMCP) 3) a need to corroborate findings of previous studies on the distribution of *An. merus* in Pemba and 4) sites covered a range of altitudes, from ≤ sea level (≤ 0 metres) to the highest elevation.

Anopheles larvae were collected, using dippers, from a diverse range of water habitats including rice fields, mangrove swamps, streams and salt production sites. *Anopheles* larvae were separated from culicine using a key (from LSHTM lecture materials) and transferred into universals containing 80% ethanol for preservation for DNA analysis. All tubes were labelled by date, population identity number and the Shehia of larval habitat. A collection site was considered negative if no larvae were encountered after 30 minutes of dipping.

Ecological features of each larval site were recorded in order to investigate their relationship to species distribution and relative abundance. The water salinity was measured using a portable refractometer (by Aquarium solution). The metre was

calibrated each morning to ensure accurate readings. The altitude and the coordinates of each larval habitat were recorded with a handheld GPS (eTrex by Garmin).

3.1.2. Adult Mosquitoes

Using a CDC light trap, host seeking mosquitoes were collected from mud and thatch huts in five villages neighbouring suspected *An. merus* breeding sites. Collections were made from a total of 10 households corresponding to the larval collection sites: PE14, PE21, PE10, PE1 and PE25. An untreated net was purchased for all participating households and homeowners were instructed to sleep under the net on the night of the collection. As exhaled carbon dioxide is a known host attractant, a CDC light trap was placed at the head of the bed of each participating household (see fig. 6a). In order to sample zoophillic species an additional CDC light trap was set up at a goat shed of one of the participating households (see fig. 6b). The shed (and the goat) were protected overnight with an untreated net. All light traps were switched on at 6.00pm and mosquito catches were retrieved early the next morning at 6.00am. The trapped mosquitoes were killed, thoroughly dried and separated by species and sex using a key (from LSHTM lecture materials). *Anopheles* females were placed in eppendorf tubes with silica gel and preserved for DNA analysis. Each tube was labelled as described above. The gravid and blood-fed status of each *Anopheles* female was noted.

6a) **6b)**

Figure 6: 6a. Set up of human baited trap with CDC light traps; 6b. Set up of animal baited trap with CDC light trap.

3.2. Molecular identification of Anopheles gambiae s.1. larvae and mosquitoes

As a part of the preliminary survey of larval breeding sites in Pemba, individual *Anopheles* larvae were analysed using a polymerase chain reaction (PCR) assay adapted from Scott *et al* (1993). This assay allows for the identification of the five most widespread species of the *An. gambiae* complex including *An. gambiae* s.s, *An. merus, An. arabiensis* and *An. quadriannulatus* species A. The assay uses diagnostic primers, which attach to species-specific nucleotides in the intergenic spacer region of ribosomal DNA (rDNA), together with a universal primer that binds to a homologous region common to all five species. The assay produces DNA fragments that differ sufficiently in size to allow unambiguous differentiation by gel electrophoresis. This

protocol was selected as recent entomological surveys and past vector population history indicate that the above vectors do occur in Pemba**.**

A total of 19 populations, representing a variety of the habitats, geographical, saline and altitudes ranges of sampled sites, were selected for analysis. Among the specimens assayed were larvae collected from rice fields, ditches, marshy depressions, salt production sites; mangrove swamps; waterlogged fields and streams. An average of 11 specimens were analysed from each population (see Table 1 (and appendix 3 for a more detailed table) and fig 7).

DNA was extracted as follows (1) using a sterile tip as a disposable pestle, individual larvae and mosquitoes (minus the abdomen) were ground up in sterile eppendorfs, 50µl STE extraction buffer was added and each tube was incubated at 95 ˚C for 12 minutes. Tubes were then centrifuged at 13,000rpm for 4 minutes. This roughly extracted supernatant was then used for PCR amplification. (2) In order to obtain a cleaner product, DNA was extracted from individual larvae and mosquitoes (minus the abdomen) using the DNeasy 96 Blood & Tissue KIT (Qiagen), following the manufacturers protocol.

PCR amplification was conducted using the following primers; UN, GA, ME, AR and QD (Scott *et al* 1993). The last four primers corresponded to the following species of the *An. gambiae complex*; *An. gambiae* s.s, *An. merus, An. arabiensis* and *An. quadriannulatus* respectively, the former is the universal primer that matches and anneals to the same position of the rDNA of all species of *An. gambiae* complex (see table 2.).

The standard reaction mix comprised 6.4µl dH_2O, 2µl reaction buffer (10x), 0.6µl $MgCl_2$ (50mM, to give 1.5mM) 1µl dNTPs (2mM), 1.3µl UN, 0.7µl GA, 2.0µl AR, 2.7µl QD, 1.1µl ME and 0.2 µl Taq polymerase. Primers were used at the working concentration of 10pmol/µl. Finally 2µl of DNA or dH_2O (negative control) was added to each individual tube. All reagents were obtained from the Bioline PCR kit. The reaction conditions were as follows: 30 cycles of denaturation at 94˚C for 30 seconds, followed by annealing at 50˚C for 30 seconds, then extension at 72˚C for 30 seconds. Positive controls, derived from *An. gambiae* s.s and *An. arabiensis* colonies maintained at the London School of Hygiene and Tropical Medicine (LSHTM), were systematically included. *An. quadriannulatus* and *An. merus* were not available.

PCR products were separated by electrophoresis at 80V (1.5% agarose- TBE gel) and visualized under short wave ultraviolet light. Specimens were identified by fragment size by comparisons with a Bioline Hyperladder™ IV: *An. quadriannulatus* 153bp; *An. arabiensis* 315bp; *An. gambiae* s.s 390bp and *An. merus* 466bp.

3.2.1. PCR optimisation

Following poor initial PCR results (PCR failures and non specific banding) amplification conditions were optimised. Optimal primer annealing temperatures were tested using a gradient of 50-58˚C. After optimisation some samples continued to fail to amplify. For these the annealing temperature was dropped to 52˚C and $MgCl_2$ was increased from 1.5mM to 2 mM and 2.5mM). Programme conditions were also varied to include an intitial denaturation step (95˚C for 5 mins) and a final extension step (72˚C for 10 mins) as used by Tsy *et al* (2003). Each failed sample was tested at least twice

Table 1: Summary details on the larval ecology of the populations analysed

Population ID.	Larval habitat	Salinity	Altitude (m)	No. analysed
PE5	Mangrove swamp	16%	-4	8
PE9	Stream margin	0%	9	12
PE11	Ditch	20%	3	10
PE13	Rice field	0%	60	8
PE14-1	Marshy depression	21%	-1	12
PE15	Ditch	0%	5	10
PE19	Ditch	0%	4	13
PE20	Ditch/pond	3%	8	25
PE21	Marshy depression	28%	5	10
PE22	Rice field	0%	30	12
PE23	Ditch	35%	-2	10
PE27	Ditch	40%	0	10
PE28	Hoof print	8%	-5	10
PE29	Ditch	28%	9	10
PE33	Pond	35%	12	10
PE35	Salt production box	20%	-7	10
PE37	Waterlogged field	0%	62	12
PE38	Rice field	0%	34	12
PE40	Rice field	0%	10	12
Total (mean)				**216 (11)**

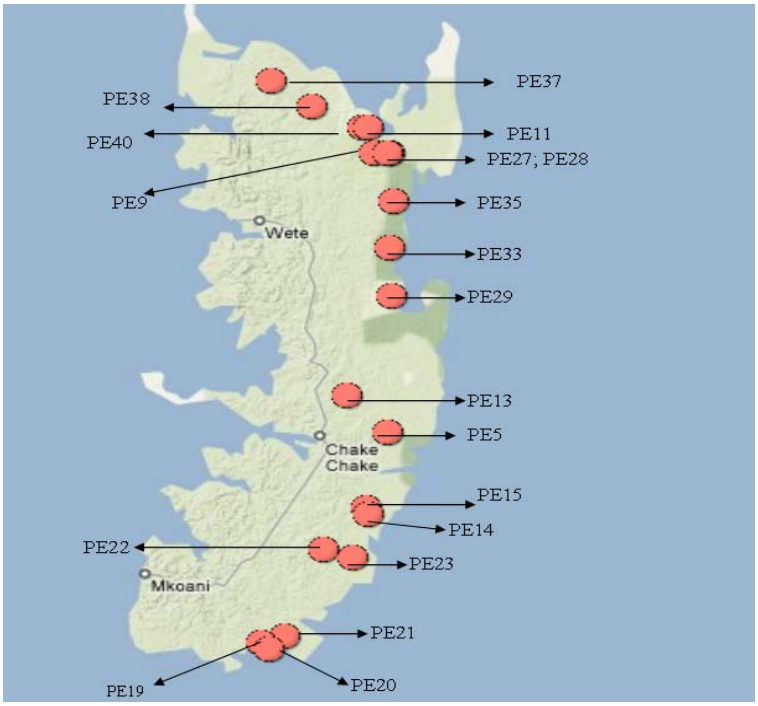


Figure 7. Map of sub-sampled populations

Table 2. *An. gambiae* complex rDNA IGS species –diagnostic primers

Primer	Primer sequence (5' to 3')	Tm (˚C)	Diagnostic band (bp)
UN	GTG TGC CCC TTC CTC GAT GT	61.4	-
GA	CTG GTT TGG TCG GCA CGT TT	59.4	390bp
ME	TGA CCA ACC CAC TCC CTT GA	59.4	466bp
AR	AAG TGT CCT TCT CCA TCC TA	55.3	315bp
	CAG ACC AAG ATG GTT AGT AT	53.2	153bp

Tm = melting temperature, bp=base pair

3.3. Data Analysis

Data was entered into MS Excel and descriptive statistics was used to summarize the data. A t test was used to determine the significance of the difference in proportions of *An. merus* larvae collected from fresh and brackish water sites. Using regression and correlation analysis the relationship between *An. merus* relative abundance and larval habitat salinity was assessed. The same analysis was repeated for altitude. Then further multiple regression analysis was performed to assess the strength of association between *An. merus*, salinity and elevation. All analysis was conducted using Stata version 10.

3.4. Ethics Approval

Ethical approval was obtained from the LSHTM. Local ethical approval was obtained from the Secretary Ethical Committee of the Ministry of Health and Social Welfare in Zanzibar and from the leader of each Shehia, (see appendix 5).

4. RESULTS

4.1. Assessing the impact of IRS and ITNs on malaria prevalence in Pemba.

It was originally proposed to review data obtained from the records kept by the ZMCP (see section 1.3 for review) and assess the impact of the recent vector control activities on malaria prevalence. However lack of the appropriate data from the ZMCP made the second part of this objective impossible.

4.2. Sampling

4.2.1. Larvae

It was originally proposed to collect larvae from >50 habitats, however sampling was not always successful. Many rice fields were noted as negative, but this may have been due to dense vegetation obstructing larval collection as opposed to an absence of larvae at these sites. Overall approximately 2, 200 larvae were sampled from 41 sites in the four districts of Pemba (see appendix 1). The number of *Anopheles* larvae per site ranged between 10 and 120, with an average of 53 per habitat. *Anopheles* larvae were observed in a range of habitats including those commonly associated with *An. gambiae* s.l such as rice and waterlogged fields, ditches, mangrove swamps, hoof prints, marshy depression and salt production sites. The waters within these larval habitats were mostly clear and their movement stagnant or slow. The opportunistic nature of *Anopheles* was demonstrated by its occurrence in temporary, semi-permanent and permanent waters. These larval sites often resulted from areas being flooded by rising tides or through human activity such as land clearage for cultivation and salt production. Culicine larvae were abundant in the majority of sites sampled, in

particular in the brackish waters of the mangrove swamps, marshy depressions and ditches at salt production sites.

Sampled habitats varied in salinity and altitude. 17 habitats were termed fresh water (0% salinity) and 24 brackish with salinity ranging from 3% to 42%. Recorded altitudes varied from -19 meters (below sea level) to 158 metres (above sea level), with landscapes ranging from plains to hills and valleys.

4.2.2. Mosquitoes

Five *Anopheles* female mosquitoes were collected in CDC light traps from 2 households, near larval sites PE10 and PE21, and the goat shed, near larval site PE14. None of the *Anopheles* mosquitoes caught were gravid or blood-fed. No *Anopheles* mosquitoes were found indoors in the remaining 8 sampled households. As with larval sampling large numbers of culicine mosquitoes were caught in the CDC traps.

As few *An. gambiae* s.l. females were found, it was not possible to assess the role of *An. merus* in malaria transmission. All *Anopheles* specimens caught were identified by molecular techniques.

4.3. Molecular identification of *An. gambiae* s.l. larvae and mosquitoes.

4.3.1. Larvae

Ribosomal DNA (rDNA) analysis was performed on a total of 216 larvae from 19 populations. Using this assay 120 larvae were positively assigned to 3 species: *An. merus* (50%), *An. arabiensis* (5%) and *An. quadrannulatus* (1%) (see table 3, fig. 8 and 9. (lane 2)).

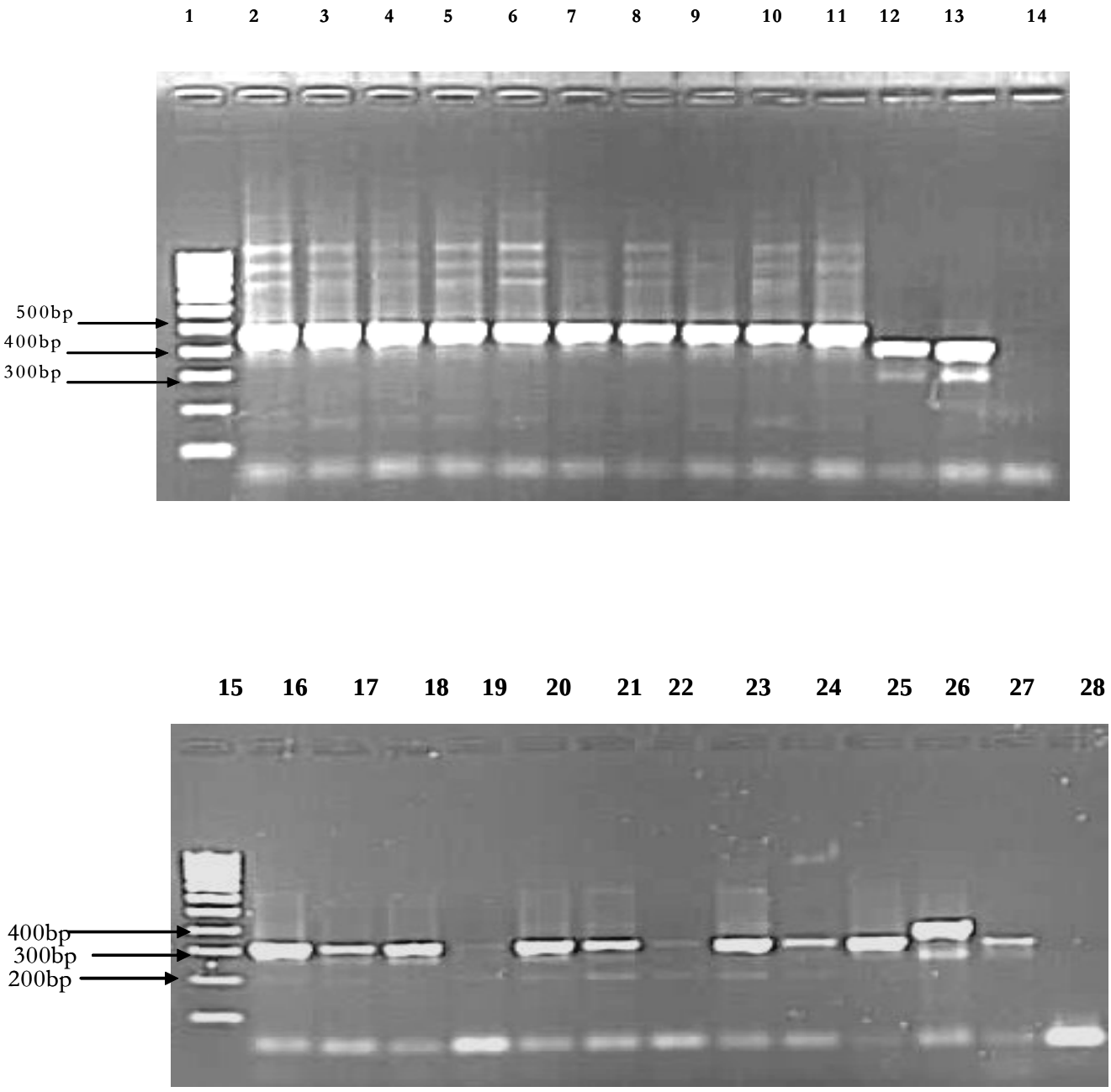


Figure 8. DNA bands produced by rDNA –PCR amplification. Lanes 2-11 show larvae from PE27 with diagnostic *An. merus* band (466bp); Lanes 16-25 show larvae from PE15 with diagnostic *An. arabiensis* band (315bp); Lanes 12,13 and 26 are *An. gambiae* s.s. (390bp) positive controls. Lane 27 is an *An. arabiensis* positive control. Lanes 14 and 28 are negative controls. Lanes 1 and 15 are the size standard Hyperladder IV

For a majority of the specimens identified as *An. merus,* a weak band diagnostic for *An. quadriannulatus* often amplified in addition to a dominant *An merus* band (see fig. 9). Initially such specimens were believed to be hybrids of *An. merus* and *An. quadriannulatus*, however previous studies have found this second band in known *An. merus* specimens (Scott *et al*. 1993; Bass *et al.* 2007). These specimens were therefore considered to be *An. merus.*

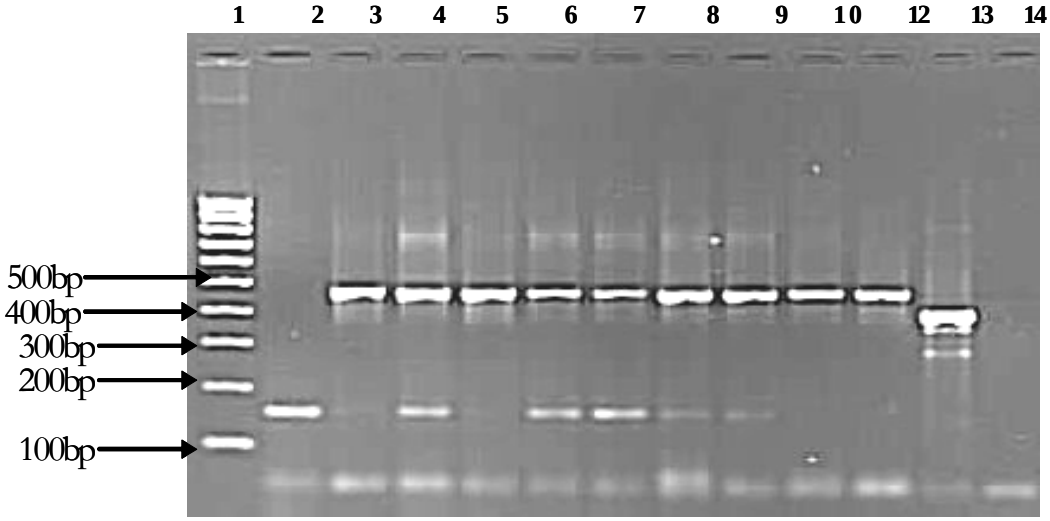


Figure 9: DNA bands produced by rDNA- PCR amplification. All lanes show larvae from PE21. Lane 2 shows diagnostic *An. quadriannulatus* band; Lanes 4 and 6-9 show the weak *An. quadriannulatus* (153bp) and the dominant *An. merus* (466bp) diagnostic band. Lane 13 is the *An. gambiae* s.s. (390bp) positive control and lane 14 is the negative control. Lane 1 is the size standard Hyperladder IV

12 specimens could not be confidently assigned to species level and were considered diagnostic failures. These specimens produced one or more bands that were too weak for accurate speciation. Example of this is population PE9; 6 of the larvae identified showed a faint *An. merus* band, with a 2nd equally faint *An. quadriannulatus* band (see fig.10)

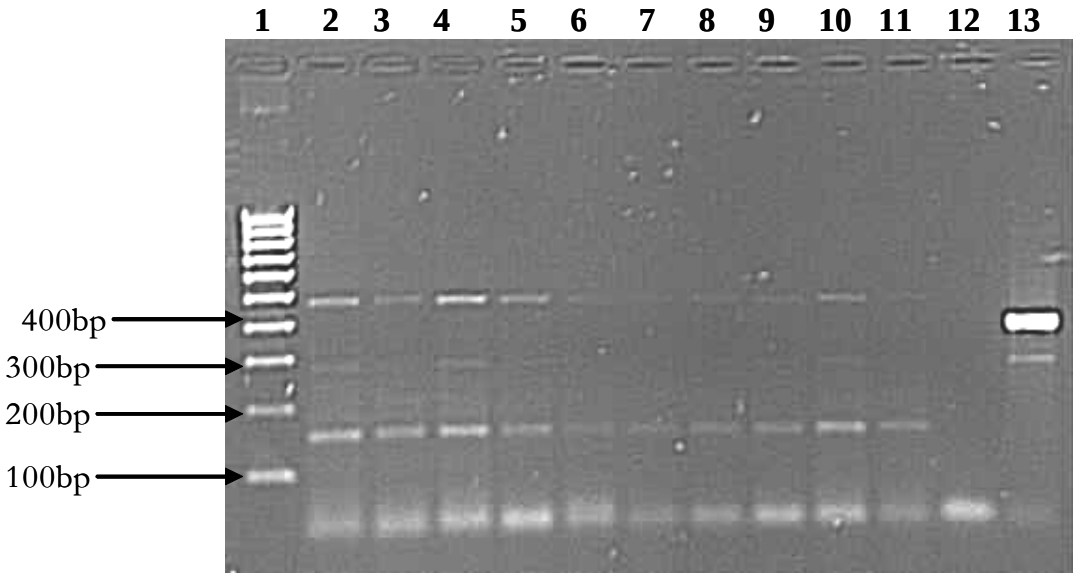
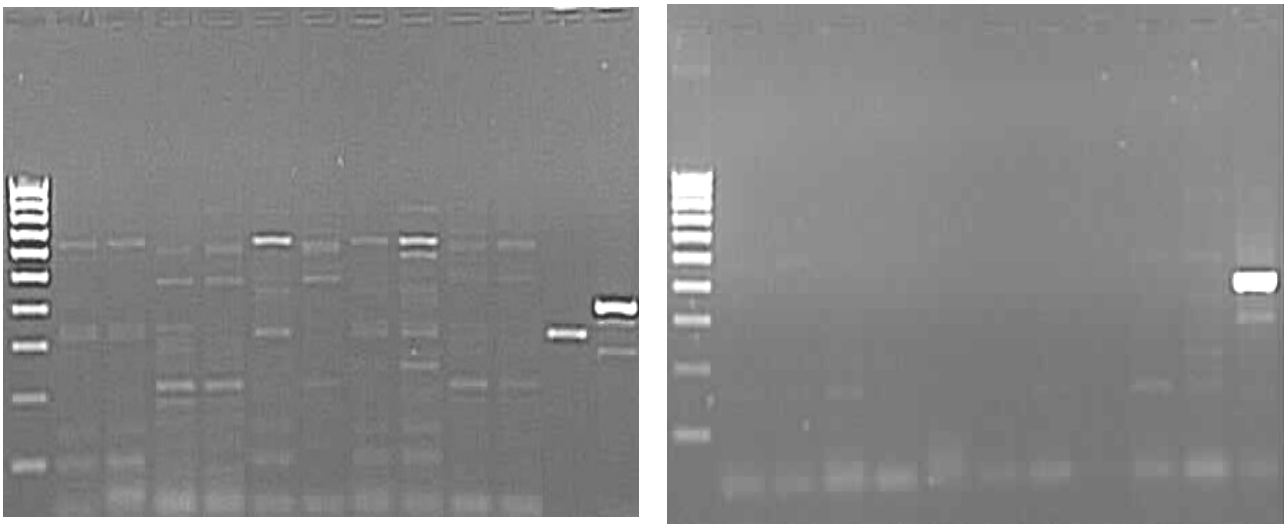


Figure 10: DNA bands produced by rDNA- PCR amplification. Lanes 6-11 show larvae from PE9 with faint *An. merus* (466bp) and 2nd equally faint *An. quadrainnulatus* (153bp) band. Lanes 12 and 13 are negative and positive *An. gambiae* s.s. (390bp) controls respectively. Lane 1 is the size standard Hyperladder IV

A total of 96 tested larvae (44%) were deemed PCR failures after a minimum of two PCR attempts (see fig. 11). The majority of these failures were from freshwater populations: 82% of the larvae in freshwater failed compared to 17% in brackish water (P= 0.0003 by t-test).



1) **2)**

Figure 11: DNA bands produced by rDNA- PCR amplification. Larvae from PE38 showing PCR failure following optimisation of conditions; Gel 1 shows multi-banding and positive *An. arabiensis* (315bp) and *An. gambiae* s.s (390bp) control respectively (in last 2 lanes); Gel 2 shows no amplification and a *An. gambiae* s.s control. Lanes 1 show the size standard Hyperladder IV

4.3.2. Adult Mosquitoes

Two of the mosquitoes tested were identified as *An. merus.* They were both from the indoor mosquito traps. The remaining 3 specimens were unidentified (see appendix 4a).

Table.3. PCR summary results

Water ecology	Population no.	No. analysed	*An. merus* (%)	*An. arabiensis* (%)	*An. quadriannulatus* (%)	Unsure (%)	Failed (%)
	PE9	12	4 (33)	0	0	6 (50))	8 (67))
Freshwater	PE13	8	0	0	0	0	8 (100)
	PE15	10	0	10 (100)	0	0	0
	PE19	13	0	0	0	0	13 (100)
	PE22	12	0	0	0	0	12 (100)
	PE37	12	0	0	0	0	12 (100)
	PE38	12	0	0	0	0	12 (100)
	PE40	12	2 (17)	0	0	5 (42)	10 (83)
Total		**91**	**6 (7)**	**10 (11)**	**0**	**11 (12)**	**75 (82)**
Brackish	PE5	8	7 (88)	0	0	0	1 (13)
	PE11	10	7 (70)	0	0	0	3 (30)
	PE14	12	9 (75)	0	0	0	3 (25)
	PE20	25	24 (96)	0	0	0	1 (4)
	PE21	10	9 (90)	0	1 (10)	0	0
	PE23	10	10 (100)	0	0	0	0
	PE27	10	10 (100)	0	0	0	0
	PE28	10	8 (80)	0	0	0	2 (20)
	PE29	10	0	0	0	0	10 (100)
	PE33	10	9 (90)	0	0	1	1 (10)
	PE35	10	10 (100)	0	0	0	0
Total		**125**	**103 (82)**	**0**	**1 (1)**	**1 (1)**	**21 (17)**
Overall total		**216**	**109 (50)**	**10 (5)**	**1 (1)**	**12 (6)**	**96 (44)**

4.4. Distribution of *An. merus* and other members of *An. gambiae* s.l. in Pemba

With the exception of larval site PE21 *An. merus* was not sympatric with other members of the *An. gambiae* complex. The is true of *An. arabiensis*;, all occurred alone in freshwater. *An. merus* showed great plasticity in its choice of larval habitat (see appendix 3). It was prevalent in brackish waters (see table 3.); accounting for 82% (103/125) of the species identified. Contrastingly, it was significantly less abundant

at freshwater sites (p=0.0000 by ttest). Regression analysis of the relationship between *An. merus* and salinity showed that the relative abundance of *An. merus* increased with each % increase in salinity (b=2.09%, P>0.001, 95% CI: 0.96%; 3.22%) (see fig 12). However, only 47.3% of the variation in the relative abundance of *An. merus* is accounted for by its linear relationship with salinity. Also it is worth mentioning that one cannot guarantee if this trend would hold for specimens outside the tested salinity range.

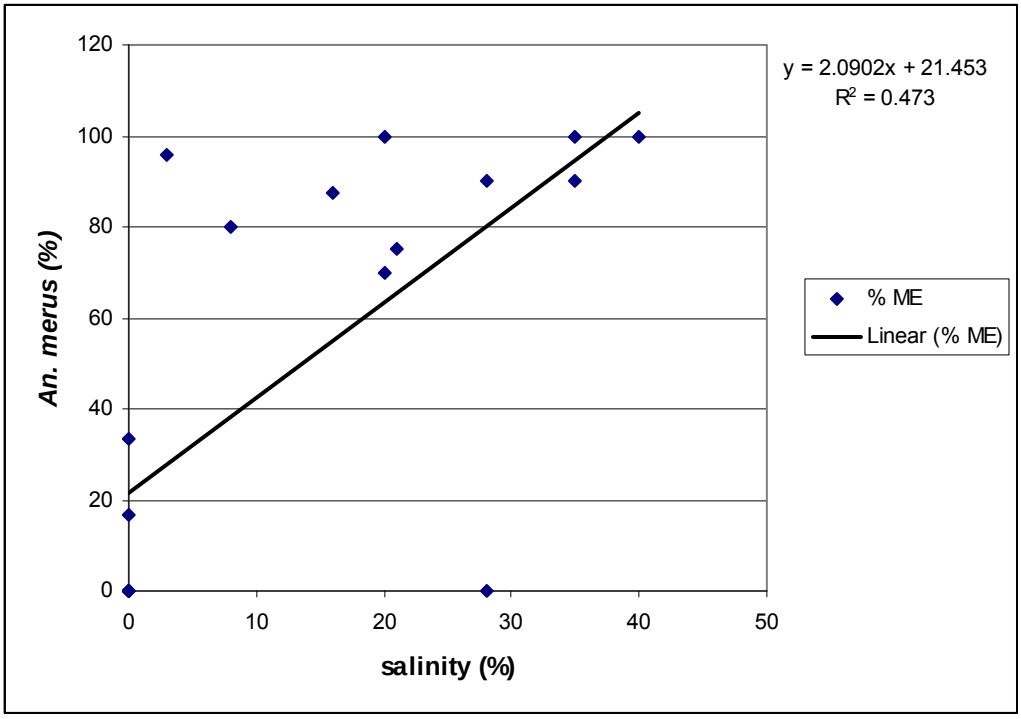


Figure 12. Relationship between salinity (%) and the relative abundance of *An. merus* in Pemba

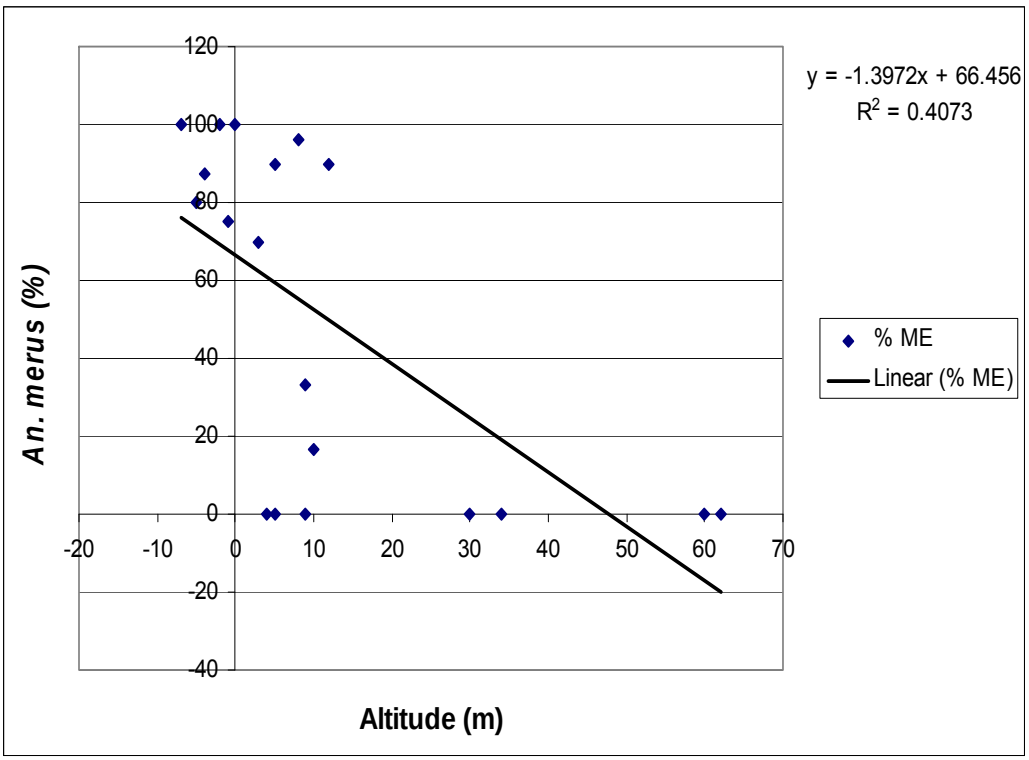


Figure 13. Relationship between altitude (m) and the relative abundance of *An. merus* in Pemba.

Regression analysis of the relative abundance of *An. merus* and altitude showed that the proportion of *An. merus* decreased as the altitude increased (b= -1.40%, P>0.003, 95% CI -2.26%; -0.53). Again, only 41% of the variation in *An. merus* relative abundance is explained by its linear relationship with altitude (see fig. 13) and the same caveats discussed above apply.

Further multiple regression analysis revealed that salinity and altitude were associated: the salinity of larval habitats decreased with every unit increase in elevation (b-0.348%, P>0.036, 95% CI -0.67; -0.025). Consequently multiple regression analysis was performed to assess the strength of association between *An. merus* relative abundance and salinity (adjusted for altitude) and *An. merus* relative

abundance and altitude (adjusted for salinity). The analysis showed that altitude did indeed confound the relationship between species abundance and salinity and after controlling for this the association between *An. merus* relative abundance and salinity lessened somewhat, although it was still significant (P>0.015). The same was true for the analysis of *An. merus* relative abundance and altitude (P>0.043).

5. DISCUSSION

5.1. Vector sampling

The aim of assessing *An. merus's* role in malaria transmission following recent vector control activities was not addressed. Attempts to collect *Anopheles* indoors and outdoors proved to be unsuccessful. Of the 10 households sampled and goat shed, only 5 female *Anopheles* species were caught. A similar situation was observed in the 1960s after the spraying campaign in Pemba. Although *An. gambiae* s.l. remained detectable it was no longer found resting indoors, though it could be caught using exit traps fitted to houses (Iyengar, 1962; Odetoyinbo and Davidson ,1968). It is thus arguable that the difficulty in catching *An. gambiae* s.l. indoors is due to the success of the IRS of 2006 to 2007. Equally it is probable that using protected human baits with CDC light traps was not a very comprehensive strategy seeing as the objective was to catch the exophilic *An. merus*. Although light traps are highly efficient, entomological monitoring of exophilic vectors requires more than one collection method, particularly when vector densities are low (Kulkarni *et al.,* 2006). Attempts were made to rectify the situation after observing the low mosquito catches; alternatives such as exit traps, pyrethroid spray catches, bait collections / indoor resting catches, and magnet traps were considered. However all these methods were ruled out for a number of reasons including: 1) lack of availability (i.e. exit traps); 2) tendency to underestimate the abundance of exophilic vectors (pyrethroid spray catches (Kulkarni *et al.,* 2006)); 3) collector subjectivity (bait collections / indoor resting catches (Shiff, 1995)) and 4) bias of attracting mosquitoes that normally would not be found present due to the attractants released by trap (magnet trap). Ideally protected human baits with CDC light traps coupled with window traps would have been best for evaluating indoor host seeking behaviour of the exophilic mosquitoes.

Mosquito trapping in livestock areas was attempted once as a large net was needed to protect animal baits from mosquitoes but unfortunately this could not be obtained from the markets. Such large nets were necessary as the animal bait being targeted was cattle (the preferred host of *An. merus* (Gillies & DeMellion, 1968)).

The over-representation of culicine mosquitoes in the mosquito catches made both indoor and outdoor implies that they are perhaps less vulnerable to the effects of spraying. Although it is unsurprising that they were found in these households, seeing as the households approximated larval sites whose ecology suite the breeding of culicine (i.e. brackish waters, water with decaying vegetation and such (Mutero and Mosha, 1984; Roberts and Irving Bell, 1997)

5.2. Molecular identification of *An. gambiae* s.l., changes to vector population and implications for malaria transmission.

Molecular identification of larvae and mosquitoes revealed that *An. merus* currently is the predominating species of the *An. gambiae* complex on Pemba. This is then followed by *An. arabiensis* and *An. quadriannulatus*. No *An. gambiae* s.s. were observed. This is a discernable deviation from the findings of the ZMCP in their most recent entomological survey (in 2005 (ZMCP, 2007)). Of the 76 species identified then, 72 were *An. gambiae* s.s., 3 *An. arabiensis* and 1 *An. quadriannulatus.* No *An. merus* were detectable. It is therefore possible that the spraying campaign has altered the population composition of *An. gambiae* s.l. by selectively eliminating the very endophilic *An. gambiae* s.s. leaving the more exophilic species (i.e. *An. merus, An.*

quadriannulatus and *An. arabiensis*) to dominate. This is not dissimilar to the observations made following previous spraying programmes in Zanzibar (Iyengar, 1962; Odetoyinbo & Davidson, 1968; Wastling, 2007). These findings imply that the current spraying campaign is very effective, especially in targeting the very endophilic *An. gambiae* s.s. which is the most efficient member of the *An. gambiae* complex. However, seeing as only a fraction of the larvae sampled were analysed and few households were sampled for adults, it sensible to be circumspect about these findings. There are other factors which partially explains these results: 1) the seasonality of species distribution within the *An. gambiae* complex means they experience shifts in their prevalence with the relative frequency of *An. arabiensis* increasing during the dry season whilst *An. gambiae* decreases (White, 1974; Coluzzi *et al.,* 1979). Seeing as this study was conducted at the end of the heavy rains (Masika) and before the light rains (Vuli) it is perhaps unsurprising that no *An. gambiae* s.s. were recorded both at the freshwater sites and indoors. 2.) variation in sampling sites may be responsible for the differences in the observations of this study and the ZMCP's: in the 2005 entomological survey, no sampling were conducted in mangrove swamp areas. However the fact that malaria prevalence has dropped (see data review section (1.2 and 1.3)) despite the presence of *An. gambiae* s.l. suggests that the species present are not very efficient at malaria transmission. Malaria transmission has not been completely interrupted though. It is likely that the remaining exophilic members are responsible for this low level transmission. Earlier studies in Pemba have not implicated *An. merus* as an efficient malaria vector and the findings of this study doesn't allow us to refute or corroborate this. However high sporozoite rates have been documented in this species thus its potential for transmitting malaria should not be ruled out (Temu *et al.,* 1998; Tsy *et al.,* 2003) It is

worth mentioning that *An. arabiensis*, which is more efficient in transmitting malaria is also likely to be responsible for this remaining transmission; Odetoyinbo and Davidson (1968) implicated this vector for the low level malaria transmission in Unguja following the spraying campaign of the 1950's.

5.3. Limitations of molecular identification method and PCR failures

No hybrids were identified among specimens assayed. Although the 2nd band corresponding to the *An. quadriannulatus* diagnostic band was often found in *An. merus* specimens.. Previous studies have documented this as a common occurrence (Scott *et al.,* 1993; Van Rensburg *et al.,* 1996 and Cornel, 1997). It has been suggested that this 2nd band occurs as a result of the *An. quadriannulatus* primer finding a homologous region within the *An. merus* genome thus leading to production of non-specific fragments. Additionally although crosses between the sibling species of the *An. gambiae* complex have been established under laboratory conditions (Davidson and Hunt, 1973) hybridisation under normal conditions is rarely observed, particularly in larval stages (White, 1974). There appears to be an innate mechanism that is predisposed towards intrabreeding with general avoidance of interbreeding. The fact that crosses result in infertile males with few sterile females explains why hybridisation rarely happens (White, 1974; Okereke, 1980). However it should be noted that true hybrids may have gone unidentified.

As previously discussed (see section 4.3.1), many PCR failures were observed despite the many steps taken to improve this. A total of 96 tested larvae (44%) were deemed PCR failures after a minimum of two PCR attempts. This is not an uncommon phenomenon with this rDNA PCR assays as confirmed by many studies including

Scott *et al.,* 1993; Van Rensburg *et al.,* 1996; Tsy *et al.,* 2003 and Bass *et al.,* 2007). However the failure rate in these studies was lower (i.e. ranging between <1% to 2% failure). Failures in this study may have occurred for the following reasons: 1.) Poor quality DNA was extracted from the larvae using unsuitable methods; 2) Ethanol used in preserving the larvae interfered with the PCR reactions; 3) These larvae are not species of the *An. gambiae* complex; 4) These larvae are *An. gambiae* s.l. but have a point mutation that prevents the PCR primers annealing successfully.

As all specimens, including those that amplified, were stored in ethanol and treated in the same manner it seems unlikely that ethanol was to blame for the PCR failures. Extraction methods used to obtain DNA were tested using *An. gambiae* s.s and *An. arabiensis* larvae from colonies maintained at LSHTM and these controls successfully amplified (see appendix 4b). Additionally whole genomic DNA, although weak, were present in both successful and failed samples visualised in a 1% TBE-agarose gel (see appendix 4c). Bass *et al.,* (2007) noted that 0.1-0.2ngs (2ng for *An. quadriannulatus*) of DNA was sufficient for successful amplification.

The fact that a majority of these failures were from freshwater populations: 82% of the larvae in freshwater failed compared to 17% in brackish water (P= 0.0005 by t-test) is interesting. Fresh water species; *An. funestus* and *An. coustani* were both identified in the 2005 entomological survey (Roberts and Irving Bell, 1997; ZMCP, 2007; Dia *et al.,* 2008). It is possible that these species were collected during larval sampling. In order to determine the exact species of the failed samples a second target, *cytochrome oxidase* I (COXI), was identified for amplification and direct sequencing. The mitochondrial gene, COXI was chosen as sequence data is available in Genbank for the species *An. gambiae* s.s., *An. arabiensis, An. quadriannulatus, An.*

funestus and *An. coustani.* Conserved primers suitable for the amplification of species of the order Diptera and the family Culicidae to which *Anopheles* belong are available (Herbert *et al,* 2003). A panel of 126 specimens were chosen for amplification including PCR failures, positive controls and specimens identified using the rDNA IGS assay. Species identity could then be confirmed by comparing sequence data obtained to positive controls and data available in GenBank. However time constraints prevented the completion of this work.

5.4. Findings relating to distribution of *An. merus* larvae and remaining members of *An. gambiae* s.l. in Pemba

Previous maps by Iyengar (1962), Odetoyinbo and Davidson (1968) on the distribution of *An. merus* following the vector control activities showed its occurrence on the east coast along a north to south transect (with some scattering on the west), not unlike the findings of this study. The plasticity of *An. merus* in its choice of larval habitat was duly noted. Although *An. merus* larvae was significantly associated with brackish water, it was found to occur in freshwater. This corroborates with previous finding of Gillies & DeMeillion, 1968; White, 1974; Gillies & Coetzee, 1987. The association between salinity and the occurrence of *An. merus* held firm as was shown by statistic analysis ($P>0.001$). *An. merus* increased in more saline waters with the highest salinity reaching 42%. However *An. merus* abundance was not solely dictated by salinity, altitude also influenced the abundance of this species with *An. merus* decreasing at high altitudes ($P>0.003$). Thus unlike the observations made by Kulkarni *et al.,* (2006) altitude was a good indicator of *An. merus* abundance. This is unsurprising as brackish water sites, the habitat favoured by *An. merus* are most

common at low altitudes due to the flooding of such sites by seawater at high tides. However as the altitude increases and larval habitats move inland and away from flooding by seawater, they become less saline and hence the decline in the abundance of *An. merus*. After adjusting for altitude (as a confounder), the association between *An. merus* and salinity was lessened although it was still significant and the same is true for altitude. These two variables could be used in predicting the distribution of *An. merus* larvae. A function which is necessary if this species is later proven to be important in malaria transmission in Pemba.

Attempts to assess the significance of *An. merus*'s occurrence in less saline waters was made impossible due to the small number of larvae definitively identified. Consequently more extensive analysis such as the Chi square test for trend or even Fishers exacts test could not be performed.

All specimens identified as *An. arabiensis* occurred in freshwater at an altitude above sea level (5 metres). Also perhaps more interesting is the observation that the *An. arabiensis* population occurred in a habitat approximating a cattle pasture. This confirms to an extent the zoophilic/ exophilic inclination of *An. arabiensis.* Edillo *et al.,* (2002) reported similar findings in Banambani village, Mali, where they found the distribution of *An. arabiensis* to be related to its distance from adult feeding sources (i.e. cattle).

The only larvae to be identified as *An. quadriannulatus* occurred in brackish water at a salinity of 28%. This is quite a deviation from the freshwater habitat that this species

usually favours. However Coetzee and Le Sueur (1988) documented *An. quadriannulatus* surviving in salinities above 25%.

6. CONCLUSION

The predominant species of the *An. gambiae* complex in Pemba now appears to be *An. merus.* *An. arabiensis* and *An. quadriannulatus* are also present though far from common. It seems that the house spraying has reduced the occurrence of endophilic members of the *An. gambiae* complex with very little effect on the very exophilic *An. merus.*

These findings are substantiated by the results of the entomological survey of 2005 and the current malaria situation: In 2005 the ZMCP documented all but one (i.e. *An. merus*)of the 4 sibling species known to occur in Pemba. Yet Wastling (2007) noted the occurrence of *An. merus* following the spraying campaign of 2006-2007. Additionally malaria prevalence has dropped substantially in spite of the presence of *An. gambiae* s.l. thus indicating the presence of inefficient vectors like *An. merus.*

The remaining transmission in Pemba however suggests one of two things:1) *An. merus* is perhaps not an inefficient vector or 2.) *An. arabiensis,* which is more efficient is responsible for the remaining transmission.

At this present juncture in the ZMCP where the focus has shifted to ensuring holoendemic malaria does not return. It would be beneficial for the ZMCP to assess the present vector populations and their role in malaria transmission now that *An. gambiae* s.s. appears to be absent. Accordingly vector control strategies must change in acknowledgement of present vector populations and their behaviour which is largely exophilic. It is hoped that the findings of this study with regards to the

occurrence of *An. merus*; its plasticity and preference of brackish waters will assist in this.

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8. **Appendix** 1: Population details; localities and larval ecology of all area sampled

Collection No.	Total larvae	Region	District	Shehia	Salinity (%)	Salinity	Altitude	latitude	longitude
PE1	120	Southern	Chake	Tibirinzi	5%	brackish	-1	-5.24295	39.76489898
PE2	90	Southern	Chake	Kichungwani	0%	fresh	4	-5.24939	39.76951028
PE3	22	Southern	Chake	Kumvini	0%	fresh	23	-5.28377	39.82137641
PE4	90	Southern	Chake	Kumvini	20%	brackish	15	-5.29843	39.81519073
PE5	110	Southern	Chake	Vitongoji	16%	brackish	-4	-5.24026	39.82257427
PE6	20	Southern	Chake	Uwandani	0%	fresh	34	-5.19821	39.8230351
PE7	27	Northern	Wete	Limbani	0%	fresh	42	-5.07821	39.76418375
PE8	31	Northern	Wete	Chwale	0%	fresh	25	-5.09916	39.81265856
PE9	54	Northern	Michenweni	Mapofu	0%	fresh	9	-5.00199	39.81254876
PE10	24	Northern	Michenweni	Sizini	39%	brackish	15	-4.9797	39.80824331
PE11	120	Northern	Michenweni	Sizini	20%	brackish	3	-4.98018	39.80844104
PE12	50	Northern	Wete	Bopwe	0%	fresh	-8	-5.04836	39.72119867
PE13	40	Southern	Chake	Nga' Mbwa	0%	fresh	60	-5.20807	39.79377639
PE14-1 (x3)	90	Southern	Chake	Dodo	21%	brackish	-1	-5.30921	39.80904495
PE14-4	30	Southern	Chake	Dodo	16%	brackish	-1	-5.30921	39.80904495
PE15	80	Southern	Chake	Dodo	0%	fresh	5	-5.30414	39.80783695
PE16	90	Southern	Chake	Pondeani	12%	brackish	-6	-5.22788	39.76150565
PE17	110	Southern	Chake	Nbagoni	8%	brackish	-19	-5.22452	39.72324478
PE18	90	Southern	Mkoani	Ngwachani	0%	fresh	41	-5.31358	39.7457651
PE19	60	Southern	Mkoani	Kengeja	0%	fresh	4	-5.41934	39.7333624
PE20	50	Southern	Mkoani	Mwambe	3%	brackish	8	-5.42529	39.73868835
PE21	90	Southern	Mkoani	Mwambe	28%	brackish	5	-5.41413	39.7494171
PE22	30	Southern	Mkoani	Chambani	0%	fresh	30	-5.33988	39.77726237
PE23	30	Southern	Mkoani	Chambani	35%	brackish	-2	-5.34662	39.79766141
PE24	35	Northern	Michenweni	Mjini-Wini	6%	brackish	68	-4.99268	39.84131335

Collection No.	Total larvae	Region	District	Shehia	Salinity (%)	Salinity	Altitude (m)	latitude	longitude
PE25	30	Northern	Michenweni	Mjini-Wini	16%	brackish	22	-4.9875	39.84102937
PE26	40	Northern	Michenweni	Mjini-Wini	14%	brackish	9	-4.98802	39.84081287
PE27	40	Northern	Michenweni	Mapofu	40%	brackish	0	-5.00173	39.82434989
PE28	30	Northern	Michenweni	Mapofu	8%	brackish	-5	-5.00244	39.82246044
PE29	50	Northern	Wete	Kambinini	28%	brackish	9	-5.1242	39.82553509
PE30	12	Northern	Wete	Kambinini	42%	brackish	2	-5.12418	39.82617924
PE31	50	Northern	Wete	Shengejuu	20%	brackish	12	-5.08289	39.82345479
PE32	50	Northern	Wete	Shengejuu	25%	brackish	12	-5.08289	39.82345479
PE33	33	Northern	Wete	Shengejuu	35%	brackish	12	-5.08289	39.82345479
PE34	50	Northern	Michenweni	Mtemani	15%	brackish	-7	-5.04312	39.82736494
PE35	40	Northern	Michenweni	Mtemani	20%	brackish	-7	-5.04312	39.82736494
PE36	34	Northern	Michenweni	Kifundi	0%	fresh	158	-4.98209	39.73616028
PE37	20	Northern	Michenweni	Konde	0%	fresh	62	-4.94101	39.74076513
PE38	58	Northern	Michenweni	Kinowe	0%	fresh	34	-4.96214	39.76860872
PE39	53	Northern	Michenweni	Tumbe	0%	fresh	4	-4.96628	39.79923226
PE40	10	Northern	Michenweni	Sizini	0%	fresh	10	-4.98017	39.80460657
TOTAL	**2183**								
Mean	**53**								
Range (min)	**10**				**0**		**-19**		
Range (max)	**120**				**42**		**158**		

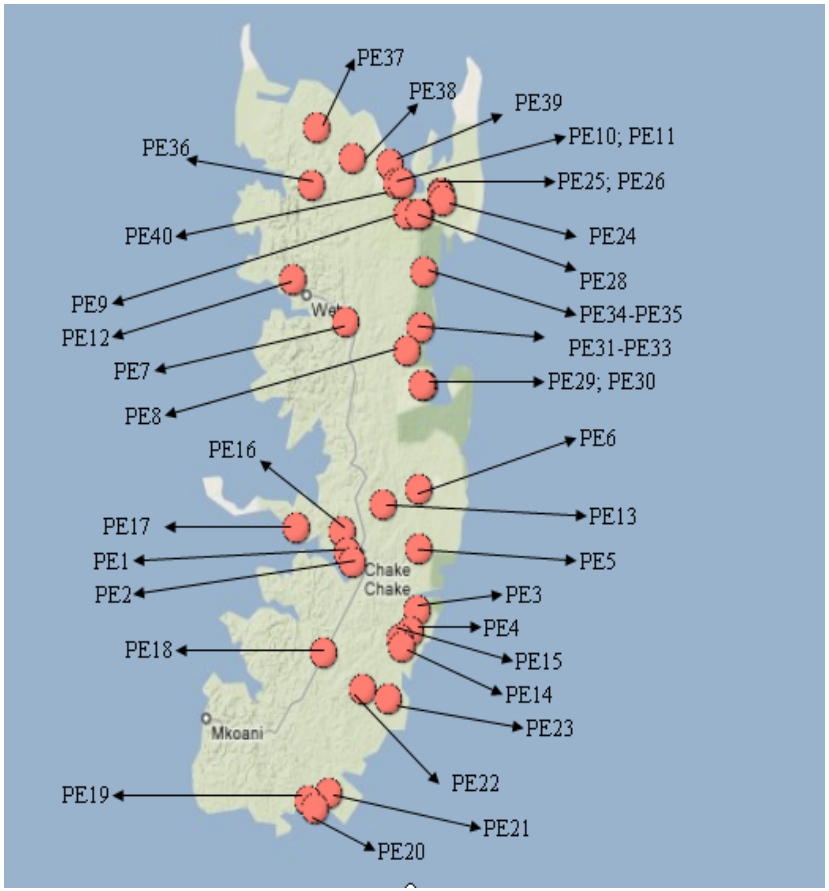
Site (N)	Larval habitat	Terrain	Climate	Environment	Water source
PE1	marshy depression, hoof & human print	valley	clear sky, no shade, light wind	village, mangroves, cultivated rice fields near; agriculture as modifier	semi-permanent and stagnant, clear
PE2	cultivated land with human prints & rice field	valley	clear sky, no shade, no wind	urban, cultivated rice field	temporary & stagnant, clear
PE3	marshy depression	plain	clear sky, no shade, light wind	village, cultivated feld; corn, rice, banana etc	temporary & stagnant, clear
PE4	stream/creek	plain	partly cloudy, no shade, light winds	village, mangrove trees	permanent (sea water appears to flow into it), stagnant, coloured and turbid
PE5	mangrove swamp	plain	clear sky, heavy shade, no wind	village, mangrove swamp, cultivated field; plantain, cassava	permanent, stagnant, clear but coloured
PE6	rice field	plain	clear sky, heavy shade,light winds	village, cultivated field:corn, rice, coconut etc	temporary,stagnant, clear
PE7	irrigated rice field & hoof prints	valley	partly cloudy, light rain, no shade and no wind	village, cultivated field: rice, plantain	permanent, stagnant, clear and coloured
PE8	rice field	plain	overcast, no shade, light winds	village, cultivated field: rice, corn, sogum etc	permanent, stagnant, clear but coloured
PE9	stream margin	valley	overcast, no shade, no wind	village, cultivated field: rice, cassava	permanent, slow, coloured but clear
PE10	marshy depression	plain	clear sky, no shade, light wind	village, mangrove swamp; modifier: former mangrove swamp cleared for salt production	temporary, stagnant, clear and coloured
PE11	ditch/beachlike depression	plain	clear sky, no shade, light wind	village, mangrove swamp, grassy vegetation, schrubs/bush; modifier: mangrove swamp clear for salt production	temporary but depends on high/low tides of the sea, stagnant, clear and coloured
PE12	irrigated rice field	valley	clear sky, no shade, gusty	village, cultivated field: rice	permanent, stagnant, clear
PE13	rice field	valley	clear sky, partial shade, gusty	village, cultivated fields: rice, banana	permanent, stagnant, clear

PE14-1 (x3)	marshy depression	plain	clear sky, no shade, gusty	village, salt production site, historically a mangrove swamp	semi-permanent; relies on flooding from salt production area; clear but turbid in some areas
PE14-4	ditch	plain	clear sky, no shade, gusty	village, salt production site, historically a mangrove swamp	semi-permanent; relies on flooding from salt production area; clear but turbid in some areas
PE15	ditch on the path of a makeshift road	plain	clear sky, no shade, light winds	village, cultivated field: rice; pastures: cattle	temporary, stagnant, clear, coloured
PE16	marshy depression/pond in a mangrove swamp area	plain	cloudy/overcast, partial shade, light wind	village, mangrove swamp	semi-perminant, stagnant, clear, coloured
PE17	marshy ditch within an area which was previously a mangrove swamp that has been cleared in preparation for rice cultivation	valley	heavy rain, no shade, no wind	village, mangrove swamp, cultivated field:rice	temporar, stagnant, clear and coloured
PE18	rice field	valley	clear sky, no shade, light winds	village, cultivated rice field	semi-permanent, stagnant, clear
PE19	ditch	plain	clear sky, no shade, no wind	village, cultivated rice field	temporary, stagnant, clear
PE20	ditch/pond; like cratters in the ground, the remanents of caves	plain	clear sky, no shade,light winds	village, mangrove trees, fishing harbour nearby	temporary, stagnant, coloured, turbid
PE21	marshy depression in a mangrove swamp area	plain	clear sky, no shade, no wind	village, mangrove swamp	permanent, stagnant, turbid
PE22	rice field	plain	clear sky, partial shade, light wind	village, cultivated rice fields	semi-perminant, stagnant, clear
PE23	ditch in a salt production site	plain	clear sky, no shade, no wind	village, mangroves	temporary, stagnant, coloured,
PE24	pond in a grass field	plain	clear sky, no shade, light winds	village, grassy vegetation, cultivated field: rice cassava	temporary, stagnant, clear and coloured

PE25	mangrove swamp	plain	clear sky, no shade, no wind	village, mangrove swamp, cultivated field: corn, cassava, banana	permanent, stagnant, turbid, rotten vegetation etc
PE26	marshy depression/ditch, some mangrove around	plain	clear sky, no shade, light wind	village, swamp; prior mangrove swamp that has been cleared, cultivated field: rice, cassava	semi-permanent, stagnant, clear, coloured
PE27	ditch close to a salt production site	plain	clear sky, no shade, light wind	village, mangroves, swamp; cleared for salt production	semi-permanent, stagnant, coloured
PE28	hoof print in a marshy depression in a rice field	plain	clear sky, no shade, light wind	village, cultivated field: rice	semi-permanent, stagnant, clear
PE29	dicth close to a salt production site	plain	clear sky, no shade, light wind	village, mangrove swamp; some cleared for salt production	semi-perminant, stagnant, coloured, clear
PE30	salt production area/box	plain	clear sky, no shade, light wind	village, mangrove swamp	permanent, slow,coloured
PE31	a pond close to a salt production area	plain	clear sky, no shade, light wind	village, mangrove swamp, cultivated rice field, salt production area	temporary, stagnant, coloured and turbid
PE32	a ditch neighbouring PE31 but quite clearly a separate entity	same as PE31			
PE33	a salt production box	same as PE32			
PE34	a salt production box	plain	clear sky, no shade, light wind	village, mangrove swamp, salt production	permanent, slow, coloured but clear
PE35	a salt production box	same as PE34			
PE36	waterlogged field	plain	clear sky, light rain later, no shade, no wind	village, cultivated field: cassava	permanent, stagnant, clear
PE37	waterlogged field	plain	clear sky, no shade, no wind	village, cultivated field:rice	temporary, stagnant, clear
PE38	rice field	plain	clear sky, no shade, no wind	village, cultivated field: rice	semi-permanent due to uncontrolled irrigation, stagnant, clear

PE39	stream pool	valley	clear sky, no shade, no wind	village, cultivated field: rice	permanent, stagnant, clear
PE40	rice field	plain	clear sky, no shade, light wind	village, cultivated field: rice	temporary, stagnant, clear

APPENDIX 2: Map of all areas sampled in Pemba



APPENDIX 3: Sub-sampled population details, localities and sampling efforts

Region	District	Shehia	Site (n)	Salinity (%)	Altitude (m)	Latitude	Longitude	Larval habitat	Terrain	Water source
Northen	**Micheweni**	Mapofu	PE27	40%	0	-5	39.82	Ditch close to salt production site	Plain	Semi-permanent, stagnant, coloured
		Mapofu	PE28	8%	-5	-5	39.82	Hoof print in rice field	Plain	Semi-permanent, stagnant, coloured
		Mapofu	PE9	0%	9	-5	39.81	Stream margin	Valley	Permanent, slow, coloured, clear
		Mtemani	PE35	20%	-7	-5.04	39.83	Salt production box	Plain	Permanent, slow, coloured, clear
		Konde	PE37	0%	62	-4.94	39.74	Waterlogged field	Plain	Temporary, stagnant + clear
		Kinowe	PE38	0%	34	-4.96	39.77	Rice field	Plain	Semi-permanent, irrigated source, stagnant, clear
		Sizini	PE40	0%	10	-4.98	39.8	Rice field	Plain	Temporary, stagnant, clear
		Sizini	PE11	20%	3	-4.98	39.81	Ditch/ beachy depression	plain	Temporary, depends on tides, stagnant, clear and coloured
	Wete	Kambinini	PE29	28%	9	-5.12	39.83	Ditch close to salt production site	Plain	Semi-permanent, stagnant, coloured, clear
		Shengejuu	PE33	35%	12	-5.08	39.82	A pond close to a salt producn. site	plain	Temporary, stagnant, coloured and turbid

Region	District	Shehia	Site (n)	Salinity (%)	Altitude (m)	Latitude	Longitude	Larval habitat	T Terrain	Water source
Southern	Chake	Vitongoji	PE5	16%	-4	-5.24	39.82	Mangrove swamp	Plain	Permanent, stagnant , clear+ coloured
		Nga'Mbwa	PE13	0%	60	-5.21	39.79	Rice field	Valley	Permanent, stagnant, clear
		Dodo	PE14-1	21%	-1	-5.31	39.81	Marshy depression	Plain	Semi-permanent, flooded by salt production water, turbid/clear
		Dodo	PE15	0%	5	-5.3	39.81	Ditch on the path of a road	Plain	Temporary, stagnant, clear, coloured
	Mkoani	Kengeja	PE19	0%	4	-5.42	39.73	Ditch	Plain	Temporary, stagnant, clear
		Mwambe	PE20	3%	8	-5.43	39.74	Pond/ditch	plain	Temporary, stagnant, coloured, turbid
		Mwambe	PE21	28%	5	-5.41	39.75	Marshy depression in a mangrove swamp	plain	Permanent, stagnant, turbid

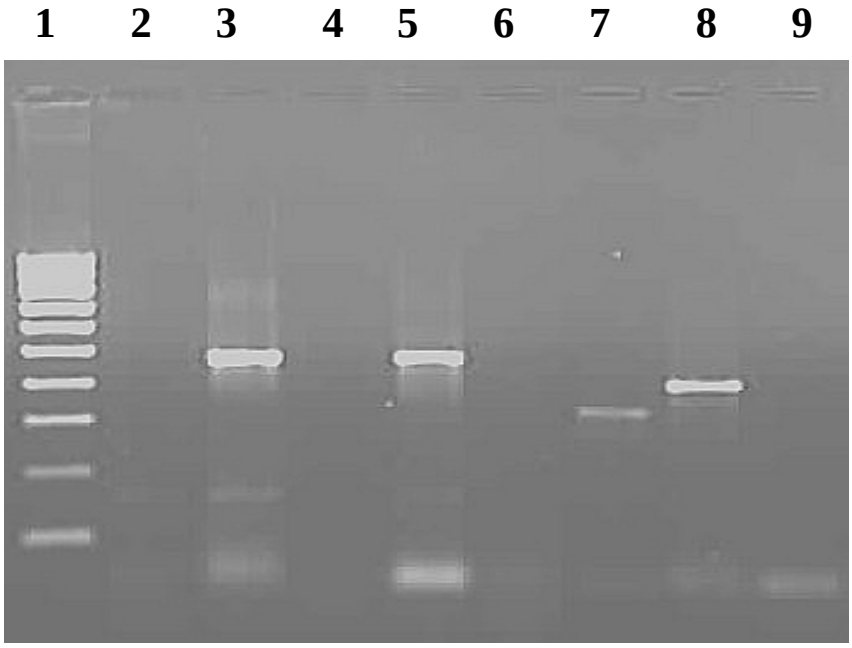
Region	District	Shehia	Site (n)	Salinity (%)	Altitude (m)	Latitude	Longitude	Larval habitat	Terrain	Water source
		Chambani	PE22	0%	30	-5.34	39.78	Rice field	Plain	Semi-permanent, stagnant, clear
		Chambani	PE23	35%	-2	-5.35	39.8	Ditch in salt production site	plain	Temporary, stagnant, coloured

Sub-Sample rDNA PCR identification results

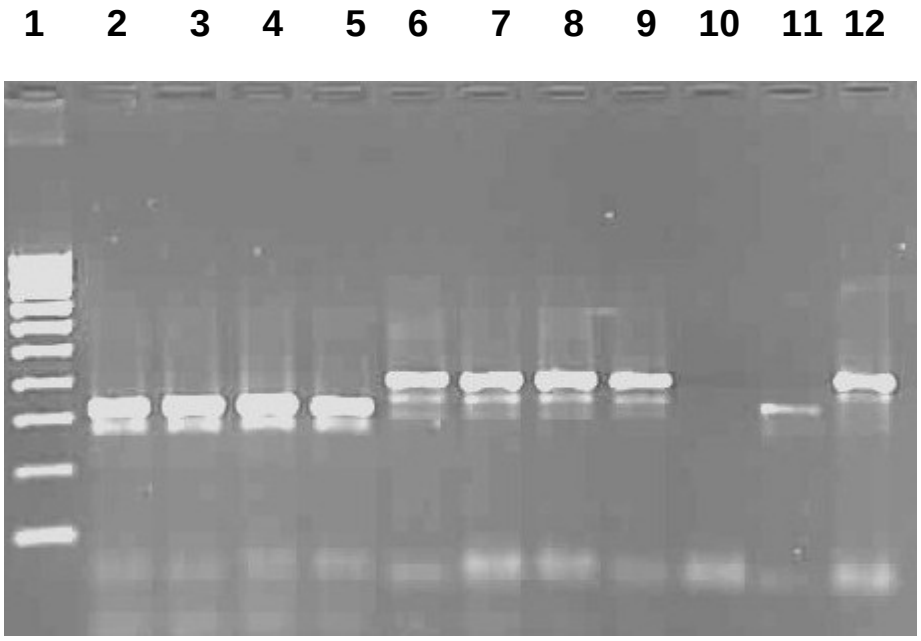
Site No.	No. of larvae collected	No. tested in PCR (sub-sample)	*An. merus* (n)	*An. quadriannulatus* (n)	*An. arabiensis* (n)	Unsure (n)	failed (n)
PE27	40	10	10	0	0	0	0
PE28	30	10	8	0	0	0	2
PE9	54	12	0	0	0	6	8
PE35	40	10	10	0	0	0	0
PE37	20	12	0	0	0	0	12
PE38	58	12	0	0	0	0	12
PE40	12	12	2	0	0	5	10
PE11	120	10	7	0	0	0	1
PE29	50	10	0	0	0	0	10
PE33	33	10	9	0	0	1	1
PE5	110	8	7	0	0	0	1
PE13	40	8	0	0	0	0	8
PE14-1	90	12	9	0	0	0	3
PE15	80	10	0	0	10	10	0
PE19	60	13	0	0	0	0	12

Site No.	No. of larvae collected	No. tested in PCR (sub-sample)	*An. merus* (n)	*An. quadriannulatus* (n)	*An. arabiensis* (n)	Unsure (n)	failed (n)
PE20	50	25	24	0	0	0	1
PE21	90	10	9	1	0	0	0
PE22	30	12	0	0	0	0	12
PE23	30	10	10	0	0	0	0

APPENDIX 4

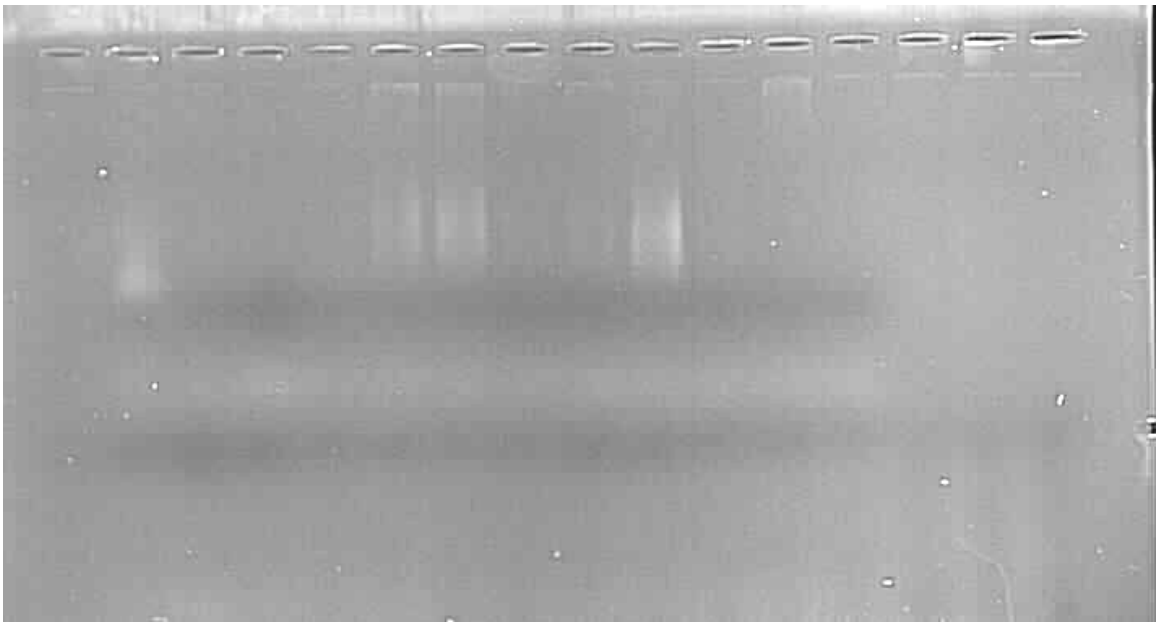


Appendix 4a: Mosquito gel: Specimens in lanes 3 and 5 are *An. merus* (466bp). Lanes 7 and 8 are controls: *An. arabiensis* and *An. gambiae* s.s. respectively

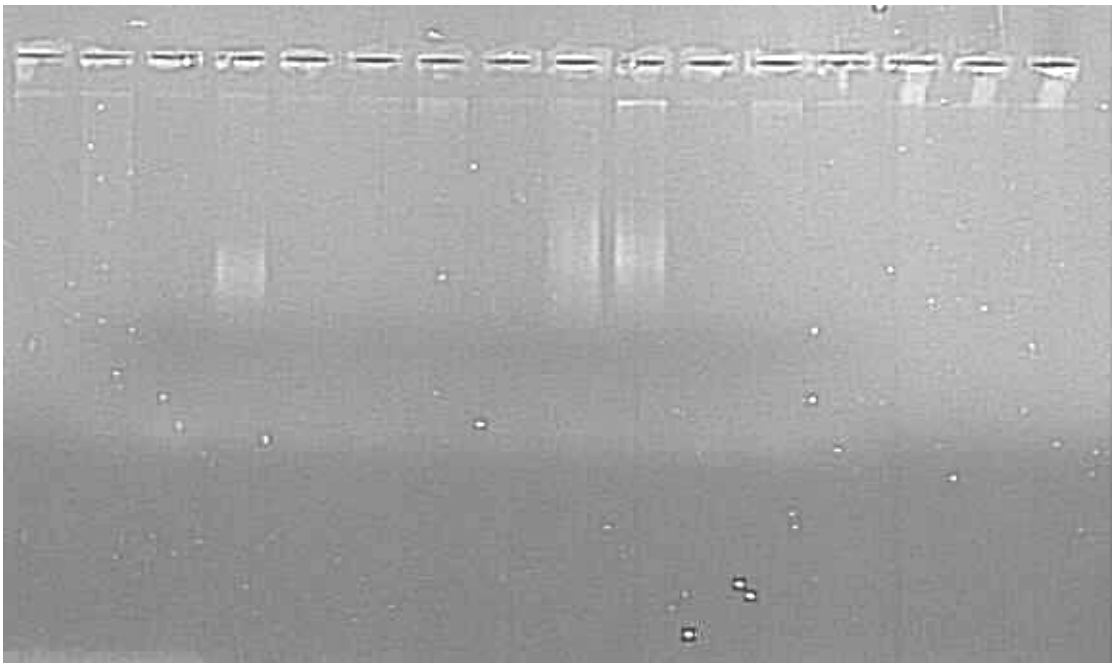


4b: Larval DNA extraction control test

4c: Whole Genome larvae DNA check



Failed Population



Identified population