The Bionomics, Population Structure

and Roles in Transmission of Malaria

Vectors in Mozambique and Angola

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This work is dedicated to my

children and their great grandparents

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ABREVIATIONS

°	Degree
°C	degrees centigrade
µg	microgram
µL	microlitre
A	*Aedes*
An	*Anopheles*
ASP	Aspiration
BB	Blocking Buffer
bp	base pairs
CSP	circumsporozoite protein
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dTTP	deoxythymidine
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
h	hour
HBI	Human biting catch indoors
HBO	Human biting catch outdoors
HC	collection with aspirators (also known as manual collection)
IAM	Infinite alleles model of mutation
IGS	Intergenic spacer
IRMA	Immunoradiometric assay
IRS	Indoor residual spraying
ITNs	Insecticide treated nets
ITS	Internal transcribed spacer
kb	kilobase pairs (1000bp)
kdr	Knock down resistance
km	kilometre
LAR	Larvae collection
LT	Light Trap
M	Molar
m^2	square meter
mA	miliamperes
Mabs	monoclonal antibodies

mg	milligram
min	minute
mL	Millilitre
mM	milimolar
mm	millimetres
MRP	Malaria Rapid Panel
mtDNA	mitochondrial DNA
no.	Number
OD	optical density
PBS	Phosphate Buffered Saline
PBS	Phosphate Saline Buffer
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
PSC	Pyrethrum spray collection
RAPD	Random amplified polymorphic DNA
RBM	Roll Back Malaria Initiative
rDNA	Ribosomal DNA
RFLP	Restriction fragment length polymorphism
RNA	ribonucleic acid
rpm	rotation per minute
S	South
sec	second
SMM	stepwise mutation model
SR	Sporozoite rate
STE	Sodium, Tris EDTA
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
u	unit
UNICEF	United Nations Children's Fund
UV	ultra-violet
vs	versus
WHO	World Health Organization
μM	Micromolar

Abstract

Studies on bionomics, population genetic structure and the roles in transmission of malaria vectors were undertaken with samples collected from 47 localities in Mozambique and 6 localities in Angola. Mosquitoes were collected using adult collection (resting and biting) and larval collection. Sporozoite rates (SR) were determined using an ELISA and mosquito identifications were performed using the latest available rDNA-PCR based techniques.

In Mozambique *Anopheles arabiensis* is widely distributed whereas *An. gambiae* S-form is uncommon in southern areas with rainfall less than 600 mm per annum, being the latitude 24° S, the southernmost record for this species. *An. merus* was found as far as 350 km inland as well as along the coast. *An. merus* showed ability to breed in both brackish and fresh water. *An. quadriannulatus* was restricted to dry areas south of 24° S. *An. funestus s.s* was common and *An. rivulorum* and *An. leesoni* were occasionally found in human dwellings.

In indoor resting collection during the wet season, the SR indicated that *An. gambiae s.s* (9.32 ± 2.19 %), *An. funestus* (5.59 ± 1.14 %) and *An. arabiensis* (3.99 ± 1.19 %) are the primary malaria vectors (χ^2=22.55, 2 df, P<< 0.001). However, the three species maintained malaria transmission equally during the dry season albeit at a lower level. *An. merus* is uncommon but plays a significant role in some localities.

An. funestus was more endophagic, *An. arabiensis* and *An. merus* were more exophagic whereas *An. gambiae* fed equally indoors and out. The SR of *An. arabiensis* indoors (1.72 ±1.37 %) and outdoors (1.98 ± 1.21 %) were similar. Also the SR of *An. gambiae s.s* was similar indoors (10.58 ± 5.94 %) and outdoor (13.46 ± 6.59 %). None of the mosquitoes caught indoors before 21:00 were infected. Outdoors 20 % of infected *An. arabiensis* and 7 % of *An. gambiae* were found before 21:00 h. The SR of *An. funestus* indoors was significantly higher (4.14 ± 1.66 %) than out (1.35 ± 1.32 %) (χ^2=4.0, 1 df, P=0.04). No infected *An. funestus* moquitoes were collected before 21:00 h. These results indicate a significant risk of acquiring malaria both in and outdoors. However, the majority of infective bites occurred after 21 h when most children and pregnant women were in bed. The use of ITN may protect this high-risk group from infective bites.

In Angola *An. gambiae s.s.* M-form was the predominant species and the S-form was recorded for the first time. Overall the proportion of S form was 3.8 % (n= 132) in coastal Benguela and 19% (n= 79) in Huambo. *An. melas* was found in one coastal area. A single specimen of *An. arabiensis* was found in a larval collection in a suburban area of Luanda, being the first record for Angola. It is not clear why both *An. arabiensis* and *An. melas* were rare. *An. funestus s.s.* (SP ≈ 1%, n=140) was the only member of the *An. funestus* complex found, and together with *An. gambiae s.s.* M-form (SP ≈ 2%, n=583) these were the main malaria vectors. The *kdr* allele was found to be present in both M and S forms of *An. gambiae*. This represents the southernmost known distribution of *kdr* resistance in continental Africa, suggesting a much wider distribution than hitherto recorded.

The population structure of *An. funestus s.s.* was assessed on the basis of twelve microsatellite loci. Results indicate a high level of genetic diversity but deviations from Hardy-Weinberg expectations were significant at most loci used. The null alleles were the main cause of heterozygote deficit although problems in resolving alleles may have led to the underestimation of heterozygosity in some gels. Significant differentiations was found in samples collected in Mozambique (pair-wise F_{ST} range: 0.0126-0.1202) as well as when these were compared with a sample from Angola 2500 km apart (pair-wise F_{ST} range: 0.0238-0.0777). These results provide some hope for management of the spread of resistance in *An. funestus* but may pose additional difficulty for those contemplating the use of transgenic mosquitoes as a means of controlling malaria.

CHAPTER I

1.1   Introduction

Despite more than a century of efforts to eradicate or control malaria, the disease remains a major and growing threat to the public health and economic development of countries in the tropical and subtropical regions of the world (Gardner *et al.* 2002). Sachs and Malaney (2002) stated: *"Where malaria prospers most, human societies have prospered least"*. The authors observed that the global distribution of per-capita gross domestic product shows a striking correlation between malaria and poverty, and malaria-endemic countries also have lower rates of economic growth. Up to USD12 billion is lost in productivity due to malaria in Africa every year (Samba, 2001).

Malaria is now on the rise again; despite global economic development, more people die from malaria nowadays than 40 years ago (Guerin *et al.*, 2002). The increase of the burden of malaria is attributed to drug and insecticide resistance and social and environmental changes (Greenwood and Mutabingwa, 2002). Another issue that has also contributed to the rise of malaria is our poor understanding of the complex biology and ecology of the vectors. It is expected that climatic changes, including global warming and associated increased precipitation, will extend vector ranges and population sizes of some species, potentially increasing malaria transmission. The most recent map of its distribution is shown in Figure 1. There are concerns for the reintroduction of malaria into European countries, especially Greece and Italy, where malaria was once highly endemic (Jetten *et al.*, 1996; Lindsay and Birley, 1996). Autochthonous *Plasmodium falciparum* malaria has been reported from Central Europe (Delmont *et al.*, 1994; Mantel *et al.*, 1995; Peleman *et al.*, 2000; Whitfield *et al.*, 1984). The most recent case of autochthonous *P. falciparum* was

observed in Germany with evidence of local transmission by an indigenous vector (Krüger *et al.* 2002).

The World Health Organization (WHO) estimated approximately 500 million malaria cases per annum resulting in more than two million deaths (WHO, 1996) but recent estimates indicates at minimum, between 700,000 and 2.7 million persons die yearly from malaria, over 75% (≈0.5-2 million) of them African children (Breman (2001). The burden of the disease in childhood (Murphy and Breman, 2001) and pregnancy (Steketee *et al*, 2001) are well discussed in a special supplement to the *American Journal of Tropical Medicine and Hygiene*, volume 64, number 1,2 of 2001.

Although four parasite species of the genus *Plasmodium* are responsible for human malaria nearly all malaria deaths and a large proportion of the morbidity is caused by *P. falciparum* (Guerin *et al.*, 2002). The parasites are transmitted by female mosquitoes of the genus *Anopheles* but can rarely be transmitted by other routes such as blood transfusion, syringe and needle, or congenital by transplacental transmission.

It was in Sierra Leone in 1899 that human malarial parasites were first observed in wild-caught *An. gambiae* and *An. funestus* by Ronald Ross (Bockarie *et al.*, 1999). In the same year Ross initiated the first antilarval measures for malaria control. Since then many methods have been employed to control or even eradicate the disease. The most important was the combination of spraying houses with residual insecticides and chemoprophylaxis, which together with other measures was able to eliminate or suppress transmission in many parts of the world, half a century ago.

Presently, malaria control tools include prompt access to treatment especially for young children, prevention and control in pregnant women, vector control, and prediction and containment of epidemics. Tools under consideration include the development of efficient drugs and vaccines (Doolan and Hoffman, 2001; Gardner *et al.* 2002) as well as

genetic control of malaria vectors to make them refractory to the parasite (James *et al.*, 1999).

While waiting for an effective vaccine or means of driving genes for malaria refractoriness in the vectors, insecticide treated nets (ITN) are an important part of the Roll Back Malaria Initiative (RBM).

The success of ITN programmes requires a profound understanding of the biology and ecology of the malaria vectors, such as species distribution and composition, breeding habits, feeding behaviour, etc. For those contemplating the use of genetically modified mosquitoes as a means to control malaria, knowledge of the population structure as well as the pattern of gene flow within a target species constitute a pre-requisite before any release of genetically modified mosquitoes. This knowledge is crucial as they determine how far introduced genes will spread, which in turn is important in determining the spatial and temporal scale required for gene introductions (Kamau *et al*, 2002).

There are, for example, concerns that large-scale use of pyrethroid impregnated nets may select for pyrethroid resistance. Because gene flow is a useful indicator of direction and rates of dispersal among populations (Slatkin, 1987), knowledge of population genetic structure is useful in predicting the spread of insecticide resistance associated with particular genes (Kamau *et al.* 1999; Kamau *et al*, 2002). This could help in development of effective strategies for insecticide resistance management (Lehmann *et al.*, 1996).

Malaria, 2002

Areas where malaria transmission occurs
Areas with limited risk
No malaria

Source: WHO, 2002
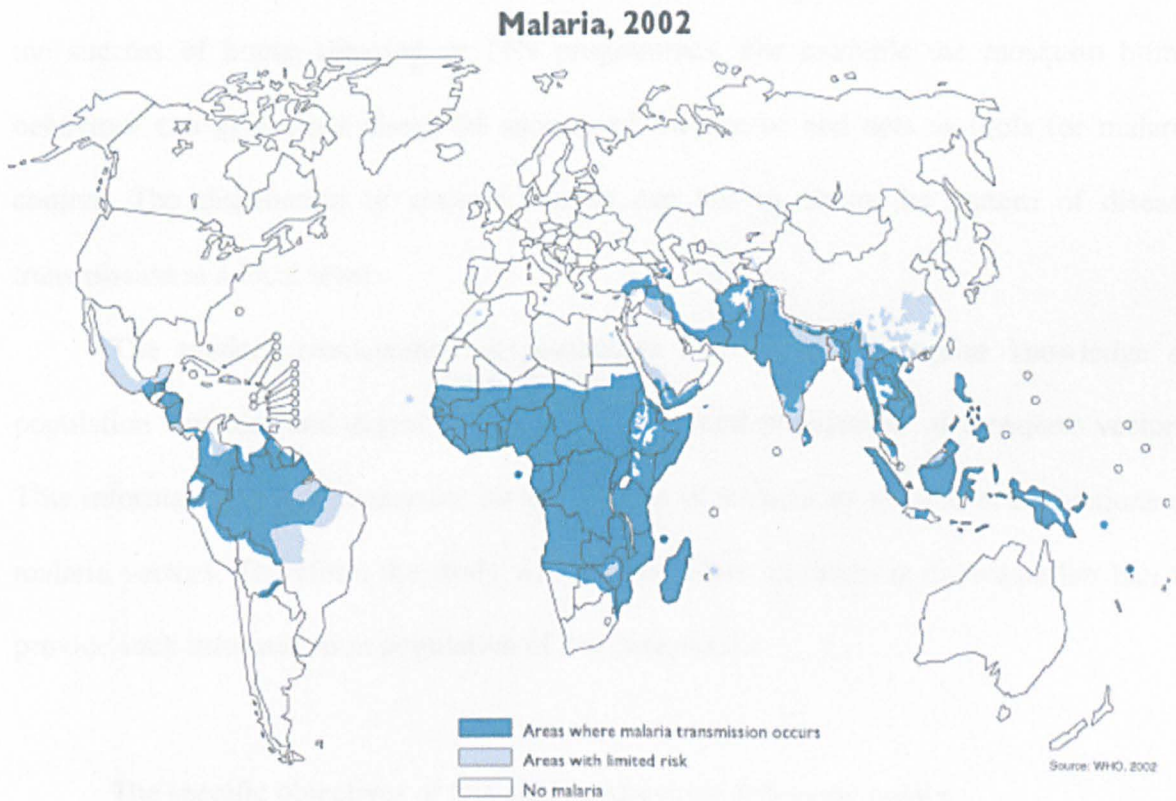
FIGURE 1.1 Geographical distribution of malaria according to the WHO (source: http://mosquito.who.int/cmc_upload/0/000/016/101/Malaria2003_map.pdf)

1.2   Aims and objectives of the study

The purpose of this study was to provide information on the bionomics, distribution and the role of malaria vectors in disease transmission, important parameters that determine the success of house spraying or ITN programmes. For example the mosquito biting behaviour can give clues about the success of the use of bed nets as tools for malaria control. The distribution of malaria vectors can tell us about the pattern of disease transmission at a local level.

The modern management of insecticide resistance will require knowledge of population structure and degree of gene flow in natural populations of mosquito vectors. This information is also important for the control of malaria by genetic manipulations of malaria vectors. Therefore, the study will use the allele variation at microsatellite loci to provide such information in population of *An. funestus s.s.*

The specific objectives of this study address the following points:

1   Distribution and role of individual species in malaria transmission within *An. gambiae* and *An. funestus* complexes in Angola and Mozambique

2   Distribution of molecular forms of the *An. gambiae s.s.* in Angola and Mozambique and their *kdr* (knock down resistance) status in relation to pyrethroid insecticides

3   The biting behaviour of principal malaria vectors, the time and location (indoors or outdoors) of major malaria transmission.

4   To test microsatellite primers loci of *An. funestus s.s.* in populations from different geographical origins and determine whether the *An. funestus* population is structured according to its geographical origins.

The last objective is derived from the hypothesis that "the limited flight range of *An. funestus* (Gillies and De Meillon, 1968) may determine some genetic distinctiveness between geographical populations of this species".

CHAPTER II

Literature review

2.1 The vectors of malaria

The vectors of human malaria all belong to the genus *Anopheles*. The genus *Anopheles* belongs to the Culicidae Family. The classification of the genus *Anopheles* has been reviewed by Harbach (1994).

Anopheles comprises 558 species (Harbach, 1994), however, only about 70 can transmit malaria to human beings and of these only 40 are important malaria vectors (Renshaw and Silver, 2001; Service, 2000). Only female mosquitoes can transmit the disease, as they need blood to complete the development of eggs. The mosquito initially acquires the malaria parasite by ingesting blood from an infected human being. After at least 10 days of a complex cycle of development in the mosquito, the parasite is passed via the saliva when the mosquito next bites someone to get blood. Once the salivary glands are infected, each subsequent bite of the mosquito is potentially infective.

The capacity of mosquitoes to transmit malaria, called vector capacity, is a measure of the efficiency with which mosquitoes carry malaria parasites from one human to another, and is an estimate of the number of secondary cases of malaria generated by one primary case (Gallup and Sachs, 2001). This capacity depends on many factors. Some mosquitoes are not attracted to humans to feed, preferring to feed on other mammals or reptiles. Some mosquitoes do not live for long enough to complete the sporogonic cycle of the malaria parasite. Others mosquitoes are just refractory to the development of the parasite.

Many of the most important malaria vectors comprise groups of morphologically similar species, which however vary in their behaviour and ecology and hence in their role in disease transmission. These groups of species are known as species complexes. For example the Gambiae Complex (Davidson *et al.*, 1966) and Funestus Complex (Gillies and Coetzee, 1987), the Culicifacies Complex in India and neighbouring countries (Subbarao and Sharma, 1997), Dirus Complex in Thailand (Bamai *et al.*, 1984) contain the most efficient malaria vectors in the world. A list of anopheline species incriminated as vector of malaria so far in the world has been given (Service, 2000; http://skonops.imbb.forth.gr/AnoBase/Species/malariaspecies.html).

The Gambiae Complex comprises at least seven biological entities of which *An. gambiae* and *An. arabiensies* are the most dangerous malaria vectors. The complex includes also *An. merus*, *An. melas* and *An. bwambae* known as localised malaria vectors and the zoophilic *An. quadriannulatus* (species A and B, see section 2.1.1) not involved in disease transmission. *An. gambiae s.s.* is now being recognised as further subdivided in M and S molecular forms of *An. gambiae s.s.* (section 2.1.3) (Favia, *et al*, 1997), thought to be incipient species.

Although, the Funestus Complex harbours several biological species only one, *An. funestus s.s.* is a major vector of malaria. *An. rivulorum* has been implicated in disease transmission only in Tanzania (Wilkes *et al.*, 1996). Other minor/local vectors includes *An. nili*, *An. moucheti*, and *An. paludis* (Fontenille and Lochouarn, 1999).

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2.2 The *Anopheles gambiae* complex

2.2.1. Historical notes

Until the early 60's *An. gambiae* was considered as a single biological entity, with some varieties breeding in fresh and others in saltwater (e.g.: De Meillon, 1941, Muirhead-Thompson, 1945, 1951). Evidence that *An. gambiae* may represent more than a single biological entity was first produced by Muirhead-Thomson (1948) in Nigeria. The author, crossing the fresh water breeding form with the salt water breeding form (called varity-*melas*), found that the male hybrids were apparently sterile and F_2 eggs from apparently normal hybrid female failed to hatch. Earlier however, evidence was presented about the specific distinctiveness of the salt water breeding members of the complex (Ribbands, 1944; Muirhead-Thompson, 1945).

Almost a decade later, Davidson (1956), studying the mode of inheritance of DDT resistance in *An. gambiae* found, unexpectedly, sterility between two freshwater breeding populations of this species. It seems, however, that the author did not at first realise the importance of his findings.

Later, Davidson and Jackson (1962) confirmed the existence of two freshwater forms (A and B) within *An. gambiae* which, when crossed resulted in the production of sterile F1 males. In the same year, Paterson (1962) demonstrated in Tanzania that the eastern salt-water breeder was partially incompatible with two strains of the complex. Discussing the nature of *An. gambiae*, based on their own experiments and available literature, Davidson (1962), stated "It would thus appear that *An. gambiae* is a complex of at least four partially incompatible forms. Whether any or all deserve rank as separate species remains to be seen".

Evidence for a fifth member of the complex, called provisionally species C, was presented by Paterson *et al.* (1963), with further direct evidence for assortative mating in

nature between forms A, B and C, therefore direct evidence that those forms are distinct species (Paterson, 1964). Later, the sixth member of the complex was presented from Uganda and named species D (Davidson and White, 1972, Davidson and Hunt, 1973).

The genetic distinctness of the six species of the complex was also confirmed by cytogenetic studies that revealed fixed inversions differences among taxa (Coluzzi and Sabatini, 1967; 1968; 1969; Davidson and Hunt, 1973).

Presently six formally named species are recognised namely *An. gambiae sensu stricto* (*s.s.*) (species A), *An. arabiensis* (species B), *An. quadriannulatus* (species C), *An. bwambae* (species D), *An. merus* and *An. melas*. The seventh member of the complex, has been discovered recently in Ethiopia, yet without formal name, is provisionally called *An. quadriannulatus* B to differentiate from the species *An. quadriannulatus* A found in southern areas of Africa. The M and S molecular forms in the *An. gambiae s.s.*, thought as being incipient species, are discussed in section 2.1.3.

The recognition that *An. gambiae* was a complex of cryptic species has helped to explain the pronounced ecological and behavioural diversity as well as the heterogeneity in vector capacities within these taxa. This heterogeneity makes it important that a population of mosquitoes in a given locality is well known, i.e. their behaviour, vector status, before any control efforts are put in place. These aspects will form the main core of the following discussion.

2.2.2 Biology and disease transmission

The *An. gambiae* complex now comprises at least seven sibling species. These species exhibit different ecological adaptations and behaviour, and consequently different degrees in their vector capacity.

Although it was known for a long time that the species A (*An. gambiae*) and B (*An. arabiensis*) are important vectors of malaria in Africa, it was first fully documented by White and Magayuka (1972). They showed that *An. gambiae* s.s. had a consistently high sporozoite rate (averaged 4.2%) while *An. arabiensis* had low sporozoite rates (0.3%). The lower sporozoite rate in *An. arabiensis* was most likely due to the greater zoophily of that species but such marked differences are not necessary typical.

An. gambiae s.s. is widely distributed (White, 1974), and the most efficient and important vector of malaria in Africa (see Gilles and De Meillon, 1968; White, 1974; Gilles and Coetzee, 1987). This exceptional vectorial capacity of *An. gambiae* s.s. is due to its close association with humans and pronounced degree of anthropophagy and endophily (see White, 1974; Gilles and Coetzee, 1987). This species is also regarded as an important vector of filariasis in rural areas of Africa (White, 1974, Mnzava *et al.*, 1989, Merelo-Lobo *et al.*, 2003).

Although with greater zoophilic tendency (reviews in White, 1974 and Gilles & Coetzee, 1987), *An. arabiensis* is also regarded as a main vector of malaria in many localities, particularly in arid or montane areas prone to seasonal outbreaks (White, 1974), and in the absence of *An. gambiae* it may act as the sole malaria vector and secondary vector of lymphatic filariasis (White, 1974).

Although with overlapping distribution, *An. gambiae* is predominant in humid, rainy areas (Lindsay *et al.* 1998, Touré *et al.*, 1998) and *An. arabiensis* becomes more prevalent in arid areas (Coluzzi *et al.* 1979; Lindsay *et al.* 1998; Touré *et al.*, 1998).

An. quadriannulatus A, thought to be exophilic has been found, in some areas, in large proportions resting inside houses. Nevertheless, it is thought to be exclusively zoophilic and, thus a non-vector of human parasites (see Gilles and Coetzee, 1987 and White, 1974). More recently, however, in a series of experiments using laboratory-reared mosquitoes Pates *et al.* (2001) have demonstrated anthropophagic behaviour in *An. quadriannulatus.* Takken *et al.,* (1999) also found in laboratory experiments that this species was susceptible to *P. falciparum* infection. In areas where this species is found in human dwellings, in the absence of cattle or game, there is a theoretical possibility that some may feed on humans and hence be involved in the transmission of malaria.

The breeding habitats of *An. gambiae s.s.* and *An. arabiensis* are generally similar, mostly temporary water, sunlit pools (Gillies and Coetzee, 1987, Charlwood and Edoh, 1996; Minakawa *et al,* 1999). Both species are dependent upon rainfall, using transient puddles, animal hoof prints and shallow depressions as principal breeding sites (Service, 1993).

In West Africa, larvae sites are even shared among the incipient species of *An. gambiae* (Coluzzi *et al.*, 1985). The only study of breeding sites in relation to M and S molecular forms is that of Edilo *et al.* (2002). However, this study failed to produce convincing evidence that the molecular forms differ in their utilisation of larval substrates.

An. merus, An. melas, An. bwambae are vectors of human malaria of lesser epidemiological significance (Service, 1985). *An. merus*, the East African brackish-water species, was found to be a vector of malaria (Muirhead-Thomson, 1951; Mosha and Petrarca, 1983) and of lymphatic filariasis (Mosha and Petrarca, 1983; Bushrod, 1981) only in Kenya and Tanzania. *An. melas*, the West African brackish-water species, is thought to play a secondary role in malaria transmission (Bryan, 1983; Akogbeto and Romano, 1999; Awola *et al.*, 2002), although Besansky (1999) has suggested it may be an important vector. *An. bwambae* is found around geothermal springs in Bwamba County, Uganda (Harbach *et al.*,

1977), is endophagic and partially endophilic. It is considered to be a local vector of malaria (White, 1974).

Members of *An. gambiae* complex have been incriminated in viral transmission of an epidemic of O'nyong-nyong fever in 1959 in Uganda (Haddow *et al.*, 1960; Williams *et al.*, 1965). Although other viruses have been isolated from members of the *An. gambiae* complex, such as Tataguine, (Tomori, 2001, Gillies and Coetzee, 1987) Chikunguya, Ngando-group, Sindbis and Germiston (Gillies & Coetzee, 1987), the information available about their natural role in the transmission is so far inconclusive.

2.2.3   Incipient speciation within *Anopheles gambiae s.s.*

Studies on chromosomal inversion polymorphisms in West Africa have showed sharp discontinuities between populations that are in sympatry within *An. gambiae s.s* (Bryan *et al.*, 1982; Coluzzi *et al.* 1985; Coluzzi *et al.* 1977; 1979; Touré *et al* 1998).

In some West African populations of *An. gambiae s.s* the karyotype frequencies were often far from Hardy-Weinberg expectations with a notable excess of homokaryotypes for inversions in the right arm of chromosome II. When samples are subdivided into certain sets of inversions, the subsets of karyotypes are in Hardy-Weinberg proportions. From these observations, five different sets of inversions (arrangements) were suggested and they were thought to be reproductively isolated units and were given a non-Linnean nomenclature: Bamako, Mopti, Savanna, Forest and Bissau (Coluzzi *et al.* 1985; Touré *et al* 1983). The polymorphic paracentric inversion sets of the chromosome II in *An. gambiae* have been correlated with adaptations to environmental conditions (Coluzzi *et al.*, 1979, 1985, Touré *et al.*, 1998).

From the middle 90s the debate on these incipient species involved studies at DNA level. Initially, Favia *et al.* (1994), found distinctive RAPD (randomly amplified

polymorphic DNA) fragments among forms. Later, Favia *et al.* (1997) found that Mopti is

consistently different from Savanna and Bamako at nucleotide sites in the intergenic spacer

(IGS) of rDNA. A diagnostic procedure was developed on the basis of PCR amplification

followed by a restriction enzyme digest that reliably distinguished Mopti from Savanna and

Bamako in samples from Mali and Burkina Faso (Favia *et al.* 1997). More recently Favia *et*

al. (2001) developed a primer-specific PCR approach for the IGS that allows the same

distinction in samples from Mali. This PCR-RFLP has been used in increased samples from

West Africa and extended to include populations from outside West Africa (della Torre *et*

al. 2001); the terms M and S were proposed to indicate the two IGS types. On the other

hand, based on internal transcribed spacer (ITS), Gentile *et al* (2001), were able to design a

PCR based technique that distinguishes *An. gambiae* s.s. in two types of rDNA, type I and

type II corresponding to molecular forms S and M in della Torre *et al* (2001), respectively.

However, these forms or types are not wholly concordant with the chromosomal forms (della

Torre *et a.l*, 2001; Gentile *et al.*, 2001; Gentile *et al.*, 2002), suggesting that chromosome

arm II inversions are involved in ecotypic adaptation rather than in mate recognition (della

Torre *et al.*, 2001). For example in some areas of Mali and Burkina Faso, there is clear

correspondence between the M molecular form and the Mopti chromosomal form, the S

molecular form with the Savanna or Bamako chromosomal form (Favia *et al.*, 1997).

However, in other areas of West Africa, this correspondence does not occur (della Torre *et al.*,

2001; Wondji *et al.*, 2002). In East Africa only the savannah chromosomal form occur, where

it is sympatric with *An. arabiensis* (Coetzee *et al.*, 2000)

The taxonomic status of the molecular forms is still a matter of intense scientific

debate. However, there are now some molecular data supporting speciation within *An.*

gambiae s.s., which includes the microsatellite loci data (Wang *et al.*, 2001; Wodji *et al*,

2002). The work of Wang *et al.* (*loc. cit*), in particular, revealed that Mopti differs

significantly from Savannah at two microsatellite loci from the proximal region of the *X*, outside the *Xag* inversion. Sympatric populations of S and M-form from Cameroon and Democratic Republic of Congo, showed statistically significant levels of differentiation throughout the genome, with the highest levels of differentiation observed on the X chromosome (Lehmann *et al.* 2003)

The debate about the reproductive isolation of molecular foms involved other types of studies. Examining the sperm in spermatheca of wild caught female mosquitoes, Tripet *et al.* (2001) found evidence of positive assorting mating, with nearly 99% intraform mating among the 250 samples analysed, but they did not observe significant hybridization between forms, indicating a lack of complete reproductive isolation. In addition the *kdr* allele in the *para* sodium channel gene, which confers resistance to pyrethroid insecticides, is found in the S form population of West Africa, but not in the M population in the same localities (Chandre *et a.l*, 1999; della Torre *et al.* 2001), but the *kdr* allele was found in both M and S forms (Fanello *et a.l*, 2000) and in Angola (Chapter 7). Fanello *et al.* (2003) suggested that this phenomenon is probably a result of gene flow from S to M-form, but divergence leading to incipient speciation can occur between populations that exchange genes (Michalak *et al.* 2001). Other evidence that may support isolation of these forms comes from the field observation that M/S heterozygotes were very rare (≤0.3%), even in sympatric populations (della Torre *et al.*, 2001).

In discussing the present developments within *An. gambiae s.s*, della Torre *et al.* (2002), states "these data suggest that we are observing speciation in its very earliest stages, with the persistence of variation shared because of recent common ancestry and with low levels of gene flow continuing to homogenise regions of the genome not directly involved in the speciation process". Whereas the molecular form S is the most common and widespread in the African continent, the exact extent of the distribution of the

molecular form M is yet to be determined. Nevertheless, recent evidence suggests that the distribution of the M molecular form extends further south to the coastal areas of Angola (Carrara *et al*, 2002). This information together with the results from the present thesis (Chapter VII) suggests that in Western Africa this form is present as far as the southern limit of the distribution of the *An. gambiae* complex. The vector capacity and insecticide resistance of the individual molecular forms requires further clarification.

2.3. The *Anopheles funestus* complex

2.3.1 Historical notes

The *An. funestus* complex comprises at least nine members, the adults of which are not easily distinguished on the basis of morphological characteristics (Gillies and De Meillon, 1968; Gilles and Coetzee, 1987). These are *An. aruni* Sobti, *An. brucei* Service, *An. confusus* Evans, *An. funestus sensu stricto*, *An. fuscivenosus* Leeson, *An. leesoni* Evans *An. parensis* Gillies, *An. rivulorum* Leeson and *An. vaneedeni* Gillies & Coetzee.

The different species of the *Anopheles funestus* complex were initially recognised in Zanzibar (Sobti, 1968) and South Africa (Gillies, 1962; De Meillon *et al.*, 1977) by their behaviour after the removal of the domestic vector species through the spraying of houses with insecticide (Green & Hunt, 1980).

2.3.2 Biology and disease transmission

An. funestus is widespread throughout the African continent (Gillies & Coetzee, 1987) and Madagascar (Mouchet *et al*, 1998). *An. rivulorum* is the second most abundant and widespread species within the group, and occasionally collected indoors along with the dominant and major malaria vector *An. funestus* (Hackett *et al.*, 2000). *An. leesoni* is

widespread, occurring from Ethiopia through to the northern parts of South Africa and across West Africa; *An. parensis* and *An. confusus* are found in Kenya and Tanzania and in the North to Kwazulu/Natal province in South Africa (Gillies and Meillon, 1968). *An. vaneedeni* occur only from Mpumalanga and Northern province in South Africa, *An. aruni* in Zanzibar, *An. fuscivenousus* in Zimbabwe and *An. brucei* from Nigeria (Gillies & De Meillon, 1968; Gillies and Coetzee, 1987).

The recognition of *An. funestus* as a formidable vector of malaria in Africa is not new. *An. funestus* is probably the most domestic of malaria vectors, preferring to feed and rest inside human houses (Gillies and De Meillon, 1968). Even in human dwellings shared with cattle and/or goats, most female *An. funestus* bite humans and congregate in sections occupied by humans (Gillies and De Meillon, 1968). In a coastal village in Ghana, where no residual insecticide spraying had yet occurred, Brady (1974) found that fed females resting outdoors had largely fed on man. These characteristics make *An. funestus* a formidable vector.

The clear importance of *An. funestus* in the transmission of malaria is demonstrated by the following few examples. In the Sahel area of Senegal, the decrease of *An. funestus* population due to scarcity of appropriate larval habitats also reduced the endemicity of malaria (Mouchet *et al.*, 1998). In Madagascar, malaria transmission ceased with the eradication of *An. funestus*, but an epidemic outbreak was observed in 1987. This was thought as a result of the reestablishment of the *An. funestus* in the country (Fontenille and Rakotoarivony, 1988).

In Western Africa the droughts that affected the area since the 1970's have affected the populations of *An. funestus* in some localities (Julvez *et al.*, 1997 Mouchet *et al.*, 1998). However, recent reports show, *P. falciparum* circumsporozoite antigen (CSA) and sporozoite rates exceeding those of other vector species (Manga *et al.* 1997, Sokhna *et al.*, 1998; Elissa *et al.*, 1999, Fontenille *et al.*, 1997), with a higher entomological inoculation rate

than *An. gambiae s.l.* (Elissa *et al.*, 1999). In some localities of the continent, this species has been found to act as the main or sole vector (Fontenille *et al.*, 1997, Lochouarn *et al.*, 1998).

As in Western Africa, in some localities of Eastern Africa, the species was found to have a higher CSA rate than members of the *An. gambiae* complex (Githeko, 1992; Githeko *et al.*, 1993; Charlwood *et al.*, 1998; Shililu *et al.*, 1998, Temu *et al.*, 1998). The picture is similar in the southeastern region, including Madagascar (Fontenille *et al.*, 1990; Severini *et al.*, 1990). In Madagascar, *An. funestus* was found in some instances to be the sole vector of malaria and was responsible for some epidemic outbreaks (Fontenille & Rakotoarivony, 1988, Ralosoa *et al.*, 1991, Randriantsimaniry, 1995).

An. funestus is also recognised as an important vector of Bancroftian filariasis under rural conditions (Gillies and De Meillon, 1968 Merelo-Lobo *et al.*, 2003). The species was found to carry O'nyong-nyong virus in East Africa (Williams *et al.*, 1965; Johnson *et al.*, 1981; Lutwama *et al*, 1999) and Tataguine virus in Nigeria and in the Central African Republic (Tomori, 2001).

An. rivolurum is the only other member of the group that has been found naturally infected with malaria parasites (Wilkes *et al.*, 1996). Although, *An. vaneedeni* was susceptible to malaria parasites in the laboratory, it has not yet been found naturally infected with parasites, although it readily feeds on humans outdoors (De Meillon *et al.*, 1977). The other members of the *An. funestus* complex are believed to be unimportant in the transmission of malaria due mainly to their zoophilic feeding preferences (Koekemoer *et al.*, 1998).

The Evans report of 1963 (cited in Gillies and De Meillon, 1968) is by far the best summary about larvae breeding sites of *An. funestus*. It stated that *An. funestus* breeds characteristically in more or less permanent clear water bodies (swamps, sides of streams and river edges, seepage, ponds etc), the existence of emergent vegetation being the most important feature. A recent study found again that *An. funestus s. l.* is associated with larger,

semi-permanent bodies of water containing aquatic vegetation and algae (Gimnig *et al.*, 2002). Until recently the lack of reliable tools for the identification of members of *An. funestus* complex limited our knowledge on breeding requirements of members of this complex. Hence it is not clear whether the breeding sites for individual species of the *An. funestus* complex show habitat partitioning.

2.4   Methods for discrimination of members of *Anopheles gambiae* and *Anopheles funestus* complexes

Because of the differences that exist in ecology and vector status of isomorphic species, it is essential to identify accurately individuals to species within any species complex for the correct evaluation of malaria vector ecology and control programmes. As stated by Besansky (1999), meaningful epidemiological studies and effective vector control programs depend upon efficient methods for discriminating among the major vectors, lesser vector and non-vectors.

Since the emergence of evidence that *An. gambiae* was a complex of cryptic species (Paterson, 1962, Davidson and Jackson, 1962), several methods for identifying them have been developed. Renewed interest in *An. funestus* complex has resulted in the investigation of methods for identification of some members of this complex.

Using a body of available literature, the most important methods are discussed from a historical and practical perspective.

2.4.1   *Anopheles gambiae* complex

2.4.1.1 Morphological and morphometric methods

In *An. gambiae* complex, much effort has been devoted to the search for morphological characters that might enable individual specimens to be identified to species. The target characters were palpal banding ratio, number of antennal coeloconic sensillae, wing marking, leg banding and the shape of the spermatheca in adults, and setae branches and pecten comb in larval stages. An exhaustive review of these efforts was produced by Davidson *et al.* (1967). It is worth mentioning that in the most comprehensive study in *An. gambiae*, *An. arabiensis*, *An. melas* and *An. merus* by Coluzzi (1964), the author was able to demonstrate that morphological characters were only more reliable between the two salt water species (*An. melas* and *An. merus*) and between these and the fresh water species. Subsequent efforts were directed at finding morphological differences between the three freshwater species in different regions of Africa (Ismail and Hammoud, 1968; Clarke, 1971; Reid, 1975a,b) but without apparent success.

2.4.1.2 Physiological tolerance of saltwater

The physiological tolerance of saltwater by larvae of *An. merus* and *An. melas* distinguishes them from the other freshwater *An. gambiae* species. A comprehensive account of the long established method for differentiating some members of the *An. gambiae* complex based on this tolerance was presented by Gillies and De Meillon (1968) and White (1974). The requirement of living larvae for such tests was an inherent complication, with the additional disadvantage that only the saline tolerant species survive.

2.4.1.3 Hybridization tests

This technique involves rearing progeny from wild females and crossing the adult offspring with laboratory strains of known identity to determine with which members of the complex they may be interfertile and therefore conspecific. Rearing mosquitoes is not easy and the time and labour involved has rendered this technique largely obsolete. However, it is worth mentioning that this technique was important in elucidating that *An. gambiae* is a complex of species (Muirhead-Thomson, 1948; Davidson and Jackson, 1962; Paterson, 1962,) as is discussed in detail in section 2.2.1.

2.4.1.4 Cytological method

A cytological technique has been devised which identifies each sibling species by its characteristic chromosomal banding pattern (Coluzzi and Sabatini, 1967, 1968, 1969; Hunt 1973). This is still the "gold standard" method and has contributed enormously to what is known today about the biology, genetics and distribution of the *An. gambiae* complex in tropical Africa (see Coluzzi *et al.*, 1979, Coluzzi, 1982). This technique requires females of a particular physiological stage (half gravid) and expertise in chromosome squash preparation and analysis (Collins *et al.*, 1987; Paskewitz *et al.*, 1993). A disadvantage according to Mahon *et al.* (1976), is that it may introduce bias into the data as many sampling methods yield dead specimens, of which only a small number is likely to be half gravid, or where mosquitoes are alive, it necessitates a holding period. The method can be used in larvae identification, although it is restricted to those in the fourth instars and hence many of the disadvantages mentioned above apply.

2.4.1.5 Biochemical methods

Early attempts to utilise biochemical techniques to distinguish members of the complex involved chromatography of pteridine pigments (see Davidson, 1967), but proved of little practical value.

Electrophoresis of soluble enzyme profiles has proven useful in the identification of members of *An. gambiae* complex (Mahon *et al.*, 1976; Miles, 1978, 1979). This technique is superior to the chromosome method because it is faster and all adults, regardless of sex and gonotrophic cycle, are identifiable (Mahon *et al.*, 1976), hence it is suitable for mass identification (Coluzzi, 1992). In addition, the diagnostic activity is concentrated in the thorax, so the abdomen and head can be removed and examined for oocysts and sporozoites, the thorax can be dissected for microfilariae and then subjected to electrophoresis to identify the mosquito (Miles, 1979). However, a major drawback is that it can only be performed on living material or freshly frozen material (Collins *et al.*, 1987, Coluzzi, 1992). The potential of this technique for misidentification of *An. gambiae* as *An. quadriannulatus* or vice-versa could have serious consequences for malaria control (Coetzee *et al.*, 1993).

Another method involves gas chromatography of cuticular hydrocarbons (Carlson and Service, 1980; Hamilton and Service, 1983, Milligan, *et al.*, 1993). However this method has never been used for routine field studies because of the need for expensive analytical equipment and a competent chemist, and the fact that it is time consuming (Hamilton and Service, 1983).

2.4.1.6 DNA based methods

DNA-based methods for the identification of mosquito vectors of malaria in the *An. gambiae* complex now contribute significantly to epidemiological studies and the control of malaria. Some sequences of genomic DNA are unique to a particular species and if these can

be isolated from the respective organism and reliably detected, this provides a system of species diagnosis (Gilles, 1993).

DNA-based techniques have in common the advantage that DNA is stable in desiccated or alcohol-preserved species, is present in all life stages, and detection methods are usually based on differences in highly repeated components of the genome.

Initially, DNA based-techniques involved hybridisation of genomic DNA with synthetic DNA probes based upon highly repetitive DNA sequences from the genomes of several species of the complex (Gale and Crampton, 1987a,b, 1988). This makes use of radioactively-labelled DNA probes that require careful hybridisation conditions and subsequent autoradiographic detection systems (Paskewitz and Collins, 1990). However, by incorporating non-radioactive labelling and simplified hybridisation procedures, these probes have been adapted for use in field laboratories (Hill *et al.*, 1991, Hill *et al.*, 1992 a,b) although they were not subsequently used. Another method used restriction fragment length polymorphism (RLFP) (Southern, 1975), which detects species-specific restriction fragment lengths in the ribosomal RNA genes (Collins *et al.*, 1987, 1988). The requirement of Southern blots that are labour-intensive and time consuming (Paskewitz and Collins, 1990) precludes the use of RFLP in field laboratories.

2.4.1.7 The PCR technique

With the possibility of expanding any desired sequence of DNA with a polymerase chain reaction (PCR) (Mullis and Faloona, 1987), the problem of limiting target DNA was overcome. PCR is a method for enzymatic amplification of specific DNA fragments using a thermostable DNA polymerase and single-stranded DNA primers derived from sequences flanking the target region (White *et al.*, 1989)

DNA amplification by PCR has been used as a diagnostic tool in several insects of medical importance. In some cases is the unique available method that can correctly identify mosquitoes to species (Charalambous *et al*, 1999 and references therein). PCR techniques for species identification have advantages of being relatively easy to perform and not stage-specific; DNA can be extracted from fresh, frozen, desiccated specimens or from mosquitoes preserved in alcohol or Carnoy's solution. Furthermore, any life stages can be used and only small amounts of mosquito DNA (such as a leg) are required for the PCR, allowing the entire body to be used for other studies such as blood meal determination, the presence of parasites, or biochemical tests for insecticide resistance. In addition, this technique is sensitive and highly reproducible between laboratories and may be used to identify both species with interspecies hybrids, regardless of life stage, using either extracted DNA or fragments of a specimen (Scott *et al.* 1993 and Paskewitz *et al.*, 1993). The potential of this method is so high that species can still be identified after several years. Recently, Townson *et al.* (1999) demonstrated the potential of PCR in identification of museum dried specimens collected about 100 years ago suggesting PCR as being a valuable tool for resolving the formal taxonomy of sibling specie complexes. Moreover, PCR based methods for identification has the advantage of being relatively inexpensive, simple to perform and the results can be visualised a few hours after.

Random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990, Welsh and MacClelland, 1990) has been used for the identification of cryptic species of medical importance, for example identification of species of *Aedes* (Ballinger-Crabtree, *et al.* 1992; Kambhampati *et al*, 1992), *An. dirus* (Manguin *et al.* 2002), *An. minimus* (Sucharit and Komalamisra, 1997; Kengne *et al*, 2001), *An. albitarsis* (Wilkerson *et al*, 1995a,b) and *An. gambiae* (Wilkerson, *et al.*, 1993) including discrimination between molecular forms of *An. gambiae s.s.* (Favia *et al.*, 1994).

A PCR-based technique for the identification of members of the *An. gambiae* complex has been developed (Paskewitz and Collins, 1990; Scott *et al.*, 1993). This is based on interspecific differences in sequences of intergenic spacer region (IGS) of ribosomal DNA. The method uses a universal primer that anneals to a sequence shared by all members of the complex, in combination with specific reverse primers that bind to unique sequences of each sibling species; *An. arabiensis, An. gambiae, An. quadriannulatus, An. merus/An. melas.* Townson and Onapa (1994) extended the method to include *An. bwambae.* More recently another primer was included for the identification of *An. quadriannulatus* B (Fettene *et al.*, 2002, Fettene and Temu, 2003). The identification of the molecular forms of *An. gambiae* *s.s.* was made available by Favia *et al.* (1997) and recently simplified by Fanello *et al.* (2002). PCR identification is now available for the majority of species complexes that involve major malaria vectors, for example *An. punctulatus* (Beebe and Saul, 1995), *An. fluviatilis* (Manonmani *et al.*, 2001) and *An. minimus s.l.* (Van Bortel *et al.* 2000).

2.4.1.8 Identification of molecular forms of *Anopheles gambiae s.s.*

In more recent years efforts have been directed in searching for molecular methods for differentiation of the chromosomal forms of *An. gambiae s.s.* These studies include the work of Favia *et al.* (1994), where the authors found among forms characteristic RAPD fragments. Later, based on nucleotide differences between molecular forms in the intergenic spacer of rDNA, a diagnostic PCR followed by restriction enzyme digest was developed (Favia *et al.*, 1997). This approach was refined to the level of primer-specific PCR (Favia *et al.* 2001). Most recently this approach was further refined to perform simultaneous identification of species and molecular forms of the *An. gambiae* (Fanello *et al.* 2002). In the previous method, mosquitoes are first identified to the species level (Scott *et al.*, 1993), and in the second step the *An. gambiae s.s.* specimens are identified to the

molecular form level. The PCR-amplification of 1.3 kb rDNA fragment containing part of the 28S coding region and part of the intergenic spacer region is followed by digestion with restriction enzyme *Hha* I (or *Tru* I) and a specific pattern is obtained for each form (Favia *et al.*, 1997). The method is based in the observation that the GCG$^\wedge$C restriction site for *Hha* I enzyme of (Favia *et al.*, 1997) lies within the *An. gambiae* specific fragment amplified by Scott *et al.* (1993).

2.4.2 Methods for discrimination of members of *Anopheles funestus* complex

2.4.2.1 Morphological methods

As in the *An. gambiae* complex, the morphological characteristics of *An. funestus* groups are variable and overlap in many aspects. However, *An. rivulorum* and *An. confusus* can be identified by larval characteristics, whilst *An. leesoni* can be identified on the basis of both egg and larval morphology (Gillies and De Meillon, 1968, Gillies and Coetzee, 1987). Examination of these characters entails holding wild-caught blood-fed females for egg laying and rearing of larvae to fourth instars, which is difficult to achieve under standard insectary conditions (Koekemoer *et al.*, 1998). *An. vannedeni* and *An. parensis* are both identical to *An. funestus* in almost all respects, and where differences do occur, they are variable and necessitate the rearing of family broods to achieve a reasonable probability of accuracy (Koekemoer *et al.*, 1998). Adult *An. vaneedeni* and *An. parensis* can be distinguished from *An. funestus* by, if present, pale scaling on the tarsomeres and male palps respectively (Gillies and Coetzee, 1987). None of the members of the *An. funestus* complex can be identified individually with absolute certainty on adult characteristics (Koekemoer, *et al.*, 1998).

2.4.2.2 Cytological methods

Cross mating has never been applied in *An. funestus* probably due to difficulties in maintaining colonies of this species in the laboratory. Up to date no known members are breeding in salt water.

Polytene chromosome banding patterns can distinguish between some members of the group. Green (1982) has shown that *An. funestus, An. rivulorum, An. parensis* and *An. confusus* each possess unique chromosome inversion rearrangements that can be used to identify them. *An. vannedeni*, however, is homosequential with *An. funestus,* differing from it only in the possession of a polymorphic inversion on arm 2 (Green & Hunt, 1980). These techniques are tedious and require a great deal of expertise. Additional disadvantages have been previously discussed in section (2.4.1.4).

2.4.2.3 DNA-based methods

Recently DNA-based techniques have been developed at the South African Institute for Medical Research (South Africa). The first technique includes the use of HpaII endonuclease that distinguishes between *An. funestus* and *An. vaneedeni* (Koekemoer *et al.,* 1998). This technique uses PCR primers developed from the D3 region in the 28s ribosomal gene, and amplified products digested with the restriction endonuclease HpaII and visualised on agarose gel. The second technique is also based on use of primers from the D3 region in the 28s gene electrophoresed on single–strand conformation polymorphism (SSCP) gels. This technique distinguishes between *An. funestus, An. vaneedeni, An. rivulorum* and *An. leesoni* (Koekemoer *et al.,* 1999). However, we are not aware of use of these techniques outside South Africa.

Based on differences in the ribosomal DNA internal transcribed spacer (ITS2) of *An. rivulorum* (approximately 380 bp) and *An. funestus* (approximately 700 bp) a rapid

PCR-based method was presented by Hackett *et al.* (2000). Based on the same region of DNA another polymerase PCR method has also been developed to accurately identify five species of the *An. funestus* complex (Koekemoer *et al.*, 2002). The species are *An. funestus*, *An. vaneedeni*, *An. rivulorum*, *An. leesoni*, and *An. parensis.*

2.5   Sporozoite rate determination

The sporozoite rate, that is, the proportion of anopheline mosquitoes harbouring sporozoites in their salivary glands, is normally expressed as a percentage. Traditionally, sporozoite infection rates were determined through direct dissection of the salivary glands of fresh mosquitoes and observing sporozoites microscopically (WHO, 1975). The technique requires skilled personnel and mosquitoes have to be fresh. Additionally, the identification of *Plasmodium* to species is impossible and the technique is not reliable in assessing mosquitoes with low numbers of sporozoites. Moreover, the technique is virtually useless in routine surveys of mosquito populations with sporozoite rates much less than 1% (Collins *et al.*, 1984).

The introduction of enzyme-linked immunosorbent assays (ELISA) has provided a simpler and cheaper tool for malaria epidemiological studies as well as malaria control. The technique, which is based on detection of the circumsporozoite protein (CSP), (epitopes that are amply expressed on the surface of sporozoites) by the highly species specific monoclonal antibodies (Mabs) (Beier *et al.*, 1988, Wirtz *et al*, 1985, 1987a,b,) was originally developed for *P. falciparum* (Burkot *et al*, 1984a, Wirtz *et al*, 1987a; Verhave *et al,* 1988), and was further extended for the detection of *P. vivax* (Wirtz *et al*, 1985) and *P. malariae* (Collins *et al*, 1988).

Prior to ELISA, an immunoradiometric assay (IRMA) was developed (Zavala *et al*, 1982) and tested in the field (Collins *et al*, 1984). The technique has the advantage, compared with traditional microscopical dissection, of providing a rapid, species-specific determination of both the presence and number of sporozoites in an infected mosquito (Zavala *et al*, 1982). IRMA can be performed on freshly caught or dry mosquitoes. ELISA was found to be as sensitive as IRMA but has an additional advantage of being safe, avoiding the use of radio-labelled chemicals and gamma counters for the analysis of results (Burkot *et al*, 1984b) and sporozoites can be detected even in mosquitoes stored at room temperature for as long as 18 months (Collins *et al*, 1988). The demonstration that the visual assessment of sporozoite ELISA is as reliable as a plate reader in determining infection rates in the field samples (Beier and Koros, 1991; Bockarie, 1993) has enhanced their potential use as a fundamental tool in malaria epidemiology studies and control.

Comparisons between ELISA and microscopic dissections, which is considered as "Gold Standard", have yielded contradictory results. For instance, ELISA was found to overestimate sporozoite rates in Cameron (Fontenille *et al.*, 2001), Senegal (Sokna *et al*, 1998) and in Kenya (Adungo *et al*, 1991; Beier *et al.*, 1988; Beier, *et al*, 1990). In contrast, Wirtz *et al*, (1987b) and Beach *et al* (1992) found results from ELISA in agreement with those of microscopic observations.

Soluble CSP can be found in different parts of the mosquito, including salivary gland tissues, hemolymph, head, abdomen (Beier and Koros, 1991) as a result of shedding of CSP from mature oocysts attached to the midgut or when sporozoites migrate through the body (Verhave *et al*, 1988). As a consequence CS antigen can be detected in mosquito thorax and other parts of its body without sporozoites being present in the salivary glands (Lombardi *et al*, 1986; 1987). This can lead to exaggeration of the sporozoite rate, if the ELISA technique is not used with caution (Ponnudurai *et al.*, 1991; Beier, 1993).

Therefore, even when the ELISA is done using just the head and thorax, (as recommended), the assay cannot differentiate between infected and infective mosquito, but rather identifies a potential infective mosquito. False positivity associated with bovine bloodmeals (Lochouarn and Fontenille 1999) and with both swine and bovine blood (Somboon *et al.*, 1993) has been reported. However, all those problems can be minimised by using only the head and thorax (Wirtz *et al.*, 1987b, Beier and Koros, 1991, Somboon *et al.*, 1993), which normally are not contaminated by the ingested animal blood.

ELISA has now become a widely used method to assess the sporozoite rates due to reasons already mentioned, associated with the fact that the techniques uses stable and relatively cheap reagents, require inexpensive equipment, and allows large scale analysis of mosquito samples (Lulu *et al.*, 1997).

Recently a rapid detection test strip (dipstick) (Malaria Rapid Panel (MRP) has been developed as an alternative to the ELISA method (Ryan *et al*, 2001). This technique uses equivalent species-specific Mabs as in ELISA to detect parasite CSP (Ryan *et al*, 2001, Bangs, *et al*, 2002). The advantages of this new method are that, the dipstick is simple to use and results can be obtained within 15 minutes compared with 4-6 hours required in ELISA (Bangs *et al*, 2002). The absence of refrigeration adds the advantages of testing mosquitoes directly under ambient conditions and mosquitoes can be tested after long period of storage over silica gel (1-3 years) (Bangs *et al*, 2002). As claimed by the authors these advantages make the technique an acceptable alternative to ELISA method. Although the authors did not mention costs, it is unlikely that the cost would be less than those involved in ELISA. This would probably prevent the MRP dipstick replacing the ELISA method in the near future. Nevertheless, MRP dipstick may have specific applications, for example, when speed is necessary or when the number of mosquitoes are few.

Alternative DNA based techniques have been developed for the detection of sporozoites in mosquitoes. These include nucleic acid hybridisation with a specific deoxyribonucleic acid (DNA) probe initially labelled with radioactive material (Delves *et al.*, 1989). The use of radioactive material can be overcome by using fluorescein (Lulu *et al.*, 1997). In this, false positives were found associated with blood. To overcome this problem mosquitoes have to be maintained for 48 hours before the assay takes place. This constitutes the major drawback of this technique.

Although DNA probes can be species-specific and allow large number of samples to be identified, sensitivity has been limited (Tassanakajon *et al.* 1993). The use of PCR-based technique to identify a specific DNA sequence of *P. falciparum* meant that the sensitivity problems associated with DNA probes would be overcome.

Originally PCR techniques were developed for the detection of the four human malaria parasite species found in human, based on the sequence of their small subunit ribososomal RNA (ssrRNA) genes (Snounou *et al.*, 1993a). The protocols were soon used in the detection of malaria parasites in mosquitoes (Snounou *et al.*, 1993b).

However, this approach is not without its problems. DNA from malaria parasites ingested in a blood meal might be present for several days in mosquitoes and detectable in PCR. Even using the head and thorax, sexual as well as asexual stages of malaria parasites trapped in the fore gut of mosquitoes might also be detected by the technique. These, together with the cost of reagents and equipment, are probably the main problems preventing the field use of PCR-based techniques in field. Nevertheless, Wilson *et al.* (1998) showed that PCR of salivary glands of mosquitoes can be more than 3 times as sensitive as microscopy in the detection of sporozoites.

2.6   Methods for the study of population genetic variation

Population genetic studies are of great importance in elucidating the population structure and pattern of gene flow between populations. Individual populations may have varying degrees of contact, from frequent genetic interchange to nonexistent. This is generally measured by examining the frequencies of different alleles, or forms of a specific gene. Mutation, genetic drift due to finite population size, and natural selection favouring adaptations to local environmental conditions will all lead to genetic differentiations of local populations, and the movement of gametes, individuals, and even entire populations - collectively called gene flow – will oppose that differentiation (Slatkin, 1987).

The control of malaria by the introduction of genes, which make the mosquitoes non-susceptible to *Plasmodium* or divert them from being attracted to bite humans will require a profound knowledge of the population structure as well as the pattern of gene flow of the target species. An understanding of genetic variation and gene flow in natural populations is crucial in determining how far introduced genes will spread which, in turn, is important in determining the spatial and temporal scale required for gene introductions (Kamau *et al*, 2002). Because gene flow is a useful indicator of direction and rates of dispersal among populations (Slatkin, 1987), knowledge of population genetics is also useful in predicting the spread of insecticide resistance associated with particular genes (Curtis and Townson, 1998; Kamau *et al*, 2002). This could help in developing effective strategies for insecticide resistance management.

Until relatively recently in the *An. gambiae* complex, techniques based on polytene chromosome inversions (Coluzzi and Sabatini, 1967; 1968; 1969; Hunt, 1973) and allozymes (Mahon *et al.*, 1976; Miles, 1978, 1979) have been used extensively in the field for species

identification and for population genetical studies. These techniques have contributed to our present knowledge of the status of *An. gambiae* complex; the number of biological entities, their biology and relation to disease transmission, and their population genetic structure. Problems related to the chromosomal markers and electrophoresis of proteins have been previously addressed (section 2.4.1.4 and 2.4.1.5).

DNA markers now play an important role in vector population genetics. Compared with isozymes, they have the advantage of being able to assess genetic variation at the nucleotide level (Tabachnick and Black, 1996).

Random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990, Welsh and MacClelland, 1990) has been used in population genetic studies in several mosquitoes of medical importance such as *Aedes albopictus* (Kambhampati *et al*, 1992) *A. aegypti* (Ballinger-Crabtree, 1992, Apostolo, 1996), *An. darling* (Manguin *et al*, 1999), including *An. gambiae* (Favia *et al.*, 1994). One of the disadvantages of RAPD is the impossibility of distinguishing whether an amplified DNA fragment is heterozygous or homozygous.

Restriction fragment length polymorphism (RFLP) indicates changes in fragment length that result from the loss (or gain) of a restriction site. RFLP are widely dispersed throughout the genome and they are often inherited as co-dominant Mendelian markers. RFLP analysis is limited because it can monitor only a part of the genome that represents the restriction sites under examination (Beckman, 1988). When comparison between 2 closely related individuals is required, RFLP may not provide sufficient markers that are discriminating (Beckman, 1988). The few cases in which RFLP have been used in African malaria vectors were when the markers were used as tool to identify members of *An. gambiae* complex (Taylor *et al.*, 1993), molecular forms of *An. gambiae s.s.* (Favia *et al.*, 1997; Fanello *et al.*, 2002), and two members of the *An. funestus* complex. (Koekemoer *et al.*, 1998).

Mitochondrial DNA (mtDNA) is primarily maternally inherited. Unlike nuclear markers, there is no recombination among genomes of different maternal clones. Studies on variation in mtDNA can be used to determine historical and phylogeographic patterns. It is generally accepted that mitochondrial gene trees are congruent with species trees (Moore, 1995) and can reveal population differentiation more quickly than nuclear DNA (Moore, 1995; Parker *et al.*, 1998). mtDNA have been the marker of choice for resolving phylogenetic relationship between species and within species complexes (Walton *et al.*, 2000).

mtDNA have been used in genetic population studies to assess the variation within and between species of anopheline mosquitoes (Conn *et al*, 1993; 1997) including *An. gambiae* complex (Besansky et *al.* 1997; Lehmann et *al*, 1997; 2000). In the past, mtDNA has been used to assess the phylogenetic relationships among members of the *An. gambiae* complex (Besansky *et al.*, 1994).

2.7   Microsatellites

Microsatellites are nuclear markers that consist of short tandem repeats, usually 1-5 bp in length, such as $(CA)_n$ or $(ATT)_n$ (Beckmann and Weber, 1992). They are found widely dispersed throughout most of the genome in many eukaryotes and can occur as frequently as once every 10 kb (Tautz, 1989; Stallings *et al.*, 1991). They are abundant in eukaryotic genomes (Hamada *et al.*, 1982; Tautz and Renz, 1984) and highly polymorphic, inherited co-dominantly and easy to score (Bruford and Wayne, 1993; Jarne and Lagoda, 1996) which makes microsatellites the most popular molecular markers for ecological and evolutionary studies (Keyghobadi *et al.*, 2002).

Furthermore, because they are non-functional, they are not subjected to strong selection and the presence or absence of a particular marker would not affect an individual's survival. Their major disadvantage, however, is that it is usually necessary to screen genomic libraries to identify and characterize microsatellite loci for each species under investigation before loci can be used in PCR. Another disadvantage includes the possibility of null alleles, which results in reduction or complete loss of amplification of some alleles due to base substitutions within the priming site. This can lead to serious underestimation of heterozygosity, compared with that expected on the basis of Hardy-Weinberg equilibrium (Callen *et al.* 1993, Paetkau and Strobeck, 1995).

Taq polymerase generates slippage during PCR and the tendency of *Taq* polymerase to add an additional dATP to PCR products can sometimes make allele scoring problematic (Ginot *et al.*, 1996). Microsatellite variation is based on length variation (in bp) of the amplified fragments. It is possible that two fragments of the same length are not derived from the same ancestral sequence, introducing the possibility of size homoplasy. Under the infinite alleles model (IAM) they should not be any homoplasy, but under stepwise mutation model (SMM) the possibility exist. Size homoplasy can lead to underestimates of population subdivision and genetic divergence between populations (e.g. Estoup *et al.* 1995; Taylor *et al.*, 1999). However, more recent review by Estoup *et al.* (2002) concluded that size homoplasy does not represent a significant problem for many types of population genetic analyses realized by molecular ecologists, and the large amount of variability at microsatellite loci often largely compensates for their homoplasious evolution. The authors observed that the situations in which size homoplasy may be problematic involve high mutation rates and large population sizes together with strong allele size constraints.

Microsatellites were first applied in African malaria vectors by Lanzaro *et al.* (1995). The authors concluded that microsatellites provide a rich source of genetic polymorphisms for the study of the population genetics of *An. gambiae* and are in many ways superior to isozymes for this purpose. Since the work of these authors significant insights on population genetics within and between species of the *An. gambiae* complex have been produced (review by Donnelly *et al.* 2002). Collectively, these studies suggests a shallow population structure and, in particular, the weak effect of distance on differentiation (Donnelly *et al.* 2002).

2.8   Genetics of *Anopheles funestus*

Contrary to *An. gambiae s.s.*, studies of the population genetic structure of *An. funestus* are scarce. The preference for breeding in patchily distributed, permanent bodies of water and the uneven distribution of this vector even before insecticide application (Molineaux and Gramiccia, 1980) suggested that *An. funestus* might be subdivided into discrete populations (Besansky, 1999).

Chromosomal studies provided unequivocal evidence of population sub-structuring within *An. funestus*. Initial evidence of heterogeneities between *An. funestus* populations from different geographical locations comes from Green and Hunt (1980). In a paper aimed at presenting a photomap of the polytene chromosomes of *An. funestus*, the authors observed significant genetic differences between samples from Zimbabwe/Rhodesia, Namibia and Nigeria. However, they gave no further details.

Green (1982) observed that the polytene chromosomes of *An. funestus* from Southern Africa (East Africa) were highly polymorphic with respect to inversions, in contrast to samples from Gambia (West). In Madagascar, Boccolini *et al.* (1992) observed significant differences in inversion frequencies between samples from different seasons and geographical areas.

Baccolini *et al.* (1994), found highly significant deviations from Hardy-Weinberg equilibrium for all the inversions in most samples from sympatric populations in Burkina Faso, and postulated that *An. funestus* in this region could represent two taxonomic units, one monomorphic standard or nearly so, and the other polymorphic. In Senegal the general pattern of karyotype distribution also suggests the presence of 2 genetically differentiated populations in an area of sympatriy (Lochouarn, 1998).

Based also on chromosomal analysis in a sample comprising more than 1000 individuals, Costantini *et al.* (1999) found: a) highly significant departures from Hardy-Weinberg equilibrium for inversions 3a and 3b in most samples, with the alternative homokaryotypes (standard and inverted) significantly more frequent than expected; b) significantly higher frequencies of the standard homokaryotypes in samples collected outdoors for arrangement 3a vs. the samples obtained from indoor. Following this observation, the authors raised the hypothesis that *An. funestus* in Burkina Faso includes two taxonomic units, one of which is mainly monomorphic standard with most inverted arrangements floating at very low frequencies, while the other taxon is nearly fixed for the polymorphic arrangement for all the other inversions at intermediate to high frequencies. The latter would be characterised by a higher vectorial capacity and would probably correspond to *An. funestus s.s.* from East Africa (Costantini *et al.*, 1999). In a recent study by Kamau *et al* (2002) based also on chromosomal inversions, the authors found that overall, the level of differentiation between population from two different sites (western and coastal Kenya) was significant, suggesting that the two populations are genetically isolated.

Mukabayire *et al.* (1999) studied populations of *An. funestus* from West Africa (Burkina Faso and Senegal) and East Africa (Kenya), using mtDNA and rDNA sequences. However, their results failed to support population heterogeneities observed in chromosomal-

based studies. From both West African countries, samples included individuals hypothesized to represent reproductively isolated taxa based upon different karyotypes and behaviours. The mtDNA cytochome b gene was variable but sequence variation was not partitioned by geographical origin or karyotype class. The most common haplotype was sampled across Africa (approximately 6000 km). The rDNA ITS2 sequences were nearly monomorphic regardless of the origin of samples, with only two rare polymorphisms detected. Whether the lack of support from mtDNA and rDNA sequences is a result of the recent origin of the presumptive taxa, or of the absence of barriers to gene flow, remains to be elucidated, using more rapidly evolving markers such as microsatellites (Mukabayire *et al.*, 1999). Locus-specific PCR primers for microsatellites of *An. funestus* have already been published by Sinkins *et al.* (2000) and more recently by Cohuet *et al.* (2002).

2.9 Malaria control methods

The most effective mean of malaria vector control is the killing of adult mosquitoes with a residual insecticide applied to bed nets or sprayed on house walls and ceilings (Curtis and Townson, 1998). The rationale of house spraying is that mosquitoes will rest on the insecticide deposit before or after biting and remain there long enough to pick up a lethal dose. During the period from the 1940s to the 1960s, the incidence of malaria was reduced to zero, or near zero in many regions of the world by spraying the inside surfaces of houses with a residual insecticide, principally DDT (Curtis and Lines, 2000).

Initially, pyrethrum, an insecticide extracted from *Chrysanthemum cinerariefolim*, was popular. This insecticide was not stable and degraded in the presence of light. Therefore, highly stable and long lasting insecticide was needed.

DDT and dieldrin were the first types of synthetic insecticide produced. DDT, in particular, was by far the most widely used insecticide, because of its cheapness and durability. However, resistance was soon observed in many *Anopheles* mosquitoes. Organophosphates such as malathion, fenitrothion and fenthion, and carbamates like carbaryl and propoxur have been used in different regions of the planet. However, these compounds are more expensive and less persistent, and resistance to one or more of these insecticides has rendered them less effective.

The introduction of synthetic pyrethroids such as deltamethrin and lambdacyhalothrin meant that the problems mentioned above were overcome. Although more expensive per unit weight compared with DDT, these pyrethroids are not much more expensive per house protected per year (Curtis, 1994). Pyrethroids are much more acceptable to householders because they leave no visible deposit on walls and because they kill nuisance insects such as cockroaches.

Bed nets have long been recognized as useful protection tool against malaria (Manson, 1900). Initially they work as physical barrier against night biting mosquitoes. When the bed net is impregnated, an insecticide treated net (ITN), it provides a cheap and effective means of control of malaria in the community. Insecticide-treated nets protect their occupants by diverting host-seeking vectors to look for a blood meal elsewhere or by killing those that attempt to feed (Lines *et al.*, 1987).

The efficacy of ITNs and other materials in reducing morbidity and mortality due to malaria has been convincingly documented (Alonso, *et al.* 1991, Binka *et al.* 1996, Choi *et al.* 1995, D'Allessandro *et al.*, 1995, Nevill *et al*, 1996), and therefore forming now the most important part of the Roll Back Malaria (RBM) strategy. However, pyrethroid resistance has been reported in *An. gambiae*, in some countries of West Africa; Benin, Burkina Faso and Côte d'Ivoire (Akogbeto and Yakoubou, 1999; Elissa *et al.*, 1993;

Chandre, *et a.l*, 1999; Martinez-Torres *et al*, 1998). In South Africa and Mozambique resistance has been shown in *An. funestus* (Brooke *et al.,* 2001). Surprisingly, there is evidence from Côte d'Ivoire that pyrethroid treated bed nets were still an effective prophylactic measure against malaria even in areas where *An. gambiae s.s.* was resistant to pyrethroids (Darriet *et al.* 2000). Much more studies are required to validate this evidence.

Bedbug (*Cimex hemipterus*) control is a motivating factor for public co-operation in anti-malaria programmes based on pyrethroid-treated bed nets (Temu *et al.* 1999). Resistance of bedbugs to pyrethroids, permethrin and alphacypermethrin has been found in villages of Tanzania (Myamba *et al.* 2002). There are therefore, concerns that the emergence of pyrethroid resistance in bedbugs will reduce the re-treatment rates of bed nets achieved in these villages of Tanzania (Myamba *et al.* 2002) and elsewhere.

Currently, effort being invested includes the genetic manipulation of mosquitoes that would modify the capacity of natural vectors to support parasite development (James *et al.*, 1999; James, 2002). Basically, the idea behind this is to transform mosquitoes with genes that render them refractory against infection by malaria parasites, then release them into natural populations and thus make mosquito populations incapable of transmitting malaria.

Other method under investigation includes the development of an efficient vaccine against one of the life stages of the malaria parasite, preferably pre-erythrocitic, but the one which has been extensively field tested only gave a limited degree of protection (Alonso *et al.*, 1994). As for the vaccine, even under the most optimistic scheme with unlimited resources, it will still be many years from now, requiring iterative testing of improved combinations and formulations until sufficient efficacy is obtained (Richie and Saul, 2002). This is also true for those contemplating control of malaria through genetically modified mosquitoes.

2.10 Malaria and its vectors in Mozambique

In Mozambique, in spite of the efforts of the National Malaria Control Program, malaria is still one of the most extensive diseases. Regrettably, epidemiological and clinical data are scarce and mainly hospital-based and invariably are limited to urban areas (Maputo and its vicinity) (Dgedge, *et al.*, 1994; Franco, *et al*, 1994; Franco, 1994; Martinenko *et al.*, 1994, Hogh *et al.*, 1995, Thompson *et al.*, 1997, Franco *et al.*, 1984; Shapira and Schwalbach, 1988; Dinis and Schapira, 1990; Hogh *et al*, 1994, 1998; Betgstrom *et al.*, 1993; Axemo *et al.*, 1995, Granja *et al.*, 1998; Saute *et al.*, 2002, Dgedge *et al.*, 2001; Varandas *et al.*, 2001). Nevertheless, the most comprehensive data available indicate that from 1993-1996 the disease was responsible for 42% of hospitalisation in paediatric services and a mortality rate of about 5.3% (Direcção Nacional de Saúde, 1998).

Mozambique is a vast country extending about 2000 km from north to south and consists of various eco-climatic zones. These areas exhibit different patterns of rainfall, and hence different malaria transmission patterns. In general however, transmission occurs throughout the year with *P. falciparum* being the most predominant species causing more than 95 % of malaria cases, others being *P. malariae* and *P. ovale* (Direcção Nacional de Saúde, 1998). However, a more comprehensive picture of the pattern of malaria distribution in Mozambique (Figure 2.1) was provided by the MARA/ARMA project.

Drug resistant malaria is a problem of increasing importance in Mozambique. Chloroquine-resistant *P. falciparum* has been reported since early 1983 (Franco *et al.*, 1984; Schwalcach and Maza 1985). The most recent survey indicates that resistance is widespread and the therapeutic efficacy of chloroquine is only 60 % (Dgedge *et al.* in prep). In Manhica, for example, 58 % of sick children brought to local hospitals had

parasites resistant to chloroquine (Mayor *et al.* 2001). Nevertheless, chloroquine is still the first-line drug for the treatment of uncomplicated malaria.

The known malaria vectors are all members of either the *An. gambiae* complex or the *An. funestus* complex. However, in studies in Mozambique these groups of species have generally been treated as a single species: *sensu lato*.

With the exception of the taxonomic works of De Meillon (1941a,b), the few other reports on malaria vectors are restricted to the Maputo area (formerly Lourenço Marques) in southern Mozambique. The work of De Meillon (1941a) clearly showed that *An. funestus* was the dominant malaria vector in areas around Maputo. This work together with the contributions of De Meillon (1940), Pereira (1958), Worth and De Meillon (1960), Worth and Peterson (1961), established the existence of 27 anopheline mosquito species in Mozambique.

The distribution of members of the *An. gambiae* complex was studied by Petrarca *et al.* (1984) and Cuamba *et al.* (1996). The work of Petrarca (*loc. cit*) is the most comprehensive survey available to date. These authors showed *An. arabiensis* widely distributed within the country; *An. gambiae s.s.* was found in central and northern areas of the country, *An. merus* was confined to coastal areas, whereas *An. quadriannulatus* was only recorded in a single collection in the most southern area of Mozambique. The role of these vectors has not yet well elucidated in Mozambique. Mnzava *et al.* (1997) found high frequencies of human blood in *An. arabiensis* and *An. merus*, suggesting that both species could be playing a role in malaria transmission in the Maputo area. A higher human blood index was also found by Thompson *et al.* (1997) in Maputo area, *i.e. An. arabiensis* with 98.5 % and *An. funestus* with 99.5 %, but the infection rates were lower. In a more thorought study in the same area, Mendis *et al.* (2000) found very low sporozoite rates but comparable between *An. arabiensis* and in *An. funestus*

An. rivulorum is the second most widespread species within the *An. funestus* complex, and is occasionally collected indoors along with the dominant and major malaria vectors *An. funestus* (Hackett *et al.*, 2000). Very recently, in houses from Ndumu area (South Africa), bordering Mozambique, Hargreaves *et al.* (2000), using an rDNA-PCR method, identified four members of the *An. funestus* complex; *An. funestus s.s., An. parensis, An. rivulorum* and *An. leesoni*. One would therefore expect to find these species in Mozambique since there are no apparent geographical or climatic barriers to prevent the spread of these species into Mozambique.

Malaria control strategies in Mozambique rely on prompt diagnosis and effective treatment of malaria cases and indoor spraying with pyrethroid insecticides in some peri-urban areas of large cities. For several years, vector control activities were confined to the southern areas of Mozambique, close to South Africa and Swaziland. These activities have relied mainly on spraying houses with residual insecticide. Until the shift to pyrethroids in the early 90's (Cuamba and Dambo, 1994) DDT was the most widely used insecticide. As a direct consequence of spraying, malaria transmission was suppressed in these localities from 1950s onwards (Soeiro, 1956; Soeiro *et al.*, 1956; Pereira, 1957; Schwalbach and Maza, 1985), with a greater impact on *An. funestus* than on *An. gambiae s.l.* (Pereira, 1957). This author also claimed that in areas of control *An. funestus* had shifted its behaviour, preferring to bite outdoors rather than indoors as a direct consequence of spraying activities. In fact, the mosquitoes biting outdoors could be other members of *An. funestus* complex, but needs confirmation.

During the 1980s, vector control activities were restricted to Maputo suburban areas (Martinenko *et al.*, 1989) and by 1985, *An. funestus* was reported to be predominant over *An. arabiensis* (Zharov, 1992). This situation still prevails in the Maputo area where, despite almost annual spraying with pyrethroids, there have been outbreaks of malaria. The situation

is similar in areas outside Maputo, which were subjected to intense vector control during the 50s to 70s. There is a need, however, to confirm whether this upsurge of *An. funestus* is a consequence of resistance to pyrethroid insecticides, already observed in areas of Mozambique (Brooke *et al.,* 2001) and South Africa area bordering Mozambique (Hargreves *et al.,* 2000) or is due to other biological or ecological phenomena.

Crook and Baptista (1995) evaluated the use the permethrin-impregnated wall-curtains on biting rates of malaria vectors (*An. gambiae s.l.* and *An. funestus*) and on the *P. falciparum* parasite rate, and morbidity due to malaria in children in the Maputo area. They found that the curtains lowered biting rates of both vector species inside curtained houses but reduced inside resting and outside biting only in *An. funestus*. Additionally the number of children with *P. falciparum* parasitaemia fell significantly in curtained houses, but malaria morbidity in under fives was unaffected.

A pilot ITN study was carried out in Boane District, Mozambique from 1996 until 1998 to determine the socio-economic factors associated with the demand for insecticide treated nets and to determine the feasibility of implementing the ITN project throughout the primary health care system (Dgedge, 2000). Bed nets have never been used before. They are now slowly becoming popular, principally in areas hit by floods during 2000 and 2001. For example, United Nations Children's Fund (UNICEF) alone, in response to the floods, supplied 45,000 insecticide-treated nets (ITNs) for use in temporary accommodation centres, which have benefited nearly 90,000 people displaced by floods (http://www.unicefusa.org/alert/emergency/mozambique/mozambique.html), but in 2002, UNICEF procured over 345,000 mosquito nets and 490,000 insecticide kits for Mozambique. http://www.unicef.org/mozambique/latest_news_27mar03.htm.
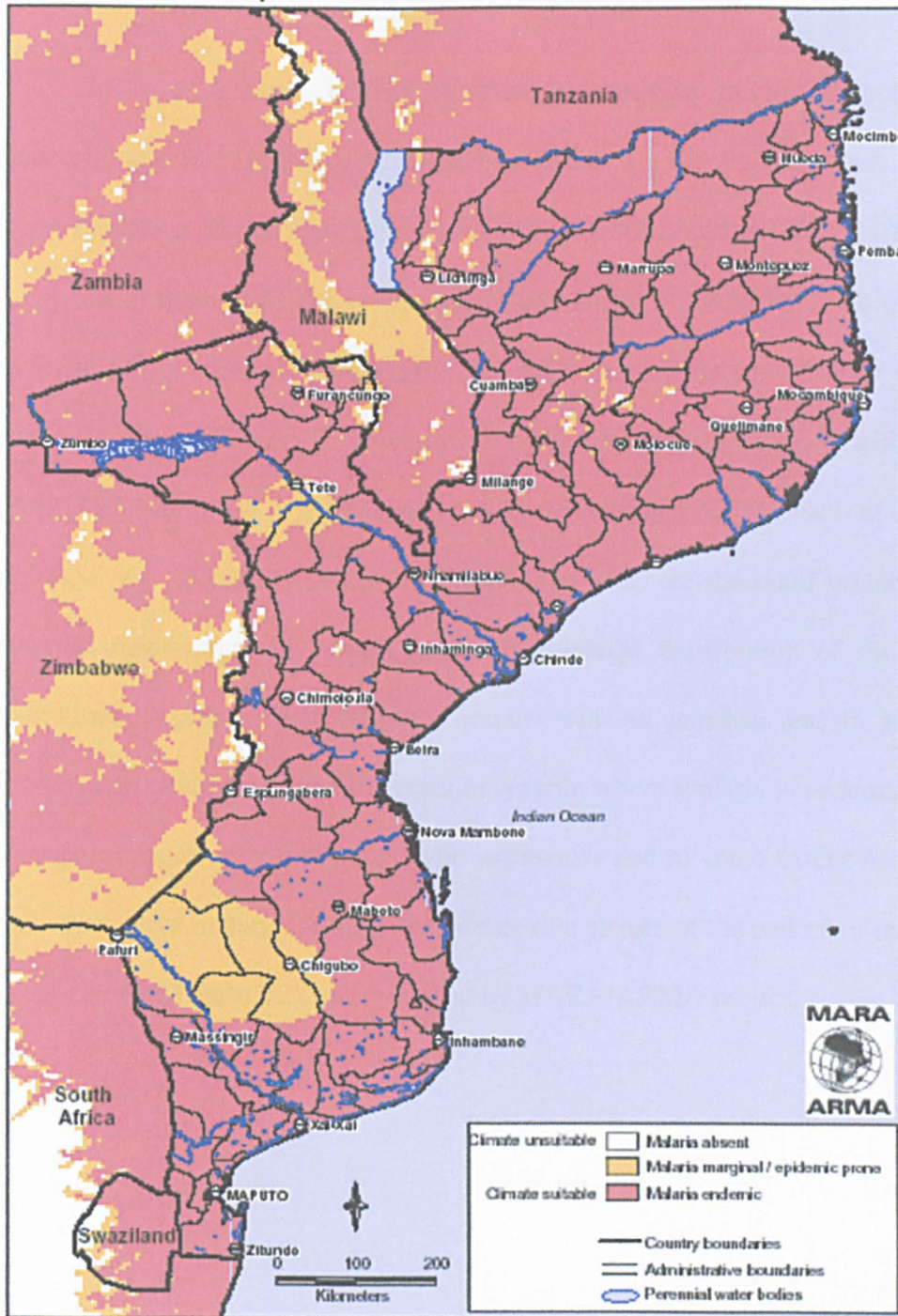
FIGURE 2.1 Distribution of endemic malaria in Mozambique.
Source: MARA/ARMA, http://www.mara.org.za/

2.11   Malaria and its vectors in Angola

In Angola, there is lack of basic information in all components of malaria epidemiology. The following information was based, except when stated, in Documento de Base- Programa Nacional de Controle de Malária, 2001 (DB-PNCM, 2001).

In Angola, malaria is the principal cause of morbidity and mortality with an estimate of 1.5 million cases a year. Of these cases, between 15-30% occur in children under the age of 5 years. *P. falciparum* is the main parasite species with a prevalence of about 90% followed by *P. vivax* and *P. malariae.* Four species of mosquitoes (*An. gambiae, An. arabiensis, An.melas* and *An. funestus*) are the main vectors responsible for malaria transmission in Angola. The geographical distribution of the vectors follows rainfall and vegetation patterns of the country with *An. gambiae* and *An. funestus* occurring in large numbers in the northern parts of Angola where malaria is endemic. In the southern drier parts malaria is unstable and *An. arabiensis* and to some extent *An. funestus* are the main vectors of malaria. A more comprehensive picture of the pattern of malaria distribution in the country (Figure 2.2) was produced by MARA/ARMA project.
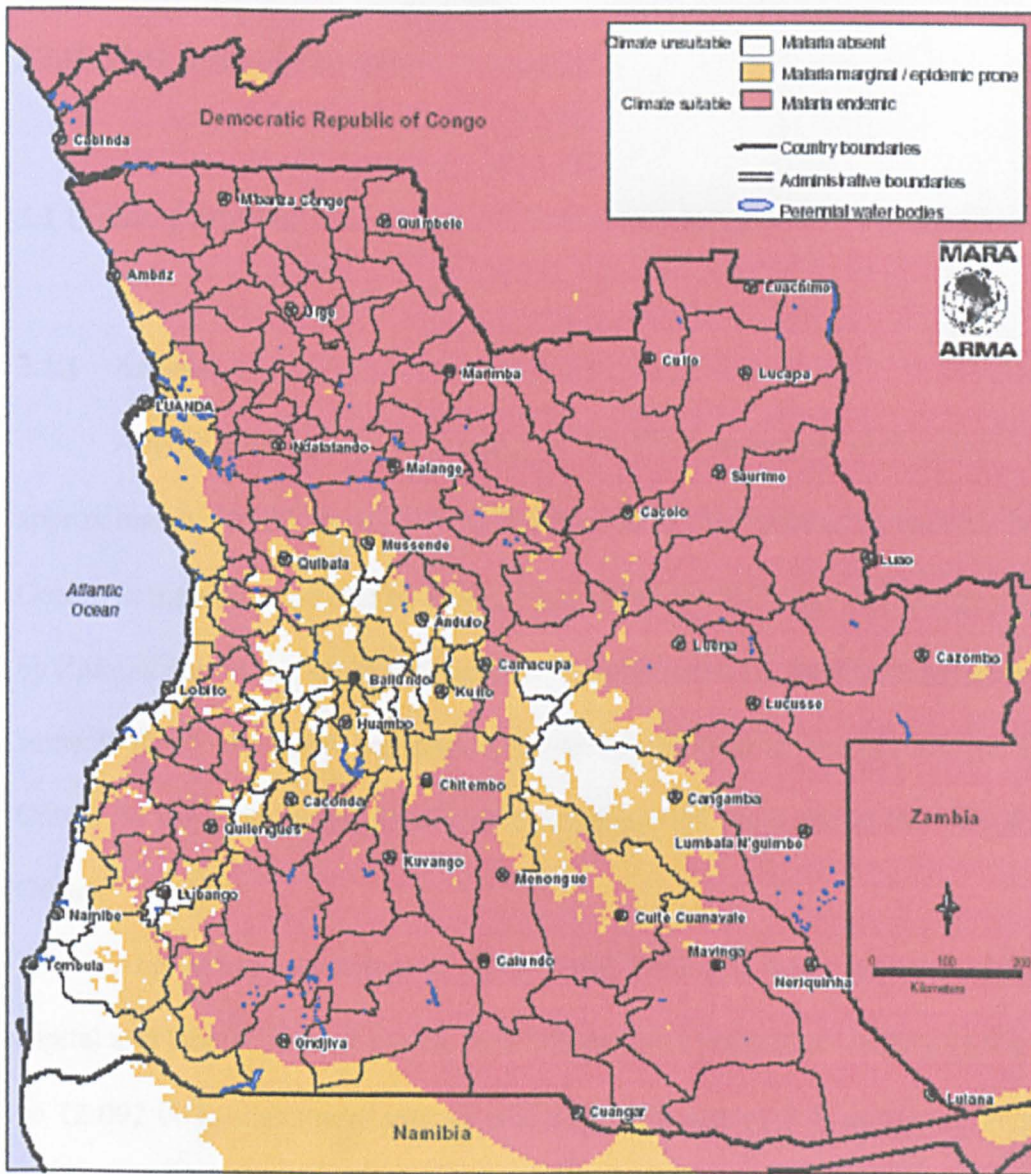
FIGURE 2.2 Distribution of endemic malaria in Angola.
Source: MARA/ARMA, http://www.mara.org.za/

CHAPTER III

Material and Methods

3.1 Country profiles

3.1.1 Angola

Angola (Figure 3.1) is located in the Western region of Southern Africa within approximately 12° 30´ S and 18° 30´ E. The country is bordered in the west by the Atlantic Ocean, in the north and east by Democratic Republic of Congo and Congo, in the southeast by Zambia and in the south by Namibia. A small enclave, the Cabinda Enclave, is located some 30 Km to the north, separated from the rest of Angola, and is bordered in the north by Congo, in the south by the Democratic Republic of Congo and in the west by the Atlantic Ocean.

Angola has a coastline of about 1 600 km and a total area of 1 246 700 km^2. The capital and largest city is Luanda. In 1998, the total population of Angola was estimated to be 12 092 000 inhabitants, with an annual growth rate of 3 % and infant mortality of 125 per thousand (WHO, 1999).

Angola gained its independence from Portugal in 1975, after more than a decade of armed struggle. However, the independence did not mean peace and prosperity for the people of Angola. Internal conflict between principally MPLA and UNITA backed by foreign countries started the most destructive war in the country. Several peace accords failed. The death of Dr Jonas Savimbi, leader of UNITA, brought another hope, with the

peace agreement between the commanders of UNITA and the government army signed in April 2002.

Despite abundant natural resources, which include oil, diamonds, gold, extensive forests and Atlantic fisheries, the economy of Angola is in disarray due to more than a quarter century of almost continuous warfare.

Subsistence agriculture provides the main livelihood for 85 % of the population. However this is a high risk activity in Angola. Millions of land mines remain and many farmers are reluctant to return to their fields. As a result, much of the country's food must still be imported. Oil production and the supporting activities are vital to the economy, contributing about 45 % to GDP and 90 % of total exports (http://www.mapzones.com/world/africa/angola/economyindex.php). According to the same sources, in 1999 the GDP was $8.5 billion, or about $690 per person. With a few exceptions (South Africa, Mauritius, Botswana) the figures are higher than those of other member states of the Southern African Development Community (SADC) (http://www.iht.com/IHT/SUP/112999/sp-SAF-3.html).

The country is characterised by an arid coastal strip stretching from Namibia to Luanda; a wet interior highland; a dry savannah in the interior south and southeast; and rain forest in the north and Cabinda (http://www.state.gov/r/pa/ei/bgn/6619.htm). To the south of Benguela from the coast to the interior is the Namib Desert. Its topography includes the narrow coastal lowland plain along the Atlantic, which rises abruptly to a vast interior plateau in the east with mountains. The highest point is the Moco Mountain at 2 620 m.

The climate of Angola is influenced by the sea in coastal areas and by altitude in inland plateau. The hotter and drier climate is along the coast. There are roughly two seasons; the rainy season lasts from November to April followed by the cool and dry season from May to October. Rainfall is high in the north and in the central highlands

(average 1 250 mm to 1 750 mm), but decreases rapidly towards the coastal area (average 250 mm to 1 000 mm). To the south of Benguela, the average rainfall is less than 100 mm a year (http://www.angola.org/REFERENC/geography/html).
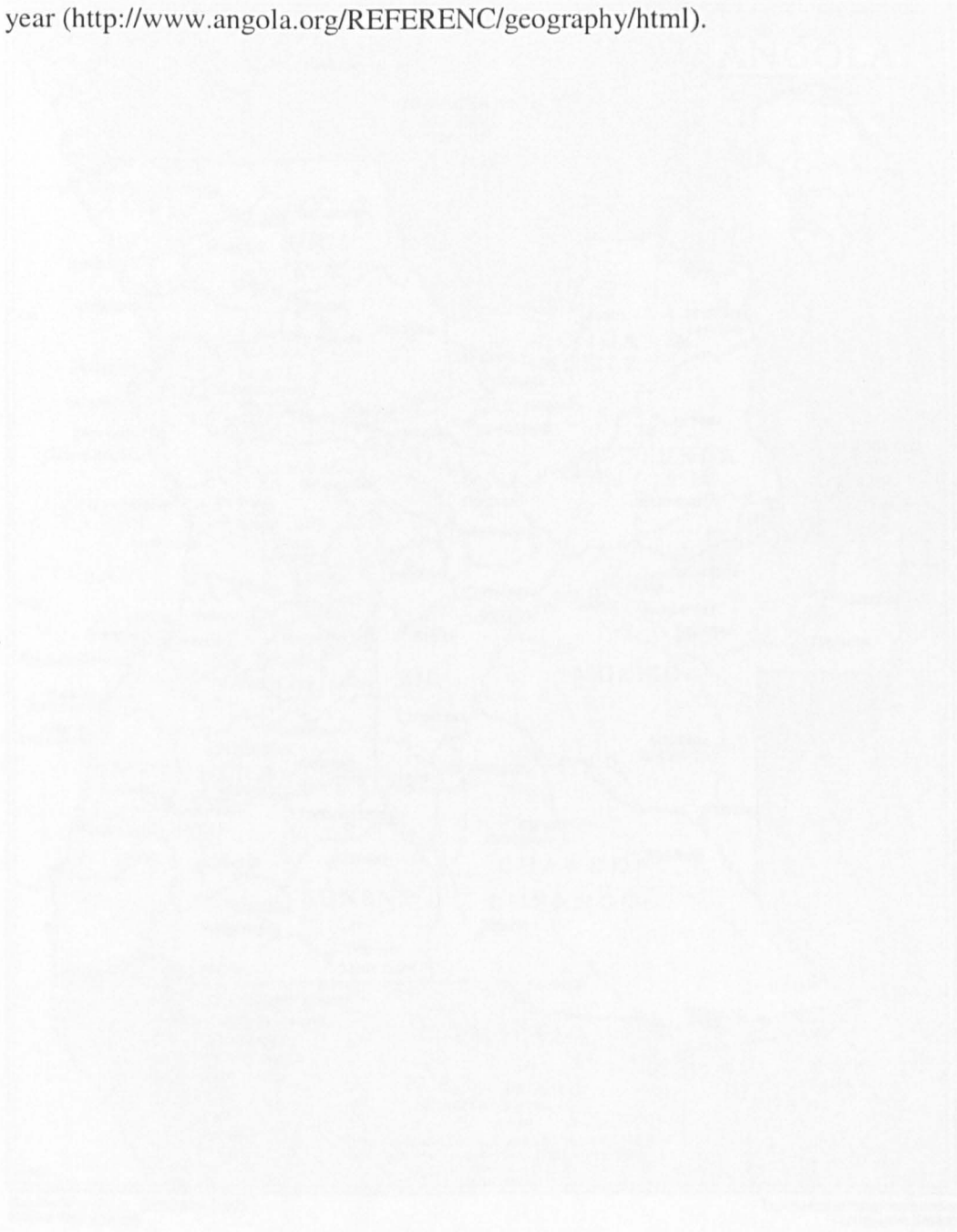
FIGURE 3.1. Geographic location of Angola.
Source: http://www.un.org/Depts/Cartographic/map/profile/angola.pdf)

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3.1.2 Mozambique

Mozambique (Figure 3.2) is a Southern African country located on the east coast of the continent between approximately 10° 27' S and 26° 52' S and 30° 12' E and 40° 41' E. The country covers an area of 799 380 km^2, is bordered by Tanzania to the north, Malawi, Zambia and Zimbabwe to the west and South Africa and Swaziland to the south. In the east its coast lies on 2 500 km Mozambican Channel a branch of the Indian Ocean, which separates the country from Madagascar.

According to the 1997 population census, the population of Mozambique was estimated to be 16.1 million with a growth rate of 2.2 % (INE, 1999). According with these statistics, the population of Mozambique is predominantly rural (71.4%) and mainly concentrated in the coastal areas. The life expectance is about 46 years and women constitute 53% of the total population.

Mozambique acquired its current geographic form in May 1891, in the Anglo-Portuguese Treaty of partition of zones of influence in Africa. The agreement was used to legitimise the Portuguese occupation of Mozambique, which began in the seventeenth century. The country gained independence in 25 June 1975, after ten years of military struggle conducted by Frelimo, which became the ruling party and adopted Marxism as the ruling political philosophy. In 1989, the ruling party abandoned Marxism and a new constitution provided multiparty elections and a free market economy. The first elections were held in October 1994, after an UN-negotiated peace agreement with RENAMO forces had ended the fighting in 1992, after nearly 15 years of civil war. The liberation war and the subsequent civil war both had a dramatic impact on the economy of the country.

FIGURE 3. 2. Geographic location of Mozambique.
Source: http://www.un.org/Depts/Cartographic/map/profile/angola.pdf)

Mozambique is essentially an agricultural country, with most of its workers engaged in subsistence farming. It is essentially rain-fed agriculture and involves growing basic staple foods such as maize, rice, cassava and sorghum, and some cash crops, principally cashew nuts, coconut palms and cotton (GTA 1990). Tobacco and tea plantations have now become important in the economy of the country, after a long period of interruption due to the civil war. Fisheries are probably the most important source of revenue for the populations living in the coastal areas, and one of the most important sources of foreign exchange.

The important natural resources include hydroelectric power, natural gas, coal, timber, sea produce and land for agriculture and cattle production. Despite its natural resources and economically strategic geographical position in Southern Africa, Mozambique, is one of the poorest countries of the World. The Gross Domestic Product (GDP) per capita in 1996 was US$185.8, and in 1998 US$236.9 (INE 2000).

In general there are two main seasons in Mozambique; the hot and wet season from November to April and the dry and cool season from May to October. The details of climate and ecological zones are given in the following section (3.2.2).

Natural disasters such as floods and drought are common in the central and southern areas of Mozambique and continue to impede the revival of the country's economy. Severe flooding occurred in February 2000, when abnormal heavy rains in southern Africa caused extensive flooding and submerged immense areas of land. This ravaged much of the country's infrastructure, and produced probably more destruction than the war itself over vast swathes of land in the south of Mozambique. Apart from the loss of life and of cattle, homes, roads, bridges, railway lines, and electricity transmission lines were washed away and crops were ruined. Subsequently during the rainy season of 2001, floods occurred in the central northern areas of the country, mainly in Púngue and Zambeze

river basins. Since 2002, Mozambique, along with five other southern African countries, has been hit by drought with consequent food shortages.

The landscape of the territory is essentially coastal lowland, with uplands in the centre, high plateaus in the northwest and mountains in the west, with Mount Binga, the highest point at 2436 m, situated on the border with Zimbabwe (Figure 3. 3). The land is covered with vegetation ranging from grasslands to closed forests, from savannah to woodlands and open or secondary forests, depending on the natural environment and the degree of human intervention.

FIGURE 3.3. Landscape of Mozambique. Source: Atlas Geográfico, Moçambique, 1986.

3.2   Eco-climatic regions

3.2.1   Angola

Mosquito samples from Angola were collected from a limited number of localities. The ecological and climatic characteristics of these sites will be discussed in chapter VII, where the characteristics of these samples are discussed.

3.2.2   Mozambique

In Mozambique, the climate contributes to a variety of natural ecosystems with differences in fauna and flora. Climate (Figure 3.4) is arguably the single most important factor in formation of the ecological zones of the countries. In general there are two seasons: the wet season from November to April and a dry season from May to October. The following subdivision of the country in to different ecological zones is based on Da Barca and Dos Santos (2000) and the Atlas Geográfico de Moçambique (1986).

3.2.2.1 Wet tropical region

This area includes almost all northern region and parts of the central region of Mozambique, with mean rainfall above 1000 mm and characterized by one effective rainy season much longer than the dry season. The rainy season is generally from November to April and dry and from May to October. The annual mean temperature varies from 24 °C to 26 °C.

In high altitude areas the climate is modified by altitude with rainfall. As matter of simplification high altitudes zones will be included within wet tropical regions. These include the high lands of Niassa, Zambeze, and Manica 500 meters above the sea level with a rainfall exceeding 1000 mm, but with mean temperatures of less than 24 -26°C.

In the south of Mozambique the climate is in general dry tropical, but there is a coastal strip 30 km to 60 km wide from the coast to hinterland, in the three provinces of south Mozambique. The annual rainfall fluctuates from 800 mm to 1400 mm and the area is classified as having a Wet tropical climate. The vegetation varies from coastal scrubs and mangroves in coastal margins to deciduous *Miombo*. At latitude 25 °S, approximately at Xai-Xai there is a large prairie separating the deciduous *Miombo* in the north and *Open Forest* in the South.

The vegetation of this region is mainly *Open Forest of Miombo* but with many spots of dense everygreen forest in the mountainous areas. Exception is found in the southern half of Tete, where the mean rainfall is between 600 mm and 800mm. In this area the vegetation is predominantly *Mopane Savannah* but with areas of *Baobab Savannah* (*Adansonia digitata*). This area has a climate characteristic of dry tropical areas.

The mangrove is part of the coastal vegetation, which is evergreen and situated within the area where the rainfall is higher than 800 mm.

Miombo makes up a mosaic with other vegetation types. Mature miombo forests typically have a 10-20 m tall, single-storey canopy. The shrub layer is discontinuous, the field layer varies from a dense and tall (1-2 m) grass stand to a sparse but continuous cover of herbaceous plants. The canopy is dominated by trees belonging to the subfamily *Caesalpinioideae*, particularly to three closely related genera *Brachystegia*, *Julbernardia* and *Isoberlinia* (White 1983, in http://www.lodestone.org/users/maria/misc/miombo1.html). The name of Mopane is derivate from the fact that *Colophospermum mopane* being the predominant tree. The mopane trees have deciduous leaves and are tolerant of poorly drained or alkaline soils and those with high clay content. This means the *Mopane* has a wide range of distribution throughout southern Africa.

3.2.2.2 Dry tropical region

The Dry tropical climate is predominant in the south, with the effective dry season longer that the wet, with temperature normally exceeding 26 °C. The hot rainy season is generally from November to April and a cold dry season from May to October. There are two distinct areas. The first is a strip occupying the Save river basin, descending to the south and became narrow from Mabote, continuing to south and terminate in Catuane region. The annual rainfall is between 600 to 800 mm, with most of the rain between January -March. The vegetation is mainly *Dry Deciduous Miombo*. The interior of Gaza province is relatively arid with rainfall less than 600 mm. The vegetation is predominantly of *Savannah of Mopane* but with large areas of savannah of baobab (*Adansonia digitata*), and various *Acacia* species.

3.2.2.3 Semi-arid region

The semi-arid region is found mainly in the south, in the interior Gaza province, between Chicualacuala and Massingir. The mean annual temperature is between 24-26°C and rainfall less than 400 mm.

FIGURE 3.4 Climatic zones of Mozambique. Green=Wet tropical, Light yellow=Dry tropical, Yellow: Semi-dry Tropical and Purple= Climate modified by altitude
Source: Atlas Geográfico, Moçambique, 1986.

3.3   Geographical origin of the samples

3.3.1   Angola

In Angola, mosquitoes sampling was carried out in Funda (8 º 51'S, 13 º 34'E, 0 m to100 m) area of Cacuaco, a town located at approximately 30 km from Luanda, the capital of Angola. Samples were also collected from a coastal central area of Benguela (12 º 34'S, 13º 24'E, 0 m to100 m) and Lobito (12º 20'S, 13º 34'E, 0 m 100 m). Other mosquitoes were collected from the high lands of Huambo (12º 47'S, 15º 44'E, 1500 m to 2000 m). The detailed account of these localities can be found in section 7.2.1. (Chapter VII). Mosquitoes were collected during a WHO mission intended to evaluate the vector control activities specially the usage of bed nets in Angola. The localities were selected by local malaria control programme accordingly to the degree of usage of ITNs and in accordance with military security. Adult mosquitoes were collected during April of 2001, corresponding to the end of the rainy season. Indoor resting mosquitoes were collected from several houses (details in chapter VII) using the pyrethrum spray collection method. Larvae were also collected in Abril using a small metal soup ladle.

3.3.2   Mozambique

The majority of the samples for this study were obtained from surveys carried out in 2000-2002 using various collection methods. Samples comprised adult resting indoors or out or collected from human bait indoors or out, or as larvae from natural or man made ground water collections. The sampled localities represent different eco-climatic regions of the country and were collected in dry and wet seasons. These included sampling from the wet tropical areas, dry tropical and semi-arid regions. Samples used for analysis in Chapter IV were collected during 1998-2000.

All samples sites are shown in Figure 3.5 and the respective samples sizes are shown in the Table 3.1. The sampling sites are numbered with the same number in Figure and in the Table, respectively. The Table presents also the names of sites and their geographical coordinates as well as altitude, season of collection and eco-climatic region.

FIGURE 3.5. Collection s sites in Mozambique as indicated by black triangles. Site numbers correspond to those in Table 3.1. Source: Atlas Geográfico, Moçambique , 1986.

TABLE 3.1. Origin of samples of *An. gambiae and An. funestus s.l.* females caught in localities of Mozambique Numbers in parenthesis represent the percentage in relation to total sample size.

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
1	Matutuine	Catuane			12/06/2000	Dry	Dry Tropical	< 100	ASP	10	90	10 (11)	80
1	Matutuine	Catuane			12/06/2000	Dry	Dry Tropical	< 100	HBI		24	13 (54)	11
1	Matutuine	Catuane			12/06/2000	Dry	Dry Tropical	< 100	HBO		36	23 (64)	13
2	Matutuine	Salamanga	26 28 21	32 36 59	12/05/2000	Dry	Dry Tropical	< 100	ASP	10	125	26 (21)	99
2	Matutuine	Salamanga	26 28 21	32 36 59	12/05/2000	Dry	Dry Tropical	< 100	HBI		254	193 (76)	61
2	Matutuine	Salamanga	26 28 21	32 36 59	12/05/2000	Dry	Dry Tropical	< 100	HBO		352	345 (98)	7
3	Maputo	Catembe			21/04/2001	Wet	Dry Tropical	< 100	ASP	10	99	0	99
4	Boane*		26 03 10	32 19 38			Dry Tropical	< 100					
5	Manhica	Maragra			27/09/2001	Wet	Wet Tropical	< 100	PSC	7	20	1 (5)	19
5	Manhica	Maragra			Nov./2000	Wet	Wet Tropical	< 100	PSC	10	82	15	67
6	Chokwe	Chihaquelane	24 47 17	33 08 08	07/04/2000	Wet	Dry Tropical	< 100	PSC	15	1677	1116 (67)	561
6	Chokwe	Chihaquelane	24 47 17	33 08 08	07/04/2000	Wet	Dry Tropical	< 100	HBI		239	200 (84)	39
6	Chokwe	Chihaquelane	24 47 17	33 08 08	07/04/2000	Wet	Dry Tropical	< 100	HBO		312	300 (96)	12
6	Chokwe	Chihaquelane	24 47 17	33 08 08	12/05/2000	Dry	Dry Tropical	< 100	PSC	15	502	258 (51)	244
7	Chokwe	Hokwe			10/06/2000	Dry	Dry Tropical	< 100	HBI		30	10 (33)	20
7	Chokwe	Hokwe			11/06/2000	Dry	Dry Tropical	< 100	HBO		178	165 (93)	13
8	Massingir	Massingir	23 53 21	32 09 02	19/07/2001	Dry	Dry Tropical	100-200	PSC	10	203	44 (22)	159
8	Massingir	Massingir	23 53 21	32 09 02	14/02/2002	Wet	Dry Tropical	< 100	PSC	10	235	54 (23)	181

TABLE 3.1. *Cont.*

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** Meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
9	Mabalane	Combomune	23 27 59	32 27 55	17/07/2001	Dry	Dry Tropical	< 100	PSC	10	61	58 (95)	3
9	Mabalane	Combomune	23 27 59	32 27 55	17/07/2001	Dry	Dry Tropical	< 100	LAR		73	73 (100)	0
10	Chicualacuala	Mapai	22 43 42	32 03 49	18/07/2001	Dry	Dry Tropical	< 100	PSC	10	0	0	0
10.1	Chicualacuala	Machichi	22 08 03	31 39 17	18/07/2001	Dry	Dry Tropical	< 100	PSC	10	0	0	0
11	Xai-Xai	Nhacutse	34 43 50	33 43 43	07/04/2000	Wet	Wet Tropical	< 100	PSC	10	99	89 (90)	10
12	Inharrime	Nhamba	24 29 30	35 00 49	16/03/2001	Wet	Wet Tropical	< 100	PSC	5	476	222 (47)	252
13	Quissico	Macome	24 43 45	34 48 07	16/03/2001	Wet	Wet Tropical	< 100	PSC	6	93	65 (70)	28
13	Quissico	Macome	24 43 45	34 48 07	16/03/2001	Wet	Wet Tropical	< 100	LAR		67	67 (100)	0
14	Homoine	Chidjinguire	23 56 32	35 10 44	13/07/2000	Dry	Wet Tropical	< 100	PSC	5	164	97 (59)	67
14	Homoine	Chidjinguire	23 56 32	35 10 44	06/07/2001	Dry	Wet Tropical	< 100	PSC	10	343	76 (22)	267
14	Homoine	Chidjinguire	23 56 32	35 10 44	06/07/2001	Dry	Wet Tropical	< 100	LAR		32	32 (100)	0
14	Homoine	Chidjinguire	23 56 32	35 10 44	17/03/2001	Wet	Wet Tropical	< 100	PSC	5	483	101 (21)	382
14	Homoine	Chidjinguire	23 56 32	35 10 44	11/04/2001	Wet	Wet Tropical	< 100	PSC	10	214	86 (40)	128
14	Homoine***	Chidjinguire2	23 56 32	35 10 44	Jul./2001	Dry	Wet Tropical	< 100	HBI		45	16 (36)	29
14	Homoine***	Chidjinguire3	23 56 32	35 10 44	Jul./2001	Dry	Wet Tropical	< 100	HBO		4	2	2
14	Homoine	Chidjinguire	23 56 32	35 10 44	02/02/2002	Wet	Wet Tropical	< 100	PSC	10	627	340 (54)	287
14	Homoine	Chidjinguire	23 56 32	35 10 44	11/02/2002	Wet	Wet Tropical	< 100	HBI		135	76 (56)	59
14	Homoine	Chidjinguire	23 56 32	35 10 44	11/02/2002	Wet	Wet Tropical	< 100	HBO		95	71 (75)	24

TABLE 3.1. *Cont.*

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** Meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
15	Maxixe***	Bemba			Nov./1999	Wet	Wet Tropical	< 100	HBI		136	69 (51)	67
15	Maxixe***	Bemba			Nov./1999	Wet	Wet Tropical	< 100	HBO		106	103 (97)	3
16	Massinga	Malova	23 24 40	35 22 48	10/04/2001	Wet	Wet Tropical	< 100	ASP	10	129	0	129
17	Vilankulos	Pambara	22 00 00	35 11 41	09/06/2001	Dry	Wet Tropical	< 100	PSC	10	964	289 (30)	675
17	Vilankulos	Pambara	22 00 00	35 11 41	09/06/2001	Dry	Wet Tropical	< 100	LAR		67	67 (100)	0
17	Vilankulos	Pambara	22 00 00	35 11 41	08/02/2002	Wet	Wet Tropical	< 100	PSC	10	442	71(16)	371
17	Vilankulos	Pambara	22 00 00	35 11 41	08/02/2002	Wet	Wet Tropical	< 100	HBI		52	12 (23)	40
17	Vilankulos	Pambara	22 00 00	35 11 41	08/02/2002	Wet	Wet Tropical	< 100	HBO		153	49 32)	104
18	Mabote	Mabote	22 02 28	34 07 50	05/07/2001	Dry	Dry Tropical	100-200	PSC	10	82	73 (89)	9
18	Mabote	Mabote	22 02 28	34 07 50	05/07/2001	Dry	Dry Tropical	100-200	LAR		3	3	0
18	Mabote	Mabote	22 02 28	34 07 50	09/02/2002	Wet	Dry Tropical	100-200	PSC	10	35	29 (83)	6
19	Machanga	R.Muari	20 56 53	34 21 07	09/06/2001	Dry	Dry Tropical	< 100	PSC	10	35	27 (770	8
20	Buzi	Guara-Guara	19 52 49	35 28 50	29/04/2000	Wet	Wet Tropical	< 100	PSC	10	666	447 (67)	219
20	Buzi	Guara-Guara	19 52 49	35 28 50	21/12/2000	Wet	Wet Tropical	< 100	PSC	10	66	20 (30)	46
20	Buzi	Guara-Guara	19 52 49	35 28 50	03/07/2001	Dry	Wet Tropical	< 100	PSC	10	1611	145 (9)	1466
20	Buzi	Guara-Guara	19 52 49	35 28 50	03/07/2001	Dry	Wet Tropical	< 100	HBI		101	20 (20)	81
20	Buzi	Guara-Guara	19 52 49	35 28 50	03/07/2001	Dry	Wet Tropical	< 100	HBO		46	15 (33)	31

TABLE 3.1. *Cont.*

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** Meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
21	Dondo	Mutua			30/04/2000	Wet	Wet Tropical	< 100	PSC	5	77	22 (29)	55
21	Dondo	Mutua			01/05/2002	Dry	Wet Tropical	< 100	HBI		141	24 (17)	117
21	Dondo	Mutua			01/05/2002	Dry	Wet Tropical	< 100	HBO		111	40 (36)	71
21.1	Dondo	Chipangara			01/05/2000	Dry	Wet Tropical	< 100	PSC	5	102	19 (19)	83
22	Caia	Caia	17 48 28	35 19 32	25/07/2000	Dry	Wet Tropical	< 100	PSC	4	67	2 (3)	
23	Manica	Machipanda	18 58 48	32 48 06	29/04/2000	Wet	C.M.A	1000-1500	PSC	10	113	2 (2)	111
24	Catadinca	Barue	18 09 21	33 09 22	11/06/2001	Dry	C.M.A	1000-1500	PSC	8	8	2	6
25	Guro	Guro	17 24 51	33 21 29	10/08/2000	Dry	C.M.A	500-1000	PSC	5	3	3	0
25	Guro	Guro	17 24 51	33 21 29	10/08/2000	Dry	C.M.A	500-1000	LAR		41	41 (100)	0
25	Guro	Guro	17 24 51	33 21 29	22/12/2000	Wet	C.M.A	500-1000	PSC	5	82	79 (96)	3
25	Guro	Guro	17 24 51	33 21 29	12/06/2001	Dry	C.M.A	500-1000	PSC	8	4	2	2
26	Changara	Mazoe	16 16 22	33 32 44	13/07/2001	Dry	Dry Tropical	200-500	ASP	10	24	24 (100)	0
27	Tete	Tete			12/09/1999	Wet	Dry Tropical	100-200	LAR		23	23 (100)	0
28	Moatize	Moatize	16 04 15	33 50 52	14/06/2001	Dry	Dry Tropical	200-500	PSC	9	47	1 (2)	46
29	Moatize	Zobwe	15 28 02	34 20 30	14/06/2001	Dry	Dry Tropical	200-500	PSC	8	2	1	0
30	Angonia	Ulongue	14 43 22	32 21 39	09/08/2000	Dry	C.M.A	1000-1500	PSC	4	22	3 (14)	19
31	Fingoe	Fingoe	15 07 17	32 10 41	13/06/2001	Dry	C.M.A	500-1000	PSC	10	6		1 5

TABLE 3.1. *Cont.*

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** Meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
32	Namacurra	Macuse	17 43 24	37 11 26	26/07/2000	Dry	Wet Tropical	< 100	PSC	3	45	26 (58)	19
33	Mocuba	Mocuba	16 50 29	36 59 25	09/05/2000	Dry	Wet Tropical	200-500	PSC	4	8	8	0
34	Ile	Mamirauane	16 02 00	37 10 48	27/07/2000	Dry	Wet Tropical	500-1000	PSC	4	36	4 (11)	32
35	A. Molocue	Chapale	15 53 25	37 37 41	28/07/2000	Dry	Wet Tropical	500-1000	PSC	5	15	9 (60)	6
35	A. Molocue	Chapale	15 53 25	37 37 41	27/06/2001	Dry	Wet Tropical	500-1000	PSC	10	84	30 (36)	54
36	Ribabwe	Ribawe	14 56 01	38 19 53	29/07/2000	Dry	Wet Tropical	500-1000	PSC	5	75	21 (38)	54
37	Nampula	Barragem			20/12/1998	Wet	Wet Tropical	200-500	PSC	5	8	8	0
38	Mossuril	Mossuril	14 57 42	40 39 32	01/08/2000	Dry	Wet Tropical	< 100	PSC	2	155	22 (14)	133
39	Namapa	Erati	13 45 36	39 43 20	02/08/2000	Dry	Wet Tropical	500-1000	PSC	4	156	55 (35)	101
40	Pemba	P. Miazi	13 06 07	40 26 53	16/05/2000	Dry	Wet Tropical	< 100	PSC	5	32	26 (81)	0
40.1	Pemba	P. Metuge			16/05/2000	Dry	Wet Tropical	< 100	PSC	5	25	25 (100)	0
40.1	Pemba	P.Metuge			22/05/2001	Dry	Wet Tropical	< 100	ASP	10	30	0	36
41	Montepuez	Montepuez	13 07 28	38 59 44	07/08/2000	Dry	Wet Tropical	200-500	LAR		11	11 (100)	0
42	M. Praia	M. Praia	11 21 03	40 21 48	23/06/2001	Dry	Wet Tropical	< 100	PSC	10	319	214 (67)	105
42	M. Praia	M. Praia	11 21 03	40 21 48	23/06/2001	Dry	Wet Tropical	< 100	LAR		173	173 (100)	0

TABLE 3.1. *Cont.*

Site No Number	District	Locality	Latitude South	Longitude East	Data	Season	Ecological Zone	Altitude** Meters	CMT	NHS	Sample size	*Anopheles gambiae s.l.*	*Anopheles funestus s.l.*
43	Mueda	Chapa	11 59 11	39 17 09	24/06/2001	Dry	Wet Tropical	500-1000	PSC	10	8	6	0
43	Mueda	Chapa	11 59 11	39 17 09	24/06/2001	Dry	Wet Tropical	500-1000	LAR		31	31 (100)	0
44	Cuamba	Cuamba			Aug./2000	Dry	Wet Tropical	500-1000	LAR		12	12 (100)	0
45	Maua	Maua	13 14 37	37 28 17	16/06/2001	Dry	Wet Tropical	500-1000	PSC	10	172	20 (12)	152
46	Marrupa	Marrupa	13 11 59	37 31 32	17/06/2001	Dry	Wet Tropical	500-1000	PSC	9	87	10 (11)	77
47	Lago	Metangula	12 41 33	34 52 25	19/06/2001	Dry	C.M.A	200-500	PSC	10	389	37 (10)	352

KEY:
* Samples used for chapter IV
** Obtained from Atlas Geográfico , Moçambique
***. Single collection
CMT- Collection methods
NHS- Number of houses sampled
ASP – Collection by aspiration (samples kindly provided by Sonia Casimiro and Ivone Rungo)
HBI- Human bait indoor
HBO- Human bait outdoor
LAR –Larvae collection
PSC- Pyrethrum spray collection

3.4 Sampling methods applied

3.4.1 Indoor and outdoor resting mosquitoes

Although only comparatively few anopheline species rest in human and animal habitations, those that do are often important vectors of malaria and filariasis (Service, 1993). Malaria vectors were collected using hand collection with aspirators (HC) or pyrethrum spray catch technique (PSC) (WHO, 1975). The PSC method is enables collection of about 80-90% of mosquitoes resting inside the houses (Service, 1977). The catches, however, are influenced by the type of structure, for example whether the house has gaps, such as at the eaves, windows or doors, which allow varying numbers of mosquitoes to escape during pyrethrum collection. Nevertheless, PSC is now used as a standard, quick and easy method of catching mosquitoes resting in huts and animal shelters (Service, 1977).

Many houses in rural localities of Mozambique consist of one room and have no conventional beds; very few houses have furniture. Before collection starts verbal consent was obtained by the householder in the presence of the political or administrative authority of the locality. In the absence of these persons, they were substituted by a teacher or a community health official of the area. Before PSC collection is performed potable water, food, and some domestic utensils and light furniture (e.g. chairs) were removed from the house. White sheets were then laid over the bed and floor. Two people went round the house spraying the eaves to prevent any mosquito escaping from the house, using a domestic commercial insecticide. Just before the two spray men complete the half-round a signal was given to the third man to start spraying indoors. After spraying, the house was left for 10 min to 15 min for the knockdown effect to take place and mosquitoes to fall on the sheets. Dead mosquitoes were collected on sheets with forceps, kept in Petri dishes and conserved in cool boxes until morphological identification. The insecticide used is known commercially as

Baygon [Cyfluthrin (Pyrethroid) 0,25g/Kg, Propoxur (Carbamate) 10,0g/Kg, Dichlorvos (Organophosphate) 5,0 g/Kg,)] from Bayer.

Hand-collection with mouth-operated aspirators (HC), generally yield fewer mosquitoes than PSC. Another disadvantage of HC is that it relies on the intensity of efforts of the collectors. Additionally, many mosquitoes rest in positions difficult to reach.

HC was applied in the Boane area during studies conducted from 1998 to 2000 and in a few collections carried out by the Malaria Control Programme teams, and mosquitoes kindly provided to this study. In Boane HC was also used during 1999 to collect mosquitoes in pit shelters. Basically in HC mosquitoes are searched for on the ceilings, walls, in hanging clothes, underside of beds and tables and others surfaces with a torch to locate mosquitoes. When located are then sucked by the tube and kept in a paper cup.

3.4.2 Blood seeking mosquitoes

The most direct way to measure the human biting rate is the human bait catch (WHO, 1975). Despite being expensive, technically difficult to replicate and unethical in areas of drug-resistant malaria, this method is unique in that it directly samples human-biting mosquitoes (Le Goff *et al.*, 1997). Blood-seeking female mosquitoes were collected by human bait catch indoors (HBI) and outdoors (HBO) by a team of two collectors rotated from indoors to outdoors on an hourly basis. This team was replaced at midnight by another team. Experienced collectors sat on stools with their legs exposed below the knees. Mosquitoes were caught with mouth aspirators just after landing and placed in paper cups, a new cup being started every hour. Prophylaxis with chloroquine was mandatory for those involved in human bait collection at laboratório de Entomologia do Instituto Nacional de Saúde, Maputo. The collection was carried out in eight localities, each locality being sampled once.

The reliability of Light Traps (LT) as a method for estimating the human vector contact is subject of some debate. For example, Lines *et al.* (1991) in Tanzania and Magbity *et al.* (2002) working in southern Sierra Leone concluded that LT combined with untreated bed-nets can be used as a surrogate HBC in estimating biting rates. However, in the coastal zone of Kenya, Mbogo *et al.* (1993) found that light traps did not provide an adequate estimate of man-vector contact.

Nevertheless LT collection was tried but was abandoned due to the difficulties of recharging the batteries, as many localities visited do not have electricity or if they do, it is just for a few hours at night.

3.4.3   Sampling immature stages

Larvae collections were performed in some localities of the country on surface water. The collections were carried out using a small metal soup ladle or a plastic dipper with long handles. They were both comparable in terms of volume. The plastic dipper with long handles was only used when the accessibility to the breeding site was difficult, mainly around bridges or in localities where it was suspected that land mines remain. The number of dips was dependent on the surface area of the breeding sites. Thus the dips, varied from just one dip in a smaller water bodies to the maximum of 20-30 dips in large water collections (e.g.: water bodies > 5 m^2). When larvae were present, all larvae instars were taken but further work was limited to the third and fourth instars.

3.5   Morphological identification and preservation of material

In the field, at the sites of collection, adult mosquitoes were first identified by the mean of morphological characters (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987) as members of the *Anopheles gambiae* complex or *An. funestus* complex and then they were stored dry over silica gel in Eppendorf tubes with a label indicating the name of the locality, day, species and numbers of specimens.

Larvae were also identified on the basis of morphological characters using the keys of the above-mentioned authors, and preserved in Eppendorf tubes in 70% ethanol.

3.6.   DNA extraction

DNA extraction was made using of three different techniques: STE method of Robertson and MacLeod (1993), the method of Collins *et al.* (1987) and the phenol-chloroform extraction of Ballinger-Crabtree *et al.* (1992). The STE and Collins methods were used in PCR-based identification to species for both *An. gambiae* and *An. funestus* complexes. These methods are rapid allowing DNA to be extracted, PCR-amplified and the results of species identification to be obtained in the same day, allows large numbers of mosquitoes to be identified in a relatively short period of time.

Although involving much more time, the inclusion of a phenol extraction step has the advantage of producing a cleaner and hence more stable DNA template, which can be stored for longer periods at 4 °C (Ballinger-Crabtree, *loc. cit*). This method was used for the extraction of DNA from mosquitoes for population structure studies and also for some samples during the optimisation of PCR for species identification of members of the *An. funestus* complex and for identification of M and S forms of *An. gambiae s.s.*

3.6.1 STE method

Of all the methods used for DNA extraction, STE is the fastest method of preparing nucleic acids for PCR. The method, developed by G. Gloor and W. Engels (Robertson and MacLeod, 1993) was used here with minor modifications. Individual mosquitoes or portions of mosquito were homogenised in 1.5 mL Eppendorf tubes with tapered glassrod or plastic pestles with 50 μL Sodium-Tris-EDTA buffer (STE) (0.1 M Nacl, 10 mM Tris Hcl pH 8.0, 1 mM EDTA pH 8.0). The homogenate was then incubated for 10 min at 95 °C, centrifuged for 3 min at 13,000 rpm and the supernatant transferred to a new tube. The DNA was then stored at –20 °C, and subsequently 0.5-1 μL used in 12.5 μL PCR reactions. In some cases DNA had to be extracted from the homogenates of mosquito heads and thoraxes used for sporozoite ELISA. For these, first 400 μL of sterile water was added to the ELISA homogenate, this was mixed throughout and then centrifuged for 5 min at 13 000 rpm. The supernatant was removed and DNA extracted from the pellet following the STE method as described above.

3.6.2 Method of Collins *et al* (1987)

This is a simplified protocol based on a modification of the protocol of Livak (1984). Individual mosquitoes or portions of mosquitoes were homogenised in a 1.5 mL Eppendorf tube, with a conical glass or plastic pestle, in 50 μL of lysis buffer (0.08 M Nacl, 0.16 M Sucrose, 0.06 M EDTA, 0.5 % SDS and 0.1 M Tris-Hcl pH 8.6) and the homogenate was then incubated at 65 °C for 30 min. 8 M potassium acetate was added to a final concentration of 1 M and immediately incubated on ice for approximately 30 min. The homogenate was spun for10 min at 13 000 rpm at room temperature. The supernatant was transferred to a fresh tube and 100 μL of cold 95 % ethanol added. This was then centrifuged again for 10 min or until the pellet was visible. The supernatant was discarded and the pellet washed twice with cold 70

% ethanol, air-dried at room temperature for approximately 1 h, re-suspended in 200 µL of TE Buffer (10 mM Tris, 1mM EDTA, pH 8.0) and stored at 4 °C.

3.6.3 Phenol-chloroform extraction

The Ballinger-Crabtree *et al.* (1992) extraction method is a modification of the protocols of Jowett (1986) and Sambrook *et al.* (1989). It was performed with further minor modifications.

In the previous afternoon or evening individual mosquitoes were homogenised in a 1.5 mL Eppendorf tube with a tapered glassrod or plastic pestle and resuspended in 270 µL lysis buffer (100 mM Tris-Hcl pH 8, 50 mM EDTA, 1% SDS, 0.15 mM Spermine, 0.5 mM Spermidine) and 5 µL of a 20 mg/ml solution of proteinase K and 30 µL of 10% SDS solution and incubated overnight at 50 °C. Next day the suspension was gently extracted twice. Equal volumes of phenol: chloroform: isoamylalcohol in the ratio of 25:24:1 was added to the suspension, gently mixed on a rotator for 15min to 20 min and spun for 10 min at 14 000 rpm. The upper aqueous layer was transferred to a new tube and extracted for a second time with chloroform: isoamylalcohol (24:1) and then the solution mixed gently and spun for 5 min at 14 000 rpm. The upper layer was removed to a new tube, and the DNA precipitated by adding 0.2 volumes of cold 10 M ammonium acetate and 2 volumes of absolute ethanol. The homogenate was mixed well and placed at –20 °C for about 15 min, then centrifuged for 30 min at 4°C at 14,000 rpm. The supernatant was removed, the pellet washed with cold 70 %, and then air dried for a minimum of 1 h at room temperature and re-suspended in 200-400 µL of TE Buffer (10 mM Tris, 1 mM EDTA). DNA samples were subsequently stored at 4°C.

3.7 PCR amplification and analysis

3.7.1 Species diagnosis

Two different PCR methods were performed in this study for species diagnosis of members of the *An. gambiae* and *An. funestus* complex. Initially, species diagnosis of members of the *An. gambiae* complex was carried out in Maputo, Mozambique. Both in Maputo and Liverpool, PCR amplifications of DNA target fragments were carried out in a Hybaid Omnigene thermocycler. In the UK, PCR reagents were mainly from Sigma but occasionally *Taq* polymerase and buffers were supplied by Qiagen. In Mozambique, with the exception of sterile water that was supplied by Sigma, all the PCR reagents including primers were from Amersham Pharmacia Biotech (Amersham Biosciences).

3.7.1.1 *Anopheles gambiae* complex

Amplification of the rDNA of members of the *An. gambiae* complex was performed with minor modifications as outlined by Scott *et al.* (1993). The reaction conditions were the following: 1 μL of 400 μL of the mosquito DNA, 2.5 μL of 10x reaction buffer, 1.2 mM of each dNTP (dATP, dCTP, dGTP and dTTP), 1 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase, 6.25 ng of primer GA, 12.5 ng of primers UN and ME, 18.75ng of primer AR, 25 ng of primer QD. Sterile H_2O was finally added to give a total volume 25 μL and overlaid with 1-2 drops of mineral oil in 0.5 mL microfuge tube. The PCR was carried out with a programme of 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s for 30 cycles and a final step of extension at 72 °C for 7 min. In Liverpool all the reagents were halved and PCR conditions included a pre-denaturation step of 95 °C and the final step of extension at 72 °C for 10 min.

After the PCR was complete, 10 µL of the reaction volume was removed from under the mineral oil, mixed with 5 µL of a standard agarose gel loading buffer (20% Ficoll in 25 mM EDTA pH8 containing small amount of orange-G), electrophoresed through a 1.2% agarose-Tris-borate-EDTA gel containing ethidium bromide in a TBE Buffer (0.089 M Tris, 0.089 M Boric acid, 0.002 M EDTA). The expected lengths of the sequences in nucleotides amplified between universal primers and the specific primers were read having as a reference a commercial molecular weight marker (2176-154 bp) supplied by Boehringer, but in Liverpool the molecular weight marker (1000-100 bp) was supplied by Promega. The amplified fragments were visualized by illumination with short wave-length ultraviolet (UV) light and photographed on black and white Polaroid film type 55 supplied by Hargreaves Photo Ltd. In Liverpool the amplified fragments were photographed using a Kodak digital imaging system Species identifications used DNA extracted through STE or Collins *et al* methods. The primers sequences and expected product size after electrophoresis are shown in Table 3. 2.

3.7.1.2 M and S molecular forms of *Anopheles gambiae s.s*

DNA extracted using the phenol-chloroform method was used for simultaneous identifications of species and molecular forms of *An. gambiae s.s.* (della Torre *et al*, 2001) as outlined by Fanello *et al.* (2002). This was performed only in Liverpool. The method uses the primers and protocol of Scott *et al.* (1993), followed by a restriction enzyme digest step. After PCR was finished, 1U of *Hha* I in 1× SuRE/Cut Buffer L (Roche Diagnostic, Basel, Switzerland) was added to the PCR products and digestion was carried out at 37 °C for 4-6 h. PCR products were electrophoresed through a 2% agarose-Tris-borate-EDTA gel containing 1.5 µL of Ethidium bromide. The digested fragments were visualized on a UV transilluminator as described above. After PCR-RFLP, *An. gambiae* S form is characterised

by two fragments of 257 bp and 110 bp. *An. gambiae* M form is characterised by a fragment with 367 bp (see Figure 7.4).

3.7.1.3 *Anopheles funestus* complex

PCR amplification of the rDNA of members of the *An. funestus* complex was performed, with minor modifications, as outlined by Koekemoer *et al.*, (2003). The reaction conditions were: 0.5µl of 50 µL of the mosquito DNA (STE method) or 0.5 µL of 200 µL of mosquito DNA (phenol method), 1.25 µL of 10x reaction buffer, 1.5 mM MgCl$_2$, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 1.2 pmol of each of primer, 0.5 U of *Taq* DNA polymerise and sufficient sterile H$_2$O to give a total volume 12.5 µL and overlaid with 1-2 drops of mineral oil in 0.5 mL microfuge tube. The PCR cycling was carried out with a programme of one cycle at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 40 s with a final step of extension at 72 °C for 5 min. After the PCR was complete, 5-10 µL of the reaction volume was removed from under the mineral oil and electrophoresed through a 2% agarose gel stained with Ethidium bromide. The primers sequences and expected approximated product size after electrophoresis are shown in Table 3.3.

TABLE 3.2. Primer sequence of species-diagnostic of *Anopheles gambiae* members, together with expected PCR product size (from Scott *et al.*, 1993).

Primer name	Primer sequence (5' to 3')	Product size (bp)
UN	GTG TGC CCC TTC CTC GAT GT	
GA	CTG GTT TGG TCG GCA CGT TT	390
ME	TGA CCA ACC CAC TCC CTT TA	466
AR	AAG TGT CCT TCT CCA TCC TA	315
QD	CAG ACC AAG ATG GTT AGT AT	153

The allopatric *An. merus* and *An. melas* are both identified specifically by ME primers, but the sequence between UN and ME is 2 nucleotides longer in *An. merus*.

TABLE 3.3. Primer sequence of species-diagnostic of *An. funestus* members, together with the approximated PCR product size (from Koekemoer *et al.*, 2002).

Primer name	Primer sequence (5' to 3')	Product size (bp)
UV	TGT GAA CTG CAG GAC ACA T	
FUN	GCA TCG ATG GGT TAA TCA TG	505
VAN	TGT CGA CTT GGT AGC CGA AC	587
RIV	CAA GCC GTT CGA CCC TGA TT	411
PAR	TGC GGT CCC AAG CTA GGT TC	252
LEES	TAC ACG GGC GCC ATG TAG TT	146

3.8   Sporozoite ELISA for *Plasmodium falciparum*

Traditionally, the determination of sporozoite infection rates is through laborious and skilled direct dissections of salivary glands of fresh mosquitoes. The introduction of enzyme-linked immunosorbent assays (ELISA) has allowed the introduction of a simpler and cheaper tool for malaria epidemiological studies, as well as for monitoring malaria control. The technique is based on detection of the circumsporozoite protein (CSP- epitopes that are amply expressed on the surface of sporozoites) by highly species-specific monoclonal antibodies (Mabs) (Beier *et al.*, 1988, Wirtz *et al*, 1985, 1987).

Only Phosphate Buffered Saline (PBS) was prepared in our laboratories. All other reagents, including Mabs, were purchased from the Centers for Diseases Control and Prevention, USA. Individual heads and thoraxes of single female mosquitoes were tested for the presence of circumsporozoite protein (CSP) of *P. falciparum* (Pf) using the technique described by Burkot *et al.* (1984) and later modified by Wirtz *et al.* (1987).

The "sandwich" enzyme-linked immunosorbent assay (ELISA) is begun by coating the 96-microtiter plate with a capture antibody directed against CSP at the concentration of 0.1 µL monoclonal antibody of Pf in 50 µl PBS. This is done in the evening before and left overnight at 37 °C. In the next day, well content was aspirated and wells filled with 200 µL of BB (Blocking buffer) to block the remaining active sites and incubated for one hour at 37 °C. At this stage homogenates of head and thoraxes of individual mosquitoes that have been prepared in advance and frozen at –20 °C was removed from the freezer and left to thaw at 4 °C. After one hour, the BB was aspirated and 50 µL of mosquito homogenates was added to the plate wells. Positive and negative controls were also added to specific wells at this time. Negative controls were known unfed females cultivated in the insectaries. After two hours incubation, the mosquito triturate was aspirated and the wells washed twice with PBS-0.05%

Tween. Bound CSP was detected with a peroxidase-linked antibody (0.05 μg/ μL) after one hour of incubation at 37 °C, completing the formation of the "sandwich. After this period the well content was aspirated, the plate washed three times. The reading was carried out 30 min after incubation in the dark after adding ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-sulphonoc acid)] as substrate on an automated ELISA reader at 405 nm.

A sample was considered positive if it produced a visually detectable green colour with an OD (optical density) value at least twice the mean of the OD of seven negative control wells on the plate (Beier *et al.*, 1988). In order to confirm the results, all positive mosquitoes were re-tested.

CHAPTER IV

The bionomics and infection rates of malaria vectors in Southern Mozambique, with special reference to *Anopheles merus* before and after the floods of 2000

Abstract

The present study examined some aspects of bionomics of *Anopheles* species in Boane, southern Mozambique. Adult mosquitoes were collected by manual aspiration, human bait catches and larvae were collected from two types of water collections from February 1998 to June 2000. Mosquitoes were identified using rDNA-PCR techniques.

Permanent brackish water bodies yielded only specimens of *An. merus,* whilst temporary fresh water bodies contained almost equal numbers of *An. merus* and *An. arabiensis.* This is the first record of *An. merus* using the same breeding pools as *An. arabiensis.*

During 1998, of those anophelines collected on human bait, 97% were *An. merus,* with a mean density of 15.1 mosquitoes/man/night outdoors and only 9.8 mosquitoes/man/night indoors. The other species was *An. arabiensis* with 0.4 mosquitoes/man/night indoor and 0.5 mosquitoes/man/night outdoors. In indoors resting collection of 1998 and 1999 *An. merus* was the most common species with proportions varying from the minimum of 79% to 100%. However after the floods of 2000 the densities of both *An. arabiensis* and *An. merus* became comparable due to a 20-fold increase in densities of *An. arabiensis* in relation to previous years. The densities of *An. funestus* increased dramatically after the rain season. These changes are attributed to the exceptional rainy season of 2000 that caused floods in the area. The exceptional abundance of *An. merus* found by all methods of collection applied, suggests that it may be a major malaria vector in the area. The delayed oocyst rates of *An. merus* (14 ± 8.0 %) compared with *An. arabiensis* (11 ± 6.7 %) and *An. funestus* (7 ± 1.8 %) confirms that *An. merus* is an important malaria vector in the area

The proportions of blood-fed: gravid *An. merus* in indoor (1.9: 1) and outdoor collections (1.8: 1) were comparable suggesting partial exophily. This result together with the almost two fold higher densities in outdoors human biting collections suggests that house spraying alone would not be able to control this species.

4.1   Introduction

The majority of malaria in Africa is transmitted by three mosquitoes species; *An. arabiensis* and *An. gambiae*, members of the *An. gambiae* complex and *An. funestus s.s. An. merus*, is one of the six formally recognised species in the *Anopheles gambiae* complex, but its role in disease transmission has only been documented in Kenya and Tanzania (Muirhead-Thomson, 1951; Mosha and Petrarca, 1983, Temu *et al.*, 1998), but in the latter country it is also an important vector of filariasis (Bushrod, 1981).

In Mozambique much of vector control activities have been implemented in southern areas of the country. However, there is no systematic and comprehensive data on bionomics and relative importance of malaria vectors in these areas. The few available studies indicate the presence of three species of the *An. gambiae* complex in southern localities of the country, namely *An. arabiensis*, *An. merus* and occasionally *An. quadriannulatus* (Petrarca *et al.*, 1984; Cuamba *et al.* 1996). In these localities *An. arabiensis* together with *Anopheles funestus* are considered the most important malaria vectors (Cuamba and Dambo, 1994; Cuamba *et al.*, 1995; Thompson *et al.*, 1997 and Mendis *et al.*, 2000). However, none of the above-mentioned authors, although working in coastal areas, reported the presence of *An. merus* in their samples, although Donnelly *et al.* (1999), reported considerable numbers of *An .merus* from samples collected by this author in nearby localities in 1996.

In a small-scale unpublished study by this author, a diagnostic PCR technique (Scott *et al.*, 1993) was used to identify *An. gambiae s.l.* to species in samples collected in Boane during March 1996, less than 26 km from Matola, where the previous studies of Thompson (*loc .cit*) and Mendis (*loc.cit*) were carried out. *An. arabiensis* and *An. merus* were found in approximately equal numbers in mosquitoes collected in day resting collection. Because of

this unexpected species composition, it was thought important to ascertain the species composition of the *An. gambiae* complex occurring in the area.

During February 2000, parts of Southern Mozambique received exceptionally high rainfall, the highest observed over a period of 50 years. This resulted in flooding in most of the river basins and low land areas. Abnormally high rainfall may modify the normal breeding places of mosquitoes, favouring some and at the same time creating adverse condition for others. It was therefore hypothesised that flooding may have changed the species composition of the *An. gambiae* complex observed during previous years in Boane. It was expected that after the floods, *An. arabiensis* would outnumber *An. merus,* as the typical breeding sites of *An. arabiensis* will increase in number as floodwaters recede. Contrariwise, it was expected that the numbers of *An. merus* would decline as their breeding places were washed away and/or the brackish water diluted by the floods. Therefore, sampling was continued during 2000 to determine the effects on the ecology of members of the *An. gambiae* complex, with special reference to *An. merus*.

4.2   Materials and methods

4.2.1   Study area

The study was conducted in the region of Boane town (25° 2′S 32° 19′E) (site no. 4 in Table 3.1 and Figure 3.5) in the basin of the Umbeluzi River, about 26 km by road from Maputo, the capital of Mozambique (Figure 4.1). The climate of this region is dry tropical with monthly rainfall fluctuating from 0 to 326 mm and an annual average of 650 mm (Donnelly *et al.,* 1999). Accordingly, there is a hot rainy season from November to April and a cold dry season from May to October, and the average monthly temperature varies from a low of 19° C to a high of 26° C

In four days in February 2000, the region received more than three fold of the normal amount of rain for the month, 393.3 mm; much more than the half of normal rainfall of one year. This caused floods and breeding sites and larvae were washed away. The breeding conditions were extensively modified, for example the brackish-water marshes were diluted with fresh water. The pattern of rainfall during 10 years (1990-99) is compared with rainfall observed during 2000 in Figure 4.2. The rainfall data were kindly provided by *"Estação Agrária do Umbeluzi"*, which is located less than 4 km from the collection sites

The human population in Boane district is about 59 000, with 14 000 in the town and nearby bairros and other localities that form the district. The economy is based on agriculture (mainly citrus and maize) and cattle production. Subsistence farming crops include maize, cassava and vegetables. The most common domestic animals were chickens and goats.
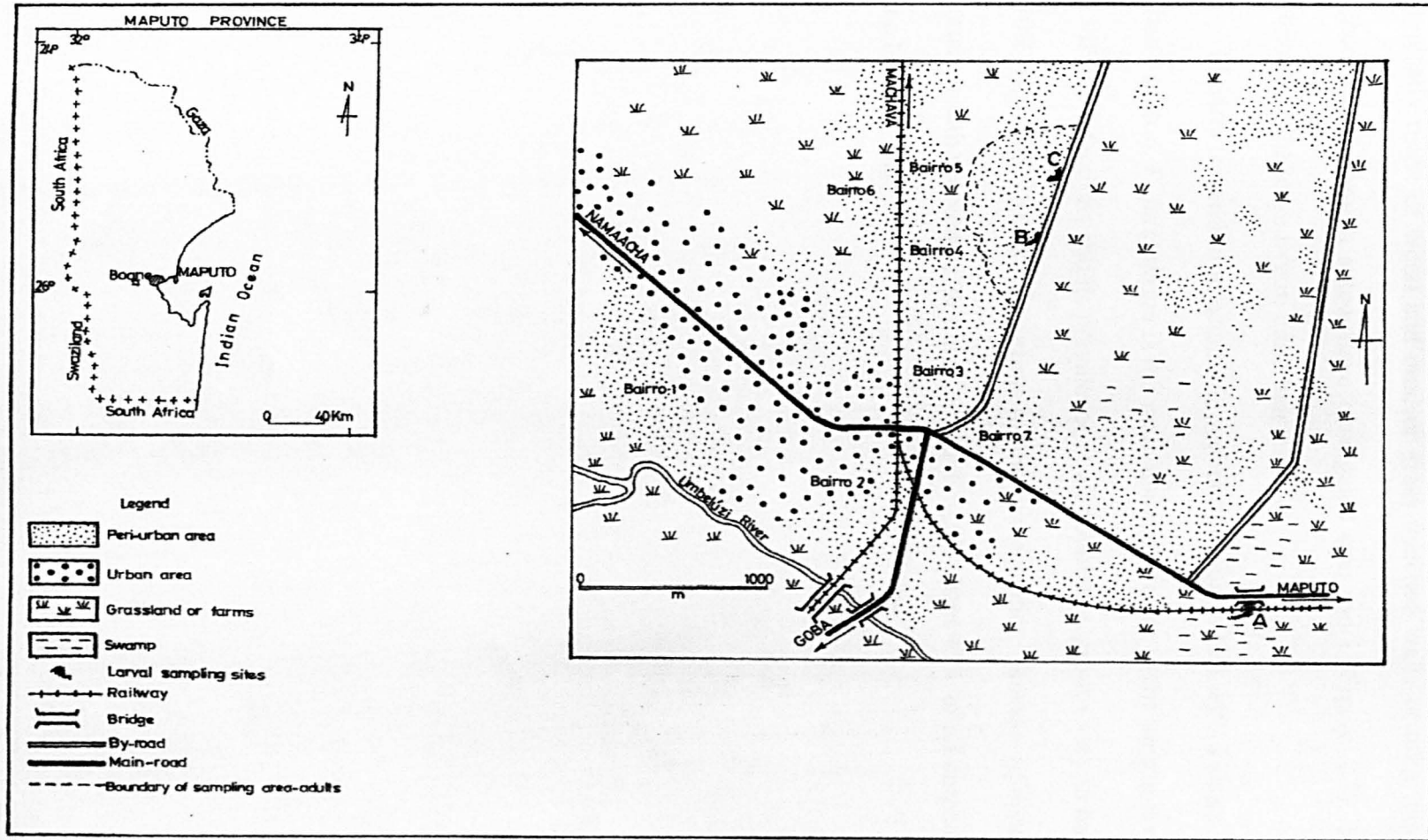
FIGURE. 4.1. Geographical location of Boane in Maputo province (top left) and detailed study site (right). A and B represents the larvae collection sites with explanation on section 4.2.2.1. The figure was adapted from maps from: Direcção Nacional de Geografia e Cadastro, Maputo.

In the centre of the town, the houses are built with bricks and cement. They generally have electricity and running water, while in the surrounding bairros the houses are typically made of wood and mud or grass thatched walls or both, mainly with grass thatched roofs although a few have corrugated iron roofs (Figure 4.3). The majority of inhabitants of the town live in these bairros.

Malaria transmission occurs throughout the year albeit with a sharp increase during the rainy season. *P. falciparum* is the main malaria species in the area accounting for more than 90% of infections and its prevalence in all population groups vary in bairros from 60% to 70% (Dgedge, 2000). According to the same author, disease (clinical) is the most important health problem in this area, accounting for about 45% of all outpatient visits to the Boane Health Centre.
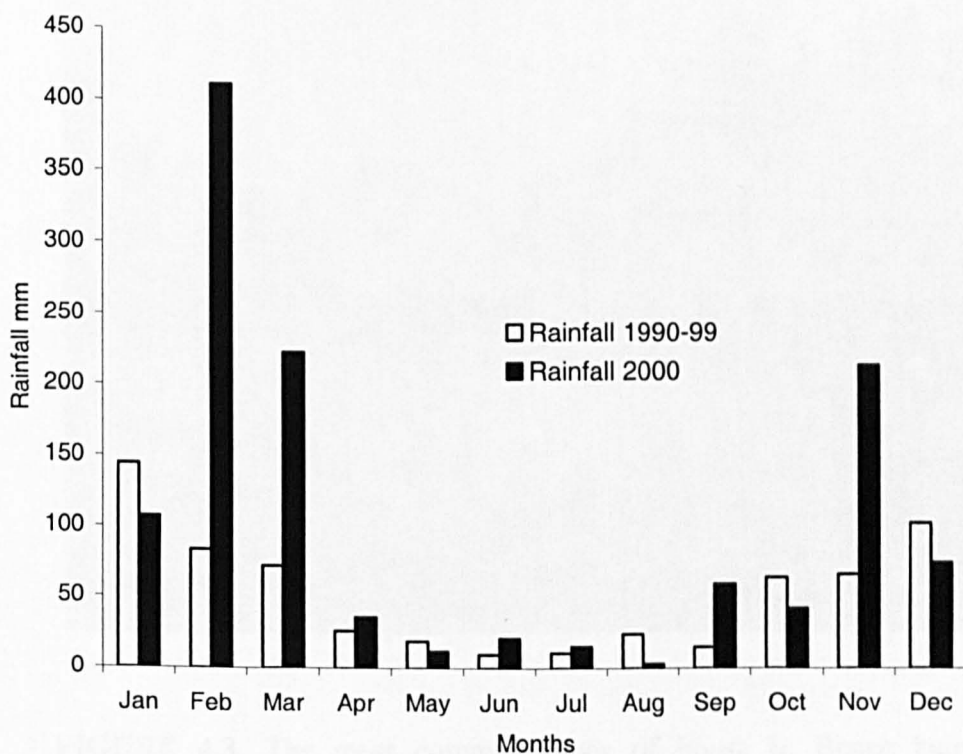
FIGURE 4.2. Average monthly rainfall observed from 1990-1999 and monthly rainfall during the year 2000 in Boane, southern Mozambique. Data were provided by "*Estação Agrária do Umbeluzi*", Boane.

FIGURE 4.3. The most common types of house in Boane bairros, southern Mozambique.

4.2.2 Mosquito collections

4.2.2.1 Larvae collection and breeding sites

Larvae were collected from 2 aquatic sites in the morning by dipping with a metallic ladle from February to May 1998. The first breeding site is a large permanent and brackish-water collection (Figure 4.4) located close to the Road Bridge from which it receives a temporary shade (breeding site A). It is fed by rainwater and just after the rains, it can reach a large dimension (c.500 m^2) that gradually reduces during the dry season and increases again during the next rain season. The second breeding site was a group of two small temporary, fresh water pools well exposed to the sun measuring each approximately 1 m^2 (B and C)). The pools were inundated with rainwater, and subsequently dried out some 3-4 weeks after the rains ceased. They were located at about 7 to 10 meters distance from the first line of houses in which adult collections were carried out. These breeding sites together with bairro 5 were separated from breeding site A by approximately 2.5 km. Between the two breeding sites there were a banana farm, some cassava farms and a grass field.

FIGURE 4.4. Overview of breeding site A (see Figure 4.1 and explanation in the text), exploited exclusively by *An. merus* in Boane, southern Mozambique

4.2.2.2 Adult mosquitoes collection

Adult mosquito collections were concentrated in bairro 5 of Boane town (Figure 4.1). During January-June 1998, eleven human landing catches were performed in parallel, indoors and outdoors, in one sentinel house, between 19.00-05.00 h. The procedures are fully described in section 3.4.2.

Mosquitoes resting in houses were hand-collected with mouth-operated aspirators from 10 houses, five times from January to February 1998. In April-May 1999, after the rainy season, seven parallel collections by manual aspirator, were performed each time in five houses and four pit shelters. In 2000, after the heavy rains, aspiration collections were carried out from April to June on 13 occasions in seven houses. In addition to these collections, three extra houses were sampled and the mosquitoes used to assess the delayed oocyst and sporozoite rates.

4.2.3 Laboratory procedures

4.2.3.1 Species identification

Adult mosquitoes were brought to the laboratory in cool boxes, killed by freezing and identified by morphological characters (Gillies and Coetzee, 1987) as members of *An. gambiae* complex or members of the *An. funestus* complex. The gonotrophic stages of female mosquitoes collected indoors and outdoors during 1999 were classified just after arrival in the laboratory (not more than 1.5 hours after collection), as unfed, fed, and gravid. These mosquitoes, together with all others collected in different years were stored dry over silica gel in Eppendorf tubes for subsequent species identifications. Larvae were also identified by morphological characters (Gillies and Coetzee, 1987), and kept in 70% alcohol. DNA from individual mosquitoes was extracted as described by Collins *et al.*

(1987) and resuspended in 200 μL of TE buffer (Sambrook *et al.*, 1989) as described in section 3.6.2. Species identifications were carried out according to the protocol and primers of Scott *et al.* (1993), described in section 3.7.1.1.

4.2.3.2 Malaria infection rates

Field fed collected *An. gambiae s.l.* and *An. funestus s.l.* mosquitoes were held in an insectarium of Instituto Nacional de Saúde (Maputo) during eight days. Mosquitoes were kept together in metallic and wire cages, in partially dark rooms at 25-27 °C and 70-80% relative humidity. The mosquitoes were allowed to feed on 10% glucose with multivitamins as a supplement. In the ninth day, all surviving mosquitoes were killed by freezing, species identified morphologically, and their midguts and salivary glands dissected for the presence of oocysts and sporozoites, respectively. All mosquitoes belonging to the *An. gambiae* complex were conserved individually in 75% alcohol for subsequent specific identification by PCR (Scott *et al.* 1993).

4.2.4 Data analyses

Oocyst or sporozoite rate is the percentage of female mosquitoes of a particular species that bear oocysts or sporozoites in their salivary glands, respectively. In the present case, we used the term "delayed" ocyst or sporozoite rate, which differ from previous concept because mosquitoes were held for eight days in the insectarium before dissection. Thus the oocyst rates and sporozoite rates are likely to be markedly lower and higher respectively, than the day of collection. Chi-square tests were used to compare the infections rates of malaria vectors. The 95% confidence interval for sporozoite rates of vectors species were calculated from the proportion of females infected with oocysts.

4.3   Results

4.3.1   Larvae collections

The permanent brackish water body yielded only specimens of *An. merus* (n=214), whereas the temporary fresh water bodies contained almost equal proportions of *An. merus* (51%) (n=20) and *An. arabiensis* (49%) (n=19). In temporary fresh water bodies, only two collections were possible since the water dried up in April. In the permanent brackish water it was possible to sample throughout the period of study (February-May, 1998).

4.3.2   Indoors and outdoors resting mosquitoes

In a small-scale study carried out on mosquitoes collected during March1996, *An. merus* (48.2%) and *An. arabiensis* were found in approximately equal proportions (n= 85). Mosquitoes collected resting indoors and outdoors in Boane between 1998 and 2000 are shown in Table 4.1. *An. merus* was, in relation to *An. arabiensis*, the most common species with percentages varying from the minimum of 79% to 100%, from indoor collection of 1998 to 1999, and outdoor collections of 1999 However after the floods of 2000 the densities of both *An. arabiensis* (2.0 mosquito/house) and *An. merus* (1.8 mosquito/house) became comparable; due to 20 fold increase in densities of *An. arabiensis* in relation to the previous year. *An. quadriannulatus* was collected in a single occasion resting indoors in 1999. During 1999 the density of *An. funestus* was 1.4 per house (n=49) but in 2000 the density was 15.7 per house (n=2043).

The gonotrophic stages of *An. merus* mosquitoes collected resting indoors and outdoors during 1999 are presented in the Table 4.2. Fed mosquitoes were the majority of both indoor and outdoor collections but with no detectable significant differences between them. Also there were no significant differences between the gravid mosquitoes indoors

and outdoors. The proportions of blood–fed: gravid females mosquitoes in indoor and outdoor collections were comparable.

TABLE 4.1. Indoor and outdoor (pit shelters) species composition and densities of *An. merus* and *An. arabiensis* in Boane during 1998-2000.

Year	Collection Site	No. samples	Sample size	*An. merus* %	*An. merus* Density	*An. arabiensis* %	*An. arabiensis* Density
1998	Indoor	5	95	79	1.5	21	0.4
1999	Indoor	7	60	93	1.6	7	0.1
	Pit shelter	7	45	100	1.6	-	-
2000	Indoor	10	266	47	1.8	53	2.0

TABLE 4.2. Gonotrophic stages of *An. merus* in indoor and pit shelters collections at Boane, during April- May, 1999.

Site	Gonotrophic stage Unfed n	%	Fed n	%	Gravid n	%	Fed: Gravid	Total
Indoor	0	-	36	66	19	34	1.9:1	55
Pit shelters	3	7	27	60	15	33	1.8:1	45

4.3.3 Night catches

Overall, *An. merus* comprised 96.8% of the combined indoor and outdoor collections (n=283). *An.merus* was 1.5 times more likely to bite man outdoors (n=166) than indoors (n=108). Outdoors *An. merus* had a mean of 15.1 mosquitoes/man/night and indoors 9.8 mosquitoes/man/night. *An. arabiensis* had a mean of 0.4 mosquitoes/man/night indoor (n=4) and 0.5 mosquitoes/man/night outdoors (n=5).

The biting activity of *An. merus* indoors and outdoors is presented in Figure 4.5. The species bite all night with numbers increasing throughout the night and decreasing after 1:00 h with only less that 1% still biting indoors at 4:00 h, whereas outdoors biting remained relatively higher during the same period (7%). The major peak were reached outdoors around midnight (12:00 to 01:00 h) and indoors one hour later.
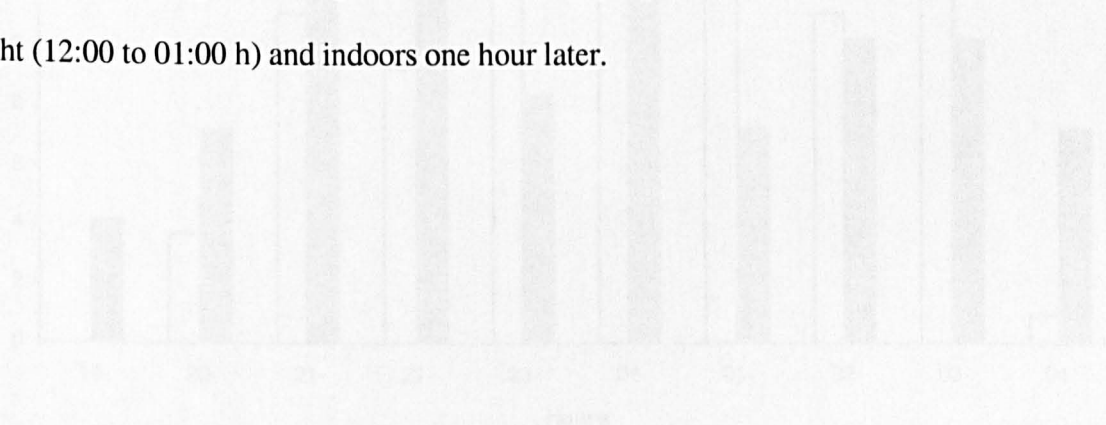
FIGURE 4.5. Hourly biting activity of *Anopheles merus* indoors (white bars, n=108) and outdoors (dark bars, n=166) at human bait in bairro 5 of Boane town, southern Mozambique.

4.3.4　Malaria infections rates

A total of 916 mosquitoes collected during April-June 2000 were dissected for the presence of delayed oocysts and sporozoites, of which 83.4% were *An. funestus*, 8.8% were *An. arabiensis* and 7.9% were *An.merus* (Table 4.3). There were no differences in the delayed oocyst rate between A*n. merus* (14 ± 8.0 %) and *An. arabiensis* (11± 6.7 %) and *An. funestus* (7 ± 1.8 %) (χ^2 =5.48 2df, *P*= 0.065). Also no significant differences were observed in delayed sporozoite rates between the three species (χ^2 =4.67, 2df, *P*= 0.1).

TABLE 4.3. Delayed oocyst and sporozoite rates of mosquitoes collected Boane during year 2000.

Species	No. dissected	Number of. Infected					
		Oocysts		Sporozoites		Both	
		n	%	n	%	n	%
An. arabiensis	83	9	11	8	10	7	8
An. merus	72	10	14	3	4	3	4
An. funestus	761	53	7	33	4	25	3
Total	916	72	8	44	5	35	4

4.4   Discussion

Three species belonging to the *An. gambiae* complex were present in different proportions in Boane; the brackish water breeding *An. merus* and fresh water breeding species, *An. arabiensis* were collected both as larvae and as adults from human bait and from indoor resting collections. *An. quadriannulatus*, another member of the complex that breeds in fresh water, which is strongly zoophilic, was only collected on a single occasion while resting in human dwellings.

Throughout East Africa, early reports suggest that *An. merus* was mostly restricted to coastal areas. The restricted coastal range of *An. merus,* was thought to be derived by strict saltwater breeding adaptation or competitive exclusion in inland areas of *An. gambiae s.s.* and *An. arabiensis* (Mosha and Petrarca, 1983). But in Boane, which is 30 Km from the sea, *An. merus* was present during the entire period of the study and was the most abundant species during all years with the exception of 2000. Elsewhere there is now enough data to support independency of *An. merus* from coastal conditions (see also discussion in Chapter 5). The observation from this study shows *An. merus* breeding not only in a permanent brackish water pool but also in temporary fresh water together with *An. arabiensis*. Fresh water breeding of *An. merus* has been observed in a locality of southern Africa (Coetzee *et al.*, 1993) but the species was found breeding together with *An. quadriannulatus*. Early in Kenya, Mosha and Mutero (1982) found that *An. merus* was able to complete its development in freshwater in the field. These observations strengthen the view of adaptation of *An. merus* to fresh water. The breeding habit together with continuous presence of *An. merus* throughout the period of the study indicates unequivocally that the species is endemic in the area. The exceptional abundance of *An. merus* observed during 1998 and 1999, leads to suggestions that this species may be a major malaria vector in the

area. With the absence of cattle in the vicinity, and within 2.5Km radius of human settlements, there is a possibility that *An. merus* will feed mainly on humans and therefore act as a major vector. Mnzava *et al.* (1997) observed higher frequencies of human blood index in *An. arabiensis* and *An. merus*, suggesting that both species could be playing a role in malaria transmission in the Maputo area.

The delayed oocyst rate of *An. funestus* of 4 % resemble those observed in an adjacent site after two days holding by Charlwood, *et al.* (1998). In Tanzania, Babiker *et al.* (1995) found a delayed oocyst rate of 11% in *An. funestus* and 21 % in *An. gambiae* after between 5-7 holding days. The rates in *An. funestus*, found by previous authors are higher than the rates of the present study and may reflect differences in malaria transmission patterns.

Up to now the role of *An. merus* in disease transmission has been studied in only a few places. Mosha and Petrarca (1983) in Kenya found a sporozoite infection rate of 3.3 % and more recently, in a coastal area of Tanzania, using an ELISA, Temu *et al.* (1998), found sporozoite rates of 6.2-11.6 %, indicating that *An. merus* plays an important role in malaria transmission. As after eight days of hold in insectarium *An. merus*, *An. arabiensis* and *An. funestus* presented comparable oocyst rates (and sporozoite rates), it is concluded that *An. merus* is an important vector of malaria in the study area. This is in accordance with earlier supposition on the role of *An. merus* in malaria transmission.

The changes in species composition in Boane after the heavy rain of 2000, is without doubt a direct effect of the rainfall/floods. Therefore, the reduction in the proportion of *An. merus* and the simultaneous increase in the proportions of *An. arabiensis* were not a surprise, as it was expected that the floods would wash away the breeding sites or dilute those of *An. merus* and thereby creating ideal breeding sites for *An. arabiensis* when the waters recede. *An. funestus* "boom" is also attributed to the rainfall/floods, which brought water in

a lowland area with plenty of grass, forming ideal breeding for *An. funestus* (see Gillies and De Meillon, 1968). *An. funestus* had be previously subject of detailed studies in adjacent area by this author and colleagues (Charlwood *et al.*, 1998).

The biting activity of *An. merus* has been previously studied in Jimbo, a coastal area of Kenya (Mutero *et al.*, 1984). The authors observed *An. merus* had a stronger tendency to bite outdoors than indoor, with outdoors and indoors biting activity peaking between 24.00 h and 01.00 h. Although with a smaller sample size the results of this study are consistent with the study from Kenya. Bed-nets are expected to be effective against mosquitoes that bite late at night indoors (Curtis, 1994). Before 21:00 h, the activity of *An. merus* was negligible indoors. Assuming that at least children and pregnant women are in bed by this time, bed-nets may be important in protecting this high-risk group from malaria.

The gonotrophic stages indoors and outdoors were comparable indicating a partial exophily of *An. merus* and suggesting the presence of a homogenous population. These results corroborate early findings in Kenya by Mutero *et al.*, (1984), although in Tanzania this species appeared to be highly exophilic (Bushrod, 1981). One of the health related objectives of "The Lubombo Spatial Development Initiative" is to reduce significantly the incidence of malaria morbidity and mortality in some localities of Southern Africa (http://www.malaria.org.za/lsdi/home.html). The programme relies on vector control through spraying houses with residual insecticides. *An. merus* being a malaria vector as showed here (4.4.2), the exophily and exophagic tendency of this species may pose a serious concern, as the species can not easily be brought under control just by spraying houses with residual insecticides.

4.4.1 Conclusions

1   In Boane, southern Mozambique, the *An. gambiae* complex was composed of *An. merus*, *An. arabiensis* and *An. quadriannulatus*.

2   Although *An. merus* was found breeding in fresh temporary water pools together with *An. arabiensis*, the main breeding site of *An. merus* occurred in permanent brackish marshes.

3   The changes in species compositions were attributed to the heavy rainfall observed during February 2000, which resulted in floods.

4   The partial exophilic nature in *An. merus* together with pronounced tendency to feed outdoors suggests that house spraying alone would not be able to control this species. But if bed nets were employed these could reduce the risk of malaria in children and pregnant women.

5   *An. merus*, together with *An. arabiensis* and *An. funestus* were vectors of equal importance in malaria transmission in Boane, southern Mozambique.

CHAPTER V

Distribution of species of the *Anopheles gambiae* and *Anopheles funestus* complexes in Mozambique.

Abstract

Mosquito collections were carried during 2000-2002 in 44 localities, representing different ecological areas of Mozambique. Mosquitoes were collected resting indoors, on human baiting catches and in larvae collections. A few samples from 1998-1999 were included in the study. The samples were collected as described, but included samples from outdoor collection in artificial pit shelters.

Identification of vector species within *Anopheles gambiae .s.l.* and *An. funestus s.l.* were carried out using rDNA-PCR techniques. Identification of the molecular forms of *An. gambiae s.s.* were based on a PCR–RFLP method. *An. arabiensis* was widely distributed, whereas *An. gambiae* was uncommon in southern areas where the rainfall was less than 600 mm per annum. *An. gambiae* occurs only as the S molecular form in Mozambique. The vegetation transition from *Deciduous Miombo* to *Open Forest* appears to prevent the spread of *An. gambiae* south of 24° S. This is the southern most record of this species and may represent its southernmost limit in continental Africa.

An. merus was found as far as 350 Km inland as well as along the coast, but in coastal areas it was always collected in areas with mangrove vegetation. *An. quadriannulatus* was found to be restricted to dry areas south of 24° S.

An. funestus s.s. was widespread and the predominant species resting indoors. The exophilic sibling species, *An. rivulorum* and *An. leesoni* were occasionally found in human dwellings, the latter also being captured on human bait.

5.1   Introduction

The knowledge of the distribution of malaria vector mosquitoes, especially those belonging to species complexes that include non-vector species, is important for the strategic planning of malaria control programmes (Coetzee *et al.* 2000). Correct identification is a pre-requisite for the establishment of the distribution of malaria vectors. One good example of the importance of correct species identification in subsaharan Africa is illustrated by work of Hunt and Mahon (1986) in the *An. gambiae* complex and Kamau *et al.* (2003) in the *An. funestus* complex. The authors found *An. quadriannulatus* and *An. parensis*, normally zoophagic and exophilic species, resting in significant numbers inside human dwellings. Another important insight comes from Southeast Asia, where *Anopheles varuna*, a non-vector of malaria, was previously misidentified as *An. minimus* and mistargeted as a malaria vector in Binh Thuan Province of Vietnam (Van Bortel *et al.*, 2001).

In Africa the principal malaria vectors are members of the *An. gambiae* and *An. funestus* complexes. The distribution of the *An. gambiae* complex has received some attention at local level and information at regional and continental level has been published (Coetzee *et al.* 1993, Coetzee *et al.*, 2000). However, very little is known on the distribution of *An. funestus* even at country level due to the lack of reliable and easy methods to differentiate their members. The recent publication of PCR-based techniques that differentiate five members of the complex (Koekemoer *et al.*, 2002) will play an important role in elucidating the biology and vector status of species of the *An. funestus* complex.

Mozambique is a vast country with different ecological conditions that may be occupied by different members of both *An. gambiae* and the *An. funestus* complexes. Petrarca *et al* (1984) using polytene chromosomal techniques, showed the presence in

Mozambique of *An. arabiensis*, *An. gambiae s.s.*, *An. merus* and *An. quadriannulatus*. The disadvantages of this technique are well known, among which, is the fact that only a small proportion of adult female mosquitoes would be half gravid and hence available to be identified without being kept alive in an insectary. Additionally the study of Petrarca *et al.* (*loc cit.*) is almost two decade old and covered few localities of the country. The number of biological species of the complex *An. funestus* in Mozambique as well as their distribution is still unknown. The present study makes use of the latest DNA-based techniques to determine the number of biological species within *An. gambiae* and *An. funestus* complexes occurring in different geographical and ecological areas of Mozambique.

5.2   Material and methods

5.2.1   Mosquito collection

Mosquitoes were collected in 44 localities of Mozambique mainly from 2000 to 2002. A few samples collected during 1998 and 1999 are included in the study. Mosquitoes were collected resting in human dwellings by either pyrethrum spray catches or by hand-collection with mouth-operated aspirators, on human bait collections and larvae collection as described in section 3.4. Larvae collections were performed in few localities and collection were made mainly during the dry season in both natural and man-made breeding sites. Artificial pit shelter collection was only performed in one locality of southern Mozambique.

Most of the sites were sampled only once but 7 localities were sampled at least twice in the same year or in consecutive years. Collected mosquitoes were identified and stored as described in the section 3.5. The number of mosquitoes collected in each locality is shown in Table 3.1 and the location in Figure 3. 5 of Chapter III.

5.2.2 Laboratory procedures

DNA from individual mosquitoes was extracted by either the STE method (Robertson and MacLeod, 1993), or the method of Collins *et al.* (1987) or using a phenol-chloroform extraction technique (Ballinger-Crabtree, 1992) as described in section 3.6. Species were identified using the rDNA-PCR of Scott *et al.* (1993) for *An. gambiae s.l.* and that of Koekemoer *et al.* (2002) for *An. funestus s.l.* At each locality with a sample size of less than 30 specimens, all mosquitoes were subjected to PCR identification, and where the sample size was over, at least 30 mosquitoes were randomly selected for the identification. Molecular forms of *An. gambiae* s.s. were identified from samples collected in a few localities using the protocol of Fanello *et al.* (2002), which involves PCR followed by RFLP. The rDNA-PCR protocols used for identification of mosquitoes species are provided in section 3.7 of Chapter III.

5.3   Results

5.3.1 *Anopheles gambiae* complex

A total of 2421 specimens of *An. gambiae s.l.* were identified to species. The geographical distribution of member species of the *An. gambiae* complex as determined from indoors, human bait and larvae collections is presented in Table 5.1. The results indicate the presence of four species of the *An. gambiae* complex: *An. gambiae, An. arabiensis, An. merus* and *An. quadriannulatus. An. arabiensis* was widely distributed throughout the country rising from the predominant to exclusive in southern, relatively dry, localities where rainfall is lower than 600 mm (Figure 5.1). *An. gambiae* is the second most widespread species after *An. arabiensis* but is only found in areas where the rainfall exceeds 600 mm. In dry tropical areas *An. gambiae s.s.* was only found in Moatize (site no.

28) in a single occasion. In northern areas of the country with abundant rainfall, *An. gambiae* was the most abundant and on many occasions exclusive species (Figure 5.2). However, south of the Save River (Figure 5.3), *An. gambiae* was only found along the coastal strip where the rainfall varies from 800-1400 mm. This pattern of rainfall persists southwards until close to Maputo city, but the distribution of species ceases at latitude 24° S. The vegetation in the above-mentioned strip is *Deciduous Miombo*, with a large *Prairie* separating this type of vegetation from *Open Forest* in the south (Figure 5.3). *An. gambiae s.s.* was not found south of this Prairie. Of 73 specimens from two localities north of Mozambique and two to the south, identified to molecular level all were as S-form.

An. merus was found about 350 km inland in Massingir (site no. 8) and Changara-Mazoe (site no 26), respectively (Figure 5.4). Along the coast, *An. merus* was collected in areas with mangrove vegetation. *An. quadriannulatus* was restricted to the dry areas south of 24° S (Figure 5. 4) and was found on some occasions.

TABLE 5.1. Distribution of *Anopheles arabiensis*, *An. gambiae*, *An. merus* and *An. quadriannulatus* in localities of Mozambique.

Site Number	Locality	Data	Collection Method	Number Identified	*arabiensis* No	*arabiensis* %	*gambiae* No	*gambiae* %	*merus* No.	*merus* %	*quadriannulatus* No.	*quadriannulatus* %
1	Catuane	12/06/2000	ASP	10	10	100	-	-	-	-	-	-
1	Catuane	12/06/2000	HBI	14	14	100	-	-	-	-	-	-
1	Catuane	12/06/2000	HBO	23	23	100	-	-	-	-	-	-
2	Salamanga	12/05/200	HBI	81	22	27	-	-	59	73	-	-
2	Salamanga	12/05/200	HBO	115	17	15	-	-	98	85	-	-
4	Boane*											
5	Maragra	11/00Wet	PSC	12	12	100	-	-	-	-	-	-
6	Chihaquelane	07/04/2000	PSC	115	115	100	-	-	-	-	-	-
6	Chihaquelane	07/04/2000	HBI	49	40	100	-	-	-	-	-	-
6	Chihaquelane	07/04/2000	HBO	48	48	100	-	-	-	-	-	-
6	Chihaquelane	12/05/2000	PSC	66	66	100	-	-	-	-	-	-
7	Hokwe	10/06/2000	HBO	71	48	68	-	-	23	32	-	-
8	Massingir	19/07/2001	PSC	24	18	75	-	-			6	25
8	Massingir	14/02/2002	PSC	31	24	77	-	-	5	16	2	7
9	Combomune	17/07/2001	PSC	56	52	93	-	-	-	-	4	7
9	Combomune	17/07/2001	LAR	56	35	62	-	-	1	2	20	36
11	Nhacutse	07/04/2000	PSC	35	35	100	-	-	-	-	-	-

TABLE 5.1. *Cont.*

Site Number	Locality	Data	Collection method	Number Identified	arabiensis No	arabiensis %	gambiae No	gambiae %	merus No.	merus %	quadriannulatus No.	quadriannulatus %
12	Nhamba	16/03/2001	PSC	50	21	42	29	58	-	-	-	-
13	Macome	16/03/2001	PSC	59	-	-	38	64	21	36	-	-
14	Chindjiguire	13/07/2000	PSC	41	22	54	19	46	-	-	-	-
14	Chindjiguire	17/03/2001	PSC	44	13	30	31	70	-	-	-	-
14	Chindjiguire	11/04/2001	PSC	45	16	36	29	64	-	-	-	-
14	Chindjiguire	06/07/2001	PSC	42	15	36	27	64	-	-	-	-
14	Chindjiguire	02/02/2002	PSC	60	16	27	44	73	-	-	-	-
14	Chindjiguire	11/02/2002	HBI	61	20	33	40	65	1	2	-	-
14	Chindjiguire	11/02/2002	HBO	44	17	39	27	61	-	-	-	-
15	Bemba	Nov.-1999	HBI	30	22	73	8	27	-	-	-	-
15	Bemba	Nov.-1999	HBO	47	45	96	2	4	-	-	-	-
17	Pambarra	09/06/2001	PSC	68	10	15	58	85	-	-	-	-
17	Pambara	09/06/2001	LAR	35	-	-	100		-	-	-	-
17	Pambara	08/02/2002	PSC	63	5	8	58	92	-	-	-	-
17	Pambara	08/02/2002	HBI	24	3	13	21	87	-	-	-	-
17	Pambara	08/02/2002	HBO	38	7	18	31	82	-	-	-	-

TABLE 5.1. *Cont.*

Site Number	Locality	Data	Collection method	Number Identified	arabiensis No	%	gambiae No	%	merus No.	%	quadriannulatus No.	%
18 Mabote	05/07/2001	PSC	44	44	100	-	-	-	-	-	-	
18 Mabote	05/07/2001	LAR	3	3		-		-	-	-	-	
18 Mabote	09/02/2002	PSC	29	29	100	-	-	-	-	-	-	
19 Machanga	09/06/2001	PSC	26	26	100	-	-	-	-	-	-	
20 Guara-Guara	2904/2000	PSC	40	28	69	12	31	-	-	-	-	
20 Guara-Guara	21/12/2000	PSC	19	14	74	5	26	-	-	-	-	
20 Guara-Guara	03/07/2001	PSC	32	23	72	9	28	-	-	-	-	
20 Guara-Guara	03/07/2001	HBI	19	8	42	11	58	-	-	-	-	
20 Guara-Guara	03/07/2001	HBO	14	10	71	4	29	-	-	-	-	
21 Mutua	01/05/2000	HBI	28	20	71	8	29	-	-	-	-	
21 Mutua	01/05/2000	HBO	44	33	75	11	25	-	-	-	-	
22 Caia	25/07/2000	PSC	2	1		1		-	-	-	-	
23 Machipanda	29/04/2000	PSC	2	-		2		-	-	-	-	
24 Barue	11/06/2001	PSC	2	-		2		-	-	-	-	
25 Guro	22/12/2000	PSC	51	9	18	42	82	-	-	-	-	
25 Guro	10/08/2000	PSC	31	-	-	31	100	-	-	-	-	

TABLE 5.1. *Cont.*

Site Number	Locality	Data	Collection method	Number Identified	arabiensis No	arabiensis %	gambiae No	gambiae %	merus No.	merus %	quadriannulatus No.	quadriannulatus %
26	Mazoe	13/07/2001	ASP	18	15	83	-	-	3	17	-	-
27	Tete	12/09/1999	LAR	23	23	100	-	-	-	-	-	-
28	Moatize	14/06/2001	PSC	1			1				-	-
29	Zobwe	14/06/2001	PSC	1			1				-	-
30	Angonia	09/08/2000	PSC	2	-		2				-	-
31	Fingoe	13/06/2001	PSC	1			1				-	-
32	Macuse	26/07/2000	PSC	26	3	11	21	81	2	8	-	-
33	Mocuba	Nov.-2000	PSC	19	4	21	15	79	-	-	-	-
34	Mamirauane	27/07/2000	PSC	4	1		3				-	-
35	Chapale	27/06/2001	PSC	28	7	25	21	75	-	-	-	-
36	Ribawe	29/07/2000	PSC	21	-	-	21	100	-	-	-	-
37	N.Barragem	20/12/1998	PSC	14	-	-	7		7		-	-
38	Mossuril	01/08/2000	PSC	22	-	-	12	55	10	45	-	-
39	Erati	02/08/2000	PSC	33	-	-	33	100	-	-	-	-
40	P.Miazi	16/05/2000	PSC	20	5	25	15	75	-	-	-	-
41	Montepuez	07/08/2000	PSC	31	-	-	31	100	-	-	-	-

TABLE 5.1. *Cont.*

Site Number	Locality	Data	Collection method	Number Identified	arabiensis No	arabiensis %	gambiae No	gambiae %	merus No.	merus %	quadriannulatus No.	quadriannulatus %
42 M. Praia		23/06/2001	PSC	74	-	-	65	100	-	-	-	-
42 M. Praia		23/06/2001	LAR	30	-	-	30	100	-	-	-	-
43 Chapa		24/06/2001	PSC	6	-		6				-	-
43 Chapa		24/06/2001	LAR	32	-	-	32	100	-	-	-	-
44 Cuamba		Aug./2000	LAR	12	10	83	2	17	-	-	-	-
45 Maua		16/06/2001	PSC	20	-	-	20	100	-	-	-	-
46 Marrupa		17/06/2001	PSC	5	2		3				-	-
47 Metangula		19/06/2001	PSC	35	11	31	24	69	-	-	-	-

KEY:
* Samples used for chapter IV
ASP – Collection by aspiration (samples kindly provided by Sonia Casimiro and Ivone Rungo)
HBI- Human bait indoor
HBO- Human bait outdoor
LAR –Larvae collection
PSC- Pyrethrum spray collection

FIGURE 5. 1. Map of Mozambique showing the pattern of rainfall and the distribution of *Anopheles arabiensis*. Sites numbers correspond to those in Table 3.1 and Figure 3.5. Source: Atlas Geográfico, Moçambique, 1986.

FIGURE 5.2. Map of Mozambique showing the pattern of rainfall and the distribution of *Anopheles gambiae s.s.* Sites numbers correspond to those in Table 3.1 and Figure 3.5. Source Atlas Geográfico, Moçambique, 1986.

Prairie

Dry Deciduous Miombo

Deciduous Miombo

Coastal scrub

Open forest of south   ▲ *An. gambiae*

FIGURE 5.3. The uppermost southern limit of *An. gambiae s.s* in Mozambique. Sites numbers correspond to those in Table 3.1 and Figure 3.5.
Source Atlas Geográfico, Moçambique, 1986

FIGURE 5. 4. Map of Mozambique showing the pattern of rainfall and the distribution of *An. merus* and *An. quadriannulaus*. Sites numbers correspond to those in Table 3.1 and Figure 3.5. Source Atlas Geográfico, Moçambique, 1986

5.3.2 *Anopheles funestus* complex

A total of 564 adults of *An. funestus s.l* were identified and 98 % were *An. funestus s.s..* The geographical distribution of member species of the *An. funestus* complex is presented in Table 5.2 and Figure 5.5. A*n. funestus s.s.* was the most abundant and widespread species of the complex predominated in human dwellings. The distribution of *An. funestus* showed no obvious relationship with either rainfall, altitude or vegetation. Others species within the complex found in human habitation included two *An. rivulorum,* one collected in Maua (site no 45) and other in Homoine (site no. 14). In Boane three *An. rivulorum* were collected from an artificial pit shelter (site no. 4). A single specimen of *An. leesoni* were found in Massingir (site no. 8), Inharrime (site no. 12) and two in Guara Guara (site 20) and Mutua (site no. 21). In Mutua three specimens of *An. leesoni* were found in human bait catches indoors.

TABLE 5.2. Distribution of *Anopheles funestus s.s.*, *An. rivulorum* and *An. leesoni* from several localities of Mozambique.

Site No.	Locality	Data	Collection Method	No. Identified	funestus No.	funestus %	rivulorum No.	rivulorum %	leesoni No.	leesoni %
3	Catembe	21/04/2001	ASP	33	33	100	-	-	-	-
4	Boane	Jul./1999	PSH	8	5		3			
5	Maragra	Nov./2000	PSC	31	31	100	-		-	-
6	Chihaquelane	07/04/2000	PSC	13	13	100	-	-	-	-
8	Massingir	19/07/2001	PSC	34	33	97	-	-	1	3
12	Nhamba	16/03/2001	PSC	44	43	98	-	-	1	2
14	Chindjiguire	17/03/2001	PSC	31	31	100	-	-	-	-
14	Chindjiguire	06/07/2001	PSC	34	33	97	1	3	-	-
16	Malova	10/04/2001	ASP	33	33	100	-	-	-	-
17	Pambarra	09/06/2001	PSC	31	31	100	-	-	-	-
20	Guara-Guara	29/04/2000	PSC	89	87	98	-	-	2	2
21	Mutua	01/05/2001	HBI	38	36	95	-	-	3	5
30	Ulongue	09/08/2000	PSC	11	11	100	-	-	-	-
28	Moatize	14/06/2001	PSC	35	35	100	-	-	-	-
40	P.Metuge	22/05/2001	ASP	24	24	100	-	-	-	-
45	Maua	16/06/2001	PSC	44	43	98	1	2	-	-
47	Metangula	19/06/2001	PSC	31	31	100	-	-	-	-

KEY:
No.- Number
ASP – Collection by aspiration (samples kindly provided by Sonia Casimiro and Ivone Rungo)
HBI- Human bait indoor
PSC- Pyrethrum spray collection

120
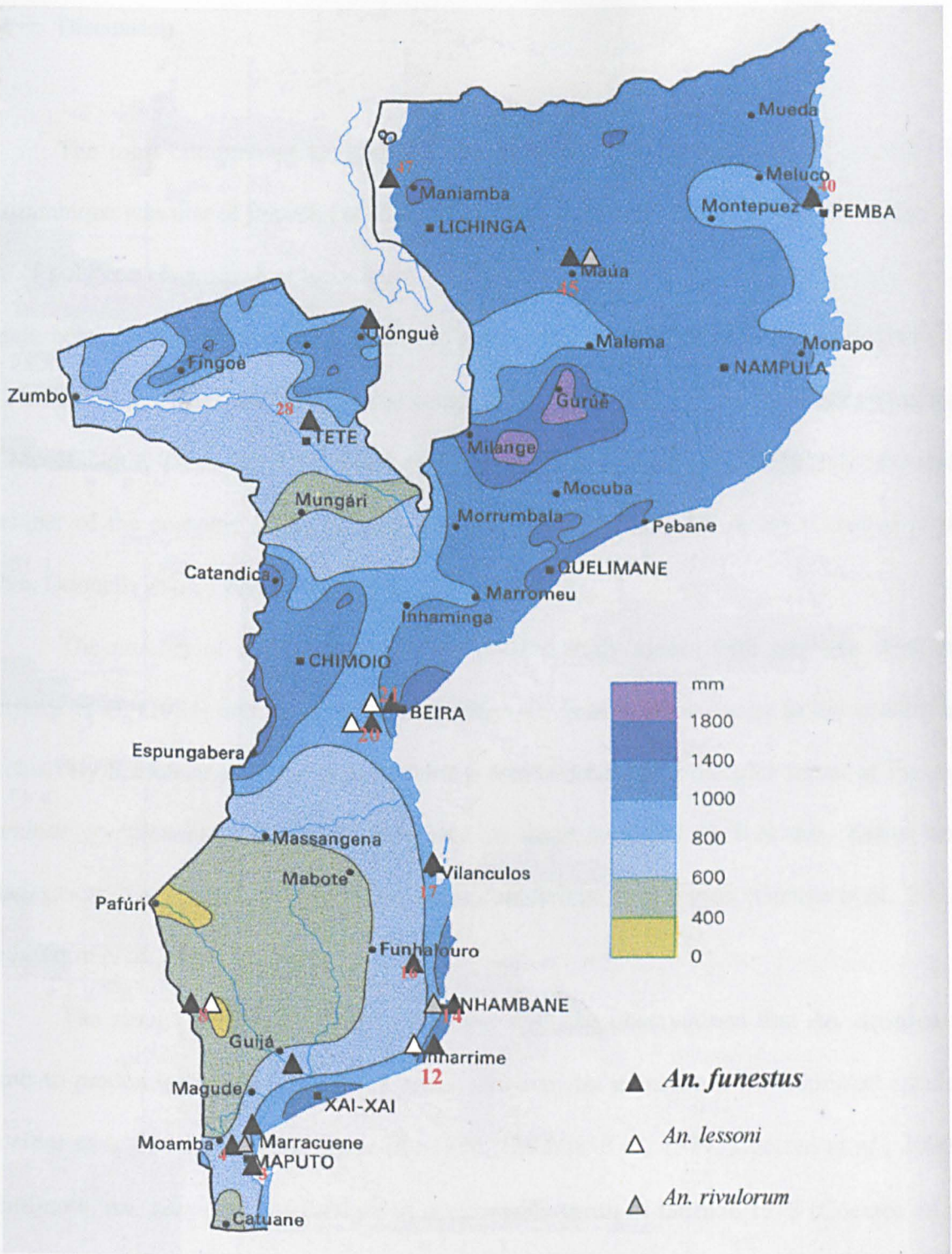
FIGURE 5.5. Map of Mozambique showing the pattern of rainfall and the distribution of three species of the *Anopheles funestus* complex. Sites numbers correspond to those in Table 3.1 and Figure 3.5. Source Atlas Geográfico, Moçambique, 1986

5.4 Discussion

The most comprehensive study on the distribution of members of *An. gambiae* in Mozambique was that of Petrarca *et al.* (1984) where mosquitoes were identified through the use of polytene chromosomal techniques. The sample sizes of Petrarca *et al.* (*loc cit.*), were small, being limited by the times it takes to read chromosomes. After the work of Petrarca *et al.* (*loc cit.*), studies on *An. gambiae* complex were carried out only in southern localities of Mozambique. These studies showed, collectively, that *An. arabiensis* is the most abundant member of the complex found in some localities together with *An. merus* (Cuamba *et al*, 1996; Donnelly *et al*, 1999; Mnzava *et al.*, 1997).

The number of species found in the present study agrees with previous work by Petrarca *et al.* (1984) and Cuamba *et al.* (1996). *An. gambiae s.s.* occurs in the country as exclusively S molecular form. In East Africa, identification of molecular forms of the *An. gambiae* complex had only been performed in some localities of Tanzania, Kenya and Madagascar. So far only the S form has been identified in these region (Gentile *et al.*, 2002; della Torre *et al.*, 2001).

The results of this study are consistent with the observations that *An. arabiensis* tends to predominate generally in dry areas, whereas *An. gambiae* is the dominant species in areas of high rainfall (Lindsay *et al.*, 1998; Coluzzi *et al.* 1979; Coetzee *et al.*, 2000). Until now, *An. gambiae* was thought to occur rarely south of latitude 19°S (Coetzee *et al.* 1993) and was apparently absent in Mozambique south of Save river (Petrarca *et al.*, 1984). In the present study, the species was found to outnumber *An. arabiensis* in most southern latitudes until close to latitude 24 °S. Annual rainfall above 600 mm was the common feature of all areas where *An. gambiae* was found. Although the rainfall is still high southward of this latitude, the vegetation transition from *Deciduous Miombo* to *Open*

Forest may be associated with the absence of *An. gambiae* south of 24° S. This is the southern most record of this species and may represent its southernmost limit in continental Africa. White (1974) has referred to the existence of four species of the *An. gambiae* complex including *An. gambiae s.s.* in southern Mozambique, but did not mention the localities nor the dates nor methods used to identify them. It appears that latitude 24°S is the southern most limit of the distribution of *An. gambaie s.s.*

Elsewhere in Africa, the relative abundance of *An. gambiae* and *An. arabiensis* are shaped by season, with *An. arabiensis* predominating during the dry season and *An. gambiae* becoming more abundant during the rainy season (Di Deco *et al.*, 1981, Rishikesh *et al.* 1985). In areas such as Pambarra (site no.17) and Chindjiguire (site no. 14) (Table 5. 1) where both dry and wet season samples were available, *An. gambiae s.s.* was the predominant species in both seasons. The seasonality of species has never been studied in Mozambique before, and these are provisional results pending further observations.

An. quadriannulatus is known to be patchily distributed in southern Africa, Zanzibar and Ethiopia (Gillies and Coetzee, 1987). In the present study, *An. quadriannulatus* was uncommon and restricted to the hot and relatively dry areas of southern Mozambique, south of 24°S. However, looking at available maps of the distribution of this species (Coetzee *et al.*, 1993, 2000), one can expect to find this species distributed further north in Mozambique as there are apparent ecological barriers that may prevent the spread of this species. A recent study in Seseo Village in Malawi at above 16°S involving both larvae and adult collection revealed the presence of *An. quadriannulatus* (Spiers *et al.*, 2002).

The brackish water species, *An. merus*, was found in Mozambique as far as 350 km inland as well as along the coast. Previously the species has been reported breeding in fresh water pools far from the sea in Kruger Park in South Africa and in the Matetsi River mouth and Hippo Valley in Zimbabwe (Coetzee *et. al.*, 1993). Elsewhere, adult *An. merus* have

been reported about 160 km inland in Buiko (Tanzania) by Mnzava and Kilama (1986). In the present studies, coastal *An. merus* was invariably found in area of mangroves vegetation. The breeding of *An. merus* in mangrove of *Avicennia* trees has been shown in Tanzania by Muirhead Thomson (1951). In the present study only adults were collected in these areas and their breeding sites remain largely to be confirmed. Studies on the breeding habits of *An. merus* in Mozambique in this study were restricted to Boane, southern Mozambique (see Chapter 4).

The distribution list of species of the *An. funestus* complex in Gillies and De Meillon (1968) shows the presence of four species in Mozambique: *An. funestus s.s.*, *An. confusus*, *An. leesoni* and *An. rivulorum*. The present study confirmed the presence of *An. funestus s.s.*, *An. rivulorum*, and *An. leesoni*. *An. confusus* is not included in the PCR method of Koekemoer *et al.* (2002) and hence it is not clear whether this species could be successfully amplified but instead is confused with one of the other nine species.

This is the first study of the distribution of *An. funestus* complex in large area using the latest rDNA-PCR techniques. The results clearly show that *An. funestus s.s.* is the widespread predominant species in human dwelling. It does not surprise to find very rarely *An. rivulorum*, *An. leesoni* in insignificant numbers in human dwellings, as these species are known as exophilic and zoophilic.

In the present study, three specimens of *An. leesoni* were collected on human bait indoors in the central region of Mozambique and in the same area another two were found resting on human dwellings. This observation invites further detailed investigations on the resting and biting behaviour of *An. leesoni* and its possible role in disease transmission. Up to now *An. rivulorum* is the only other member of the *An. funestus* complex that have been found to be infected with malaria parasites in studies in Tanzania (Wilkes *et al.*, 1996).

Although the majority of sampling was performed during the dry season, they yielded higher number of both members of *An. gambiae* and *An. funestus* complexes. *An. funestus* breeding is less affected by rainfall as it breeds in permanent water bodies such as swamps and slow-moving streams with vegetation as previously described by Gillies and De Meillon (1968). The breeding of *An. gambiae* complex is mostly dependent on rainfall. The persistence of members of the *An. gambiae s.l.* during the dry season in many of the localities surveyed is due to their ability to exploit the human made breeding sites, but breeding in pools (sandy or rocky) left in the river belt when waters recede seems to be the most important during the dry season.

5.4.1 Conclusions

1   The *An. gambiae* complex in Mozambique comprises four biological species; *An. arabiensis*, *An. gambiae s.s.*, *An. merus* and *An. quadriannulatus*. *An. gambiae s.s.* and *An. arabiensis* are the most widespread, with *An. merus* being common in coastal areas but also occurring as far as 350 km inland. *An. quadriannulatus* is uncommon and restricted to dry areas of southern Mozambique.

2   The rainfall pattern is the primary ecological factor that determines the distribution of the *An. gambiae s.s* and *An. arabiensis*, but vegetation seems to limits the expansion of *An. gambiae s.s.* to the south of latitude 24 °S. This latitude represents the southernmost limit of *An. gambiae s.s.* in Mozambique and may represent its southernmost limit in continental Africa.

3   In *An. funestus* complex three species were found in the study, *An. funestus* s.s., *An. rivulorum*, and *An. lessoni. An. funestus s.s.*, was the most abundant, widespread in indoors resting collections regardless of the ecological conditions.

CHAPTER VI

Studies on the role of individual *Anopheles* species in malaria transmission in Mozambique.

Abstract

Malaria is transmitted by anopheline mosquitoes, which can bite either indoors or outdoors. However, the contribution of individual vector in each site is not well known. In Mozambique, there are no comprehensive data on the relative importance of each vector in malaria transmission. Vector control is based on the assumption that mosquitoes of the *An. gambiae* complex are the primary malaria vectors during the wet season, with *An. funestus* maintaining transmission in the dry season, albeit at a lower level. Therefore an entomological study was conducted in Mozambique between 2000 and 2002. Mosquitoes were captured by pyrethrum spray catches and human bait catches.

During the rainy season, in day collections, the ELISA-*Plasmodium falciparum* sporozoite rates (SR) of *An. arabiensis* (3.99 ± 1.19 %) and *An. funestus* (5.59 ± 1.14 %) were comparable, but they were both lower compared with those observed in *An. gambiae* (9.32 ± 2.19 %) (χ^2=22.55, 2 df, P<< 0.001). In the dry season the SR of *An. gambiae* (3.46 ± 1.39 %), *An. arabiensis* (2.82 ±1.29 %) and *An. funestus* (3.20 ± 0.78 %) were lower and similar (χ^2= 0.44, 2 df, p=0.8).

In night collections the SR of *An. arabiensis* indoor (1.72 ±1.37 %) and outdoors (1.98 ± 1.21 %) were similar. Also the SR of *An. gambiae s.s* was similar indoor (10.58 ± 5.94 %) and outdoor (13.46 ± 6.59 %). This suggests similar risk of acquiring infection from each species vector. None of the mosquitoes caught indoors before 21:00 were infected; and outdoors 20 % of infected *An. arabiensis* and 7 % of *An. gambiae* were found before 21:00 h. The SR of *An. funestus* indoors was significantly higher (4.14 ± 1.66 %) than out (1.35 ± 1.32 %) (χ^2=4.0, 1 df, P=0.04). No infected *An. funestus* moquitoes were collected biting before 21:00 h. These results are discussed in relation to acquiring malaria and community preventive measures.

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6.1   Introduction

The sporozoite rate, the prevalence of anopheline mosquitoes harbouring sporozoites in their salivary glands is the most sensitive and powerful parameter for describing the epidemiology of malaria in a particular area (Macdonald, 1952). Furthermore, the sporozoite rate is indicative of the importance of an individual vector in malaria transmission in a certain locality. This parameter can be used as an indicator of the impact of malaria control activities, principally those aimed to reduce transmission by reducing the longevity of vectors.

The sporozoite rate has traditionally been determined by direct dissection of the salivary glands of fresh mosquitoes and detecting sporozoites microscopically (WHO, 1975). The development of ELISA (Burkot *et al*, 1984a) meant that the tedious microscopic dissection procedures, whose reliability varies with individual skills, have been overcome, thus allowing large numbers of mosquitoes to be examined.

The most important vector control activities are based in indoor residual spraying (IRS) and use of insecticide-treated bed nets (ITNs) (Curtis and Townson, 1998). The rational application of these tools requires a profound knowledge of malaria vectors, principally their behaviour such as resting and biting habits. In Mozambique, there is a complete lack of information about the role of individual *Anopheles* species in malaria transmission. Elsewhere in Africa the majority of malaria transmission studies have been carried out mainly on mosquitoes collected using human bait catches or light traps indoors, resulting in lack of information on the role of malaria vectors outdoors. This chapter sets out to determine the roles of different species of malaria vectors in transmission and to determine when and where people are getting infective bites. In turn, this determines the appropriateness of ITNs and IRS or other control measures in malaria transmission.

6.2   Material and methods

6.2.1   Mosquito collection

Moquitoe collections were carried out during the wet and dry seasons from 2000 to 2002 in several localities of Mozambique between 1998 and 2002. Details such as the geographic coordinates, climate, altitude and sample sizes are summarised in Table 3.1. House-resting mosquitoes were collected by pyrethrum spray collection or by mouth-operated aspirators. Blood-seeking mosquitoes were captured using human biting catch technique both in indoors and outdoors. These methods have been described before in section 3.4. Due to logistical problems, night biting catches were only carried out in eight localities of southern Mozambique. Collected mosquitoes were processed and stored as described in section 3.5.

6.2.2   Laboratory analyses

In the laboratory, the infection by *P. falciparum* was determined by ELISA using the head and thorax of individual mosquitoes, as described by Burkot *et al.,* (1984) and Wirtz *et al.* (1987). The procedures have been detailed in section 3.8. Species were identified using the rDNA-PCR of Scott *et al.* (1993) for *An. gambiae s.l.* and that of Koekemoer *et al.* (2002) for *An. funestus s.l*, as described in section 3.7.1.

6.2.3   Data analyses

The sporozoite rate (SR), the prevalence of anopheline mosquitoes harbouring sporozoite in their salivary glands, is expressed as the percentage of mosquitoes found positive in ELISA against the total analysed. A proportion of mosquitoes analysed for ELISA-spororozoite and all sporozoite positive specimens were later identified to the

species level. The species-specific infection rates were estimated by extrapolating from the results of the species-specific identification data, based on identification of all samples or at least 30 mosquitoes when the sample size was higher than 30 mosquitoes.

Differences in sporozoite rates were tested by the Chi-square test (with Yates correction, when df=1). The 95% confidence interval for sporozoite rates of vectors species were calculated from the proportion of females found positive.

Man biting rate (MBR) was calculated directly by counting the total species coming to bite upon collectors indoors and outdoors in all collections sites and ratios between indoor and outdoors calculated for a complex and individual species within a complex.

6.3 Results

6.3.1 Indoor day collections

The number of mosquitoes of *An. gambiae s.l.* and *An. funestus* collected between 2000 and 2002 are presented in Table 3.1 of Chapter 3.

The number of mosquitoes ELISA-tested for *P. falciparum*, the number positive and their SP (with 95 % confidence limits) of mosquitoes collected resting in human dwellings in localities of Mozambique are shown in Table 6.1.

The origin of the samples tested during the wet and dry seasons are shown in appendix 1 and 2. Overall, 4.5 % (302/6695) of mosquitoes ELISA-tested were positive for *P. falciparum*.

During the rainy season the SR of *An. arabiensis* (3.99 ± 1.19 %) and *An. funestus* (5.59 ± 1.14 %) were comparable, but they were both lower compared with that in *An. gambiae* (9.32 ± 2.19 %) (χ^2=22.55, 2 df, P<< 0.001). In the dry season the SR of *An.*

gambiae (3.46 ± 1.39 %), *An. arabiensis* (2.82 ± 1.29%) and *An. funestus* (3.20 ± 0.78 %) were not significantly different. *An. merus* was uncommon but has a SR of 0.8 % (n= 131).

TABLE 6.1. The number of mosquitoes tested, the number found positive with *P. falciparum* circumsporozoite antigen (+ve), respective sporozoite rates (SR) and 95% confidence limits (values in parentheses) for *An. gambiae, An. arabiensis* and *An. funestus* collected resting indoors in localities of Mozambique.

Season	An. gambiae			An. arabiensis			An. funestus		
	No.	+ve	SR	No.	+ve	SR	No.	+ve	SR
Wet	676	63	9.32 (7.11 - 11.49)	1177	47	3.99 (2.87 - 5.11)	1573	88	5.59 (4.46 - 6.74)
Dry	664	23	3.46 (2.07 - 4.85)	638	18	2.82 (1.53 - 4.11)	1967	63	3.20 (2.42 - 3.98)
Total	1340	86	6.42 (5.11 - 7.73)	1815	65	3.58 (2.73 - 4.43)	3540	151	4.27 (3.60 - 4.94)

6.3.2 Human bait collections

6.3.2.1 General

The numbers of *An. gambiae s.l* and *An. funestus s.l.* captured in eight human bait catches in eight localities indoors and outdoors in eight localities are shown in the Table 6.2. Of 2,375 vectors collected, 64.2 % (n=1524) were *An. gambiae s.l.* and 35.8 % *An. funestus s.l.* The MBR indicates that *An. gambiae s.l.* is 1.6 times more likely to bite man outdoors (n=928)than indoors n=596). The SR in *An. gambiae s.l.* were similar indoors (2.85 ± 1.34 %) and out (2.69 ± 1.04 %). None of 25 specimens of *An. gambiae* infected were found biting indoors before 21:00 h, but outdoors 12 % of infective bites was found before 21:00 h (3 out of 25). *An. funestus s.l.* was 1.9 times more likely to bite man indoors (n=555) than out (n=296). Twenty-three specimens of *An. funestus s.l.* collected indoors and 4 outdoors were infected but none of them were collected biting before 21:00 h (Figure 6.1).

TABLE 6.2. Proportion and ratio between indoor and outdoor of vector species collected all night by human landing catch in eight human bait catches in eight localities of Mozambique.

Species	Indoor collection		Outdoor collection		In:Outdoor ratio
	Number Collected	Proportion as %	Number collected	Proportion as %	
Complex					
An. funestus	555	65.2	296	34.8	1: 0.53
An. gambiae	596	39.1	928	60.9	1: 1.56
Partitioned					
An. arabiensis	348	40.7	506	59.3	1: 1.45
An. gambiae	104	50.0	104	50.0	1 :1.00
An. merus	144	31.2	318	68.8	1: 2.21
Total	1151	48.5	1224	51.5	1: 1.06

6.3.2.2 Biting habits and malaria transmission

An. gambiae s.l. when partitioned into its component species (Table, 6.2 and Fig 6.1) showed that: a) *An. arabiensis* was 1.5 times more likely to bite man outdoors (n=506) than indoors (n=348), b) *An. gambiae s.s.* bites equally indoors (n=104) and out and c) *An. merus* was 2.2 times more likely to bite outdoors (n=318) than in (n=144). *An. merus* was found only once in Salamanga (site no. 2, Figure 3.5 and Table 3.1). Specific identification of *An. funestus s.l.*, confirmed that *An. funestus s.s.* is the almost exclusive species. *An. funestus s.s* was 1.9 times more likely to bite man indoors than out.

The SR of *An. arabiensis* indoors (1.72 ±1.37 %) and outdoors (1.98 ± 1.21 %) were similar, also as in *An. gambiae s.s* (10.58 ± 5.94 % indoors V. 13.46 ± 6.59 % outdoors). This suggests similar risk of acquiring infection from each species vector indoors and outdoors. None of the mosquitoes caught indoors before 21:00 were infected; and outdoors 20 % (2/10) of infected *An. arabiensis* and 7 % (1/14) of *An. gambiae* were found before 21:00 h. The SR of *An. funestus* indoors was significantly higher (4.14 ± 1.66 %) than out (1.35 ± 1.32 %) (χ^2=4.0, 1 df, P=0.04). No infected *An. funestus* moquitoes were collected biting before 21:00 h. *An. merus* was collected in a single locality (Salamanga, site no. 2 in Table 3.1 and Figure 3.5) with single infected mosquitoes at 21:00hrs (SR= 0.3 %; n= 318).

6.3.2.3 Biting activity

Figure 6.1 shows the biting activity of *An. arabiensis* and *An. gambiae*. Biting activity of both species occurred throughout the night with biting peak varying between species and within species indoors and outdoors. There main biting peak in *An. arabiensis* were observed 02:00- 03:00 h indoors and at 23:00- 24:00 h outdoors collections. The indoors main biting peaks in *An. gambiae* was observed 23:00- 24:00

h, 01:00-02:00 h and outdoors at 22:00-23:00. In *An. funestus* the biting activity was observed throughout the night with peak indoors at 24:00- 01-00 h and outdoors at 04:00- 05:00 h).

Anopheles funestus
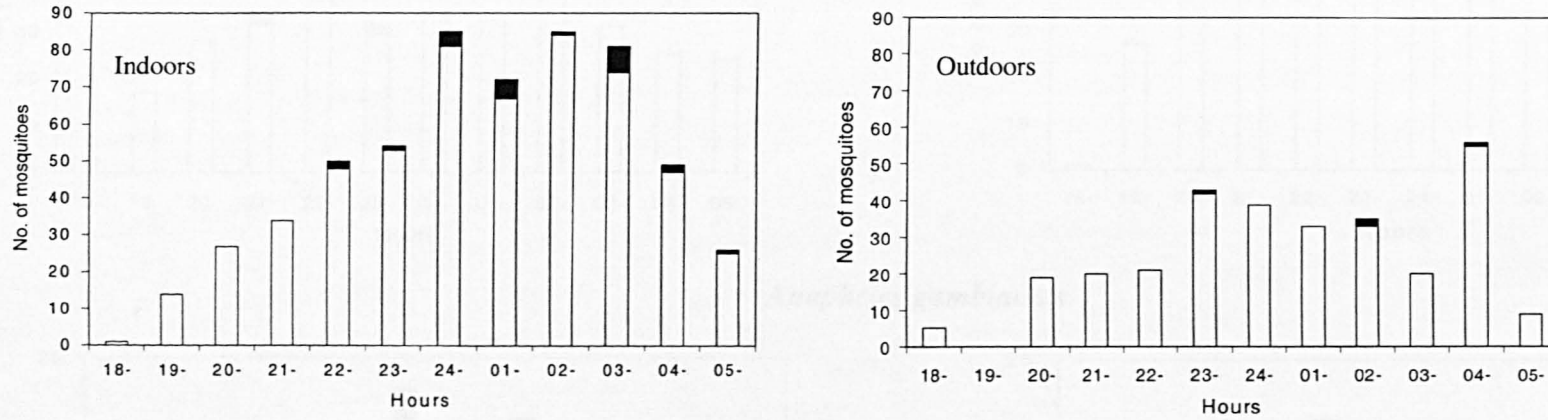
FIGURE. 6.1:Hourly biting activity of uninfected mosquitoes (white bars) and infected mosquitoes with *P. falciparum* (dark bars) collected from 18:00 to 06:00 hours in human landing catches in eight localities of Mozambique

FIGURE.6.1: *Continued*

Anopheles arabiensis
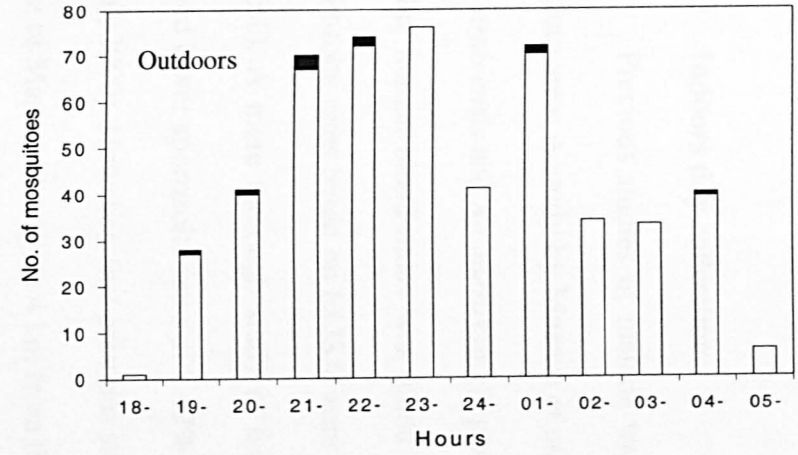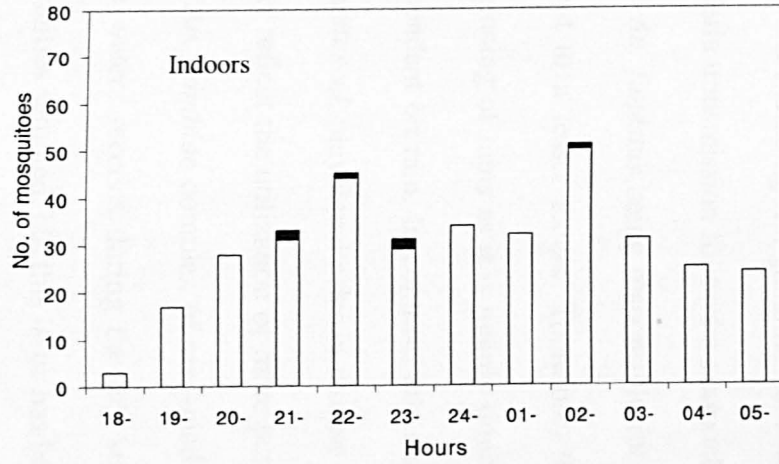
Anopheles gambiae s.s.
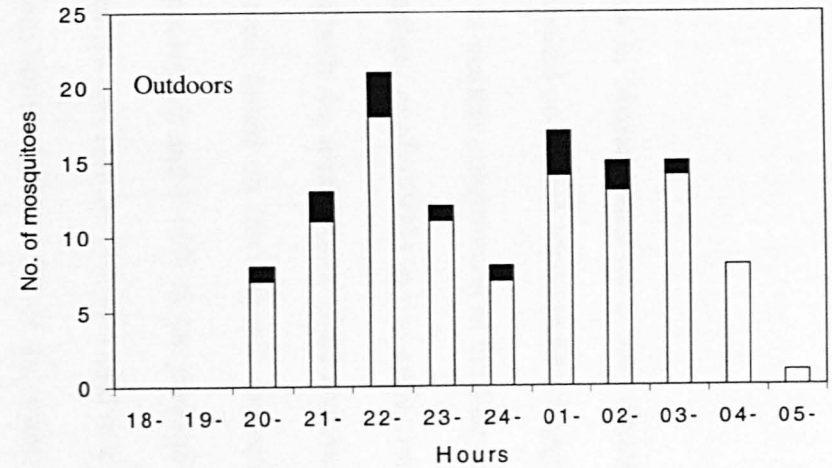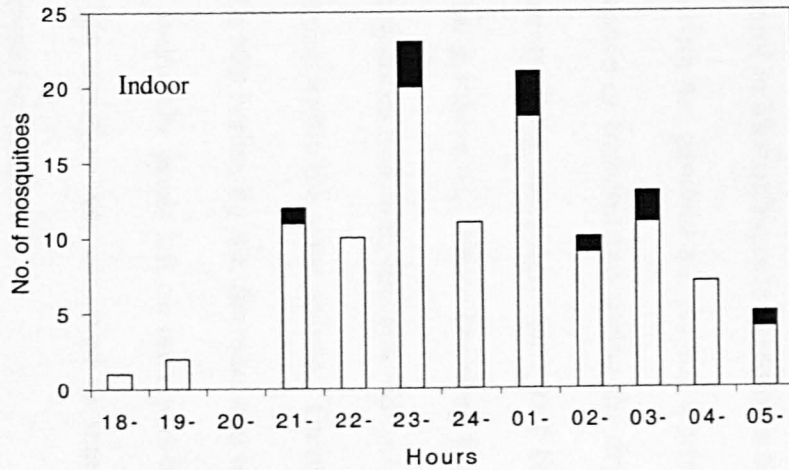
6.4 Discussion

6.4.1 Indoors day collections

Previous studies of malaria transmission in Mozambique have been limited to the Maputo area. A study by Mnzava *et al.* (1997) based on human blood indices suggested that *An. arabiensis* and *An. merus* might play a role in malaria transmission in the Maputo area. A higher human blood index was found by Thompson *et al.* (1997) in the same area, but the sporozoite rates based on ELISA were lower in both *An. arabiensis* (0.8%) and *An. funestus* (1.6%). A more thorough study in the same area, based on microscopic dissections also found lower sporozoite rates of 2.42% in *An. arabiensis* and 1.11% in *An. funestus* (Mendis *et al*, 2000). However, in a previous study, Cuamba and Dambo (1994) found in a suburban area of Maputo (less than 4 km from the previous authors), that 4.5% of *An. arabiensis* were sporozoite positive in mosquitoes collected on human bait catch.

This study provides the first results involving samples collected outside the Maputo area and including *An. gambiae s.s.* Malaria control in Mozambique is based on a belief that malaria transmission follows a seasonal pattern with *An. gambiae s.l.* playing a primary role and *An. funestus* being responsible for maintenance of transmission during the dry season, albeit to a lesser extent. Therefore, the indoor spraying campaign starts just before the beginning of rains as it is mainly directed at *An. gambiae s.l.*, whose breeding is primarily dependent on rain. In contrast, this study demonstrates that both *An. gambiae s.l.* and *An. funestus s.l.* play equal roles in disease transmission within the same season. These patterns may reflect the utilization of more permanent water bodies by *An. funestus* and utilization by *An. gambiae* complex of man-made pools and/or the pools left on the rivers-bed when the waters recedes during the dry season. This situation was observed in many of the localities sampled, but this issue has been addressed in Chapter V.

When *An. gambiae s.l.* is partitioned, the results indicate that both *An. gambiae s.s.,* *An. arabiensis* together with *An. funestus s.s.* are responsible in maintaining malaria transmission throughout the year. Although, *An. gambiae* is responsible for the major role during the rain season, in the dry season the role of three species were uniformly lower. The similar role of *An. arabiensis* and *An. funestus,* have been previously shown in a southern locality of Mozambique (Mendis *et al.* (2000). Nevertheless, comparisons with *An. gambiae s.s* were not possible as the authors worked in area where the species does not exists. Elsewhere in Western Kenya the ELISA based sporozoite rate were higher in *An. gambiae,* followed by *An. funestus* and *An. arabiensis* (Taylor *et al.*, 1990). A similar order was also observed by Dia *et al* (2003) in Ngari, Senegal. This order is consistent with the results of the present study. Takken and Knols (1999), states that the combination of anthropophily, endophily and the relatively high survival rates make *An. gambiae s.s.* and *An. funestus* responsible for most of the malaria transmission in Africa. The role of these species in disease transmission has been extensively reviewed in sections 2.2.2 and 2.3.2 of Chapter II. It is clear from such review that the role of *An. funestus*, in particular, as a malaria vector is considerable and can be greater than that at of *An. gambiae s.s.* in many localities.

The perennial pattern of malaria transmission in Mozambique, justify two rounds of indoor residual spraying. Nevertheless, the economic conditions of the country combined with the fact that most malaria transmission occurs during the rainy season justify spraying once just before the rainy season. This study demonstrates the importance of correct species identification of malaria vectors, where the *An. gambiae* complex is composed of several species, rather than conducting control activities based on assumptions.

6.4.2 Night collections

The tendency to feed on human blood determines the efficiency of the malaria vector. The most direct way to measure the human biting rate is the human bait catch (WHO, 1975). Despite being expensive, technically difficult to replicate and unethical in areas of drug-resistant malaria, this method is unique in that it directly samples human-biting mosquitoes (Le Goff *et al.*, 1997).

The indoor biting tendency of *An. funestus* is very similar to those found by Mendis *et al.* (2000) and more recently by D. Charlwood (personal communication) in a southern locality of Mozambique. Elsewhere, in Kenya *An. funestus* was found highly endophagic (Githeko *et al.*, 1996). Interestingly, Wanji *et al.* (2003), found *An. funestus* with variations in their biting behaviour according to the season, being more endophagous during the rainy season and more exophagous during the dry season. The authors suggested that the change may be related to the weather conditions in the study area (Mount Cameroon, southern Cameroon). This situation seems unlikely in the present study area. This author and colleagues (unpublished results), based on sample size comprising 32-night collection per month from November 1994 to May 1996, did not find variation in biting tendency of *An. funestus* in southern Mozambique.

Various studies suggest that the differences in biting activity of members of the *An. gambiae* complex are not significant (Gillies and Coetzee, 1987). This generalization, however, is unwarranted given that most of the papers cited by the above authors have considered *An. gambiae s.l.* as a single biological entity.

The outdoor biting tendency in *An. arabiensis* is no surprise. This behaviour has been previously observed in southern localities of Mozambique (Cuamba and Crook, 1994, Mendis *et al.*, 2000) and elsewhere in Kenya (Githeko *et al*, 1996). Moreover, *An. arabiensis* have been collected biting in open field in an area uninhabited by humans (Braack *et el.*,

1994). Outdoors biting activity of *An. arabiensis* has been studied only by Braack (*loc cit*) in South Africa, Dukeen and Omer (1986) in Sudan and in Kenya. Lemasson *et al.*, (1997) provided information on indoor behaviour of *An. arabiensis* in a locality of Senegal. Collectively these studies showed that the biting activity of this species occurs throughout the night with peaks observed just before sunrise.

The finding of *An. gambiae s.s.* having a similar tendency of biting humans indoors and outdoors was not expected. Elsewhere in Tanzania, *An. gambiae s.s.* is known to have endophagic tendency (Dr A. Mnzava, personal communication). It is unclear whether the behaviour of *An. gambiae*, here presented, is typical of this species or may be due to the bias related to small sample size. Assuming it is typical, one may argue that the similarities between indoor and outdoor biting tendencies are indicative of the presence of a homogenous population. In Barkedji, in Senegal, night biting activity indoors was similar for *An. gambiae* and *An. arabiensis*, and did not vary during the year (Lemasson *et al.*, 1997). According to the same authors the biting peak was observed consistently between 03:00 to 05:00 h. The biting habit of members of *An. gambiae* complex depends on host availability; in the area where blood meals were only available outdoors, all anophelines were exophagic (Faye *et al.*, 1997). These finding may explain in part the discrepancies of results of different authors.

Promoting increased ownership and use of Insecticide Treated Netting (ITN) material is one of the principal technical approaches of the RBM initiative and a key component of malaria control strategies in many African countries (http://www.rbm.who.int). The efficacy of ITN and other materials in reducing morbidity and mortality due to malaria has been convincingly documented (Alonso, *et al.* 1991, Binka *et al.* 1996, Choi *et al.* 1995, D'Allessandro *et al.*, 1995, Nevill et al, 1996). In Mozambique the use of pyrethroid-impregnated bed nets and curtains has already been demonstrated at Matola (Crook and

Baptista, 1995) and their distribution was shown to be feasible through the primary health care system (Dgedge, 2000). However, the success of the application of bed-net programmes remains dependent on mosquitoes (such as biting habits) and human behaviour (sleeping habits, time, indoor versus outdoors).

Theoretically bed-nets are expected to be more effective against mosquito species for which the infective bite occurs late at the night. In *An. gambiae s.s.*, Dossou-Yovo (1991) showed that malaria transmission by this species occurs essentially at the moment when villagers are in bed. Similar situation was observed in *An. punctulatus* in Papua New Guinea (Bockarie *et al.*, 1996). Nevertheless, the situation of the present study is more complex as it involves three species; *An. funestus*, *An. gambiae* and *An. arabiensis* with different susceptibility to infection and different biting habits.

In all species both indoors and outdoors, the majority of biting activity occurred after 21:00 h, when most of the inhabitants in rural areas are asleep, predominantly the children and pregnant women, the malaria vulnerable group. Fortunately there were no infected mosquitoes indoors before 21:00h. Even outdoors the infected mosquitoes before 21:00 represent only a small fraction, being *An. gambiae* and *An. arabiensis*. The results indicate a limited risk for contracting malaria before this period. Therefore, the use of bed nets could provide a high level of protection against infective bites of *An. funestus*. Additionally bed nets may still protect the children and pregnant women against early infective bites by the *An. gambiae* complex.Outdoors as well indoors the risk of getting bitten by infected mosquitoes is higher after 21:00 h. Outdoors the risk is due mainly by *An. gambiae s.s.* followed by *An. arabiensis*. The risk due to *An. funestus* is relatively small. The results indicate a complex vector system in the study area and raise difficulties in the control malaria principally in the areas where *An. gambiae s.s.* and *An. arabiensis* are sympatric. Nevertheless, as suggested before, bed nets may provide protection for those who go to the

bed early; mainly children and pregnant women. Other behavioural studies such as resting habits are sorely needed. These well help to design others anti-vector measures necessary to synergies the bed nets in controlling malaria.

6.4.3. Conclusions

1   The sporozoite rates indicate, in descending order, that *An. gambiae s.s, An. funestus s.s* and *An. arabiensis* are the primary malaria vectors in Mozambique. The malaria transmission is perennial, but higher during the rainy season, with *An. gambiae s.s.* having the leading role. During the dry season the transmission is equally maintained by the three species albeit at lower level. The results underline the need for correct identification of species members of the *An. gambiae* and *An. funestus* complexes in epidemiology and control activities in Mozambique, rather than conduct control activities based on assumptions.

2   Although the results of the present study rejected the hypothesis on which residual indoor spray is made in Mozambique, one round residual spray per year is the most ideal in the present economic context of the country.

3   Malaria is transmitted indoors as well outdoors by the three species and underlines difficulties in control the disease. However, as the majority of transmission both indoors and out occur when most of the people are in bed, the use of bed nets can provide desirable protection at least in the high risk group.

CHAPTER VII

Studies of malaria vectors in Angola

Abstract

Studies of malaria vectors in Angola are scarce. During April 2001, indoor resting mosquitoes were collected in ecologically different areas of Angola. The samples were collected to gather information on:

a) the number of biological species of *Anopheles funestus* and *An. gambiae* complexes including the molecular forms of *An. gambiae s.s*,

b) the relative importance of individual species in malaria transmission and

c) the presence of *kdr* mutations in *An. gambiae s.s.*

Using an rDNA-PCR technique, *An. funestus s.s.* was found to be the only member of the *An. funestus* complex. Within *An. gambiae s.l.*, a diagnostic PCR technique confirmed the presence of *An. gambiae s.s.*, *An. arabiensis* and *An. melas*, with *An. gambiae s.s.* being the most common species in all the localities. *An. gambiae s.s.* M-form is the predominant species and the S-form is recorded for the first time. Overall the proportion of S form was 3.8 % (n= 132) in coastal Benguela and 19% (n= 79) in Huambo. *An. melas* was found in one coastal area. A single specimen of *An. arabiensis* was found in a suburban area of Luanda. This is the first record of the presence of *An. arabiensis* in Angola. *An. melas* was found only in a coastal area of Funda. The *kdr* allele was found to be present in both M and S forms of *An. gambiae s.s.* This represents the southernmost known distribution of the *kdr* in *An. gambiae*, suggesting a much wider distribution than hitherto recorded.

The enzyme-linked immunosorbent assay (ELISA) indicated that both *An. gambiaes.s* (SP ≈ 2%, n=583) and *An. funestus s.s.* (SP ≈ 1%, n=140) are the vectors of *Plasmodium falciparum. An. melas*, which is a known malaria vector in others areas of West Africa, was not found infected in our samples but the samples size was small.

7.1   Introduction

In West Africa, members of the *Anopheles gambiae* complex, namely *An. arabiensis*, the different chromosomal forms of *An. gambiae s.s.* together with *An. funestus s.s* constitute the main malaria vectors. *An. melas,* another member of the *An. gambiae* complex, is known to be a malaria vector in the coastal areas (Bryan, 1983; Akogbeto and Romano, 1999; Awola *et al.*, 2002).

The known chromosomal forms of *An. gambiae s.s.* includes five different sets of paracentric inversion from the right arm of chromosome II (Coluzzi *et al.* 1985), designated with a non-Linnean nomenclature as Bamako, Mopti, Savannah, Forest and Bissau. Recent studies at the DNA level have shown that the *An. gambiae s.s.* exists as S and M molecular forms, based on intergenic spacer sequencing (della Torre *et al.*, 2001) or as types I and II, based on internal transcribed spacer sequencing (Gentile *et al.*, 2001). The S-form is equivalent to type I and M-form is equivalent to type II, but these are not always in concordance with the chromosomal forms (della Torre *et a.l*, 2001; Gentile *et al.*, 2001). While the molecular form S is known to be the most common and widespread in the African continent, the exact extent of the distribution of the M-form is still not well known. Similarly the distribution of *kdr* alleles in Africa is not well known. The majority of surveys have been confined to the localities of West Africa.

Malaria is endemic throughout much of Angolan territory, but due to the successive wars, malaria vector control activities and operational studies had been interrupted for decades with consequent lack of basic information on malaria vectors. The lack of such information is now evident as the war is finished and the local Malaria Control Programme has adopted the use of insecticide treated nets as the main strategy for the control of

malaria. There is a need for more information on vector species composition, behaviour and susceptibility of vectors to insecticides used in malaria control.

During April of 2001 I was fortunate to travel through some localities of Angola under a World Health Organization (WHO) mission whose aim was to evaluate the current status of malaria vector control, with special attention to the implementation and promotion of insecticide impregnated bed net programmes. During this mission samples of mosquito were collected from a limited number of localities in Angola, representing different ecological conditions. The samples were collected to provide basic information on species composition within the *An. gambiae* and *An. funestus* species complexes, their potential role in malaria transmission and to provide information on the status of susceptibility of *An. gambiae s.s* to pyrethroids.

7.2   Material and methods

7.2.1   Sites and mosquito collection

A map of Angola showing the collection sites is shown in Figure 7.1 and further details of sites are given below and summarised in Table 7.1. During April of 2001, corresponding to the end of the rainy season, indoor resting mosquitoes were collected from houses in localities of Angola using the pyrethrum spray collection method. Larvae were collected using a small metal soup ladle. The methods of mosquito collection area as described before in the session 3.4 (Chapter 3). Larvae collection was only carried out in Samba, a densely inhabited suburb of Luanda, the capital city. The adult collection sites were in Funda, Benguela and Lobito all in coastal areas of the Atlantic Ocean, and in Huambo, which is located in the interior high central plateau know as the Huambo plateau. The numbers of houses sampled varied from a minimum of 24 in Benguela to 37 in

Huambo. Samples of adult mosquitoes were stored dry over silica gel in Eppendorf tubes and larvae preserved in 80% ethanol.

Funda is a rural area of Cacuaco, a town located approximately 30 km from Luanda. It lies in the semi-arid Sudan savannah area, with rainfall between 400- 600 mm (Carrara *et al.*, 2002). The population of the area consists mainly of war-displaced people. The principal sources of income are from fishing and subsistence agriculture.

Benguela is relatively arid, being situated at the northern limit of the Namib Desert. The climate is influenced by the cold Benguela current and the mean rainfall is less than 400 mm (Atlas of the World, 1994). Adult mosquitoes were collected in the bairro of Cawango, a peri-urban area and in Bela-Vista a semi-urban area, 4.5 km from Benguela city. In Lobito city which is approximately 30 Km from Benguela, mosquitoes were collected in the suburban area of São Pedro. Figure 7.2 shows one of the common type of house in around the Lobito city. Mosquitoes were collected mainly in this type of house. Fishing is one of the major economical activities in both these areas, but it seems that tourism was previously important. Additionally, Lobito has a harbour linked with the Benguela Railway, which crosses the high plateau of Huambo and leads eventually to southern Zaire. However, due to the war, the harbour has lost its economic importance and is now being used for local demands (mainly for Lobito and Benguela areas).

Huambo is located in the temperate central plateau of Huambo. The climate is typically tropical, of high altitude (http://www.angola.org/referenc/provinces.html) with rainfall between 1000-1500mm (Atlas of the World, 1994). In Huambo, samples were collected in the bairros of Cazenga and São José. Both are peri-urban areas being about 3-4 Km apart. Huambo was virtually destroyed in the war (eg. Figure 7.3 and following explanations) and is one of the most saturated with land mines. Formerly, Huambo was one of the most important industrial areas in Angola and one of the most prosperous

agricultural areas. Due to the war the industry has collapsed and the agriculture activities reduced to subsistence level.

FIGURE 7.1: Map of Angola showing the collection sites (triangle). Cacuaco is approximately 30 km north of Luanda.
Source: http://www.un.org/Depts/Cartographic/map/profile/angola.pdf)

7.2.2 Identification of species, molecular forms and *kdr* mutations

Mosquito DNA was extracted as described in section 3.6. Identification of *An. gambiae* species was performed, with minor modifications, using the primers and protocol outlined by Scott *et al.* (1993) and species of *An. funestus* with primers and protocol outlined by Koekemoer *et al.* (2002). The full procedures of both PCRs have been described in section 3.7.1. Identifications of molecular forms of *An. gambiae s.s.* (Favia *et al*, 1997) was performed as outlined by Fanello *et al.* (2002) and described in section 3.7.1.1 using DNA extracted using phenol-chloroform method.

The knockdown resistance (*kdr*) allele is involved in pyrethroid resistance. The presence of the *kdr* alleles was carried out using the primers (Table 7.2) described in Martínez-Torres *et al.* (1998). Cycling conditions were: 94°C denaturation for 15 min, followed by 43 cycles of 94°C for 30s, 55°C for 60s and 72°C for 60s, with a final 72°C extension of 10 min. The cycling conditions and the aliquots of *kdr* positive control were kindly provided by A. Egyir-Yawson (Liverpool School of Tropical Medicine). Amplified fragments consisted of an internal control band (293 bp), a band representing the susceptible allele (137 bp) and the band representing the resistance allele (*kdr*) (195 bp).

The sporozoite rates for *P. falciparum* were determined by an ELISA using the head and thorax of individual mosquitoes as described by Burkot *et al.* (1984a) and Wirtz *et al.* (1987b). The procedures have been fully described in section 3.8.

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TABLE 7.1. Location and some ecological features of sampling sites. The numbers and densities of anophelines caught are presented in Table 7.3.

Municipality	Locality	Location	Altitude m	Latitude S	Longitude E	Rainfall mm	Climate
Benguela	Bela Vista	Coast	0-100	12° 34'	13° 24'	< 400	Dry-Trop.
	Benguela	"	"	"	"	"	"
Cacuaco	Funda	Coast	0-100	8° 51'	13° 34'	400-600	Dry-Trop.
Lobito	Sao Pedro	Coast	0-100	12° 20'	13° 34'	< 400	See 7.2.1
Huambo	Cazenga	Inner	1500-2000	12° 47'	15° 44'	1000-1500	Trop.-Altitude
	Sao Jose	"	"	"	"	"	"

Trop. = Tropical

TABLE 7.2. Sequence of the primers used for the detection of *kdr* allele (from Martinez-Torres *et al.*, 1998)

AGD1	ATA GAT TCC CCG ACC ATG
AGD2	AGA CAA GGC TGA TGA ACC
AGD3	AAT TTG CAT TAC TTA CGA CA
AGD4	CTG TAG TGA TAG GAA ATT TA

FIGURE 7.2. One of the houses where mosquitoes were collected in Lobito, Angola

FIGURE 7.3. Huambo: A stunning colonial style building destroyed by the war. Huambo now lies in ruins and has one of the most concentrations of land mines in Angola. Huambo was at the epicenter of the civil war and almost every building has been scarred by bullets and shells (Paulo Kassoma, Huambo's Governor, http://www.washingtonpost.com/wp-adv/specialsales/spotlight/angola/article19.html).

7.3   Results

The numbers of mosquitoes caught in the localities of Angola are presented in Table 7.3. *An. gambiae s.l.* was present in all localities surveyed but *An. funestus s.l.* only in Funda (Cacuaco). The densities of *An. gambiae s.l.* per house were higher in Lobito than elsewhere.

The number of individual species within the *An. gambiae* complex and the molecular forms of *An. gambiae s.s.* in different localities are presented in the Table 7.4. *An. gambiae s.s.* was the most common species in all collections. The restriction-enzyme digestion of the PCR products (Figure 7.4) showed that the An. *gambiae s.s.* M-form was the most common in all localities. The S-form was only found in Benguela and Huambo. The proportions of the molecular forms varied between sites (X^2 =35.3, df=3 P<<0.001). No M/S heterozygotes were found. *An. melas* was only present in the coastal area of Funda. In the larva collection, one *An. arabiensis* was found in Samba along with *An. gambiae s.s.* (n= 22). *An. funestus s.s.* was the only member of *An. funestus* complex found in Funda (n= 46).

The *kdr* allele was present in both Huambo and Benguela localities in both M and S molecular form. In Bela Vista (Benguela) one M and one S forms presented both resistant and susceptible alleles but no samples presented *kdr* genotypes in homozygotic. In Cawango (Benguela), both heterozygotic and homozygotic genotypes for *kdr* resistance were present as also found Casengue in Huambo. Nevertheless there were fewer heterozygotes. In Lobito and Funda where M-form was exclusive, none of the mosquitoes analysed presented the *kdr* allele. These results are summarized in Table 7.5.

The ELISA results indicated *An. funestus* and *An. gambiae s.s.* were infected with *P. falciparum* parasites (Table 7.6). The overall infection rate were 0.7 % in *An. funestus s.s.* (n= 140) and 1.9 % in *An. gambiae s.s.* (n= 583) In the last species infection rates varied from 0.9 % in Benguela to 2.5 % in Lobito and only *An. gambiae s.s.* M molecular form was found infected.

TABLE 7.3. The number and densities of adult mosquito specimens of two anophelines and species of *Culex* collected resting indoors in 6 sites in Angola.

Municipality	Site	No. of Houses	An. gambiae		An. funestus		Culex sp.	
			No.	No per. House	No.	No. per. House	No. house	No per .
Benguela	Bela Vista	24	124	5.2	0	-	-	-
	Cawango	33	255	7.7			133	4.0
Cacuaco	Funda	29	29	1.0	315	7.9		
Lobito	Sao Pedro	35	824	23.5	0	-	69	1.9
Huambo	Cazenga	34	31	0.9	0	-	105	3.1
	Sao Jose	37	121	3.3	0	-	313	8.5

TABLE 7.4. Species of *Anopheles gambiae* complex, and frequencies of M and S molecular forms of *An. gambiae s.s.* collected resting indoors in Angola.

Muncipality	Site	n	An. arabiensis	An. melas	An. gambiae		
					M-form	S-form	Total
Benguela	Bela Vista	66	-	-	62 (94 %)	4	66
	Cawango	71	-	-	70 (99 %)	1	71
Lobito	Sao Pedro	91	-	-	91(100%)	0	91
Luanda	Cacuaco*	26	-	13	13 (100%)	-	13
	Samba**	22	1	-	21 (100%)	0	21
Huambo	Sao Jose	64	-	-	60 (94 %)	4	64
	Cazengue	30	-	-	19 (63 %)	11	30

n = total number identified
* Total sample size, 3 out of 29 specimens did not amplify
** Sample from larvae collection

TABLE 7.5. Presence of *kdr* alleles in M and S molecular forms of *Anopheles gambiae s.s.* collected in some localities of Angola during April 2001.

		M or S Form	*kdr* alleles			
			SS	RS	RR	Total
Benguela	Bela Vista	M	7	1	-	8
		S	1	1	-	2
	Cawango	M	5	2	4	11
		S	-	1		1
Lobito	Sao Pedro	M	32	-		32
Luanda	Samba	M	21	-		21
Huambo	Casengue	M	3	1	3	7
		S	5	-		5
	Sao Jose	M	13	-		13
		S	1	-		1

TABLE 7.6. *Plasmodium falciparum* infection rates based on ELISA in mosquitoes of *Anopheles gambiae* species and *An. funestus s.s* collected Angola.

Municipality	Locality	*An. gambiae s.s.*		*An. melas*		*An. funestus*	
		tested	+	tested	+	tested	+
Benguela	Bela Vista	118	1 (0.9%)	-		-	
	Cawango	132	2 (1.5%)	-		-	
Lobito	Sao Pedro	317	8 (2.5%)	-		-	
Luanda	Cacuaco	13	0	13	0	140	1 (0.7%)
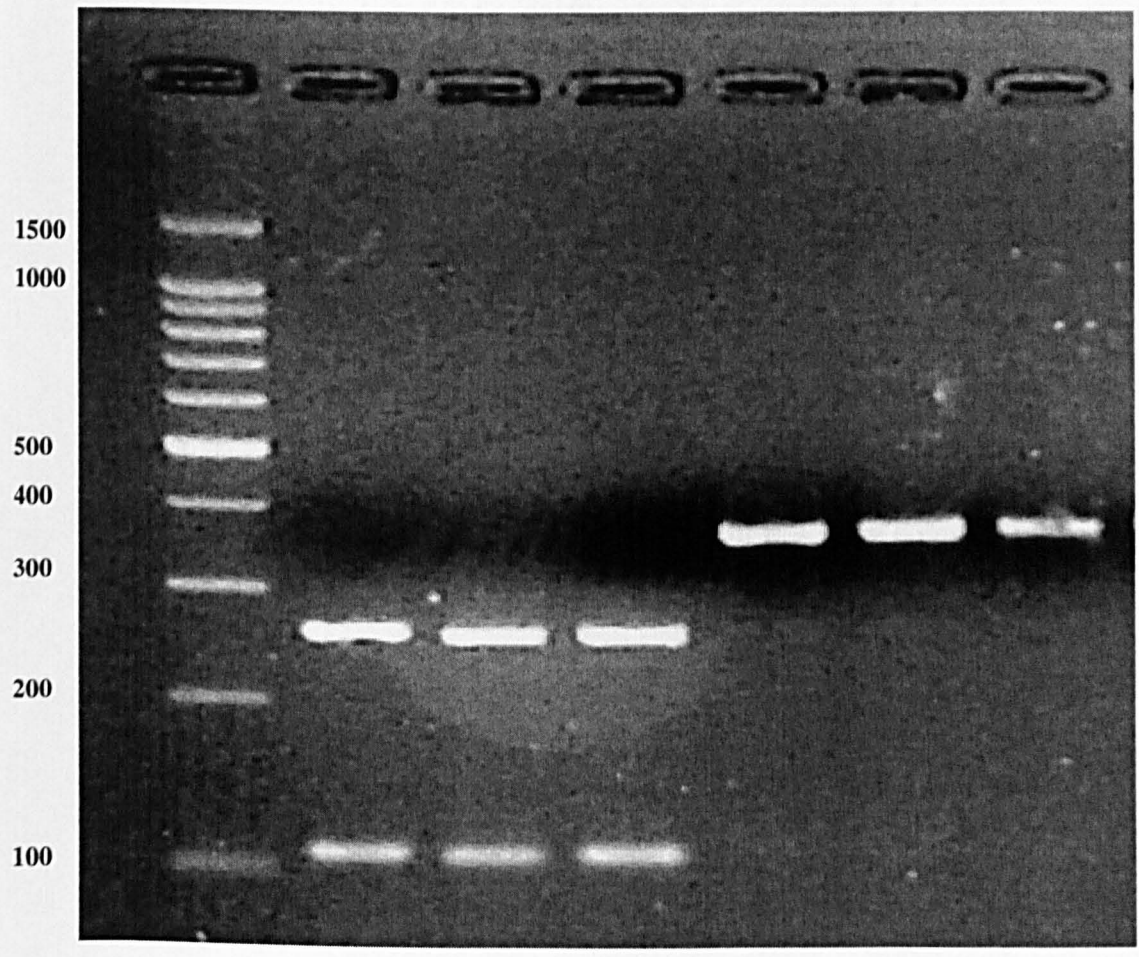
FIGURE 7.4: DNA bands after PCR-RLFP: lanes 1= 1.5 kb ladder size standard (from Promega); lanes 2-4= *An. gambiae* S-form; lanes 5-7 *An. gambiae* M-form. The *An. gambiae* S-form digestion profile is characterized by two fragments, 257 and 110 bp long, which are due to the presence of the *Hha* I restriction site. The *An. gambiae* M form does not have this restriction site and thus is characterized by a single 367 bp pair fragment .

7.4 Discussion

Based on eco-climatological data, three species of the *An. gambiae* complex are expected to occur in Angola: *An. arabiensis*, *An. gambiae* and *An. melas* results from this study confirm their presence. *An. gambiae s.s.*, molecular form M is the most common species throughout the sampled localities, as was also found by Carrara *et al.* (2002) in Cacuaco and Namibe. Our sampling, however, was extended to Benguela and Huambo where the *An. gambiae* complex was studied for the first time. In these localities *An. gambiae* molecular form M was found together with molecular form S. Our sampling included also Lobito where *An. gambiae* molecular form M was the exclusive species. It was interesting to observe that the S molecular form was more common in the high altitude and cool areas of Huambo, consistent with the observation that is the most widely distributed (see Gentile *et al.*, 2001).

Up to now, the southernmost collection of the M form was from Namibe in Angola (Carrara *et al.*, 2002). In this study S form was found in low frequency in coastal areas around Benguela. Its frequency increased in the high plateau of Huambo. No M and S heterozygotes were found, consistent with the observation that hybrids occur at a low frequency ($\leq0.3\%$) as reported by della Torre *et al.* (2001) and Wondji *et al.* (2001). The sample sizes in our study are relatively small and larger samples from more sites collected throughout the year would be required to demonstrate the distribution of these forms. Nevertheless, from the results of the present study together with those of Carrara *et al.* (*loc. cit*), one can infer that the molecular form M is most common in coastal areas of Angola, but in the interior of the country the picture may be different, as shown by the results from Huambo. The M molecular form has been recently detected in the Democratic Republic of

Congo by Lehmann *et al.,* (2003). Therefore, it is reasonable to state that the M molecular form has a wide distribution, and may be present in Namibia.

An. melas was found only in Funda, where early records have shown the existence of this species (Coetzee *et al.,* 2000 and see details at http://www.mara.org.za/downl_anoph/AnGamb_spp.xls). It is unclear, whether the absence of *An. melas* in our samples in the coastal areas of both Benguela and Lobito is due to mainly sampling in urban areas (Carrara *et al.,* 2002) or their largely exophilic habits (Bryan *et al.,* 1987), or the seasonality of this species, as observed elsewhere (Bryan *et al.,* 1987; Fonseca *et al.,* 1996). More sampling involving over seasons and several collection methods would be required to draw a more convincing picture on the distribution this species.

An. arabiensis was only found on a single occasion in larvae collections in Samba, being the first reported in Angola. Finding this species in Angola was not surprising as there is no apparent ecological barrier that may preclude its presence there. Moreover, the semi-dry conditions in Luanda, Benguela and Lobito would favour this species over its sibling *An. gambiae* (Coetzee *et al*, 2000, Lindsay *et al*, 1998, White, 1974, Rishikesh *et al.,* 1985). *An. arabiensis* is known to be zoophilic in some localities and has the tendency to rest outdoors (Githeko *et al.,* 1996). In Nigeria, *An. arabiensis* showed sharp seasonality, being abundant in the dry season (Rishikesh *et al.,* 1985). As the samples in Angola were taken only during the rainy season, indoors and at one point in time, there is a possibility that sampling has missed this species. These observations invite further research, with sampling involving several collection methods in both dry and wet seasons.

An. funestus s.l. was only found in Luanda were it was previously recorded by Ribeiro and Ramos (1975) as cited by Carrara *et al.,* (2000). In the present survey, *An. funestus* s.s. was the only species of the complex found in human dwellings. The apparent

absence of *An. funestus s.s.* from Benguela, Lobito and Huambo, where appropriate breeding places such as slow water streams and swamps with emergent vegetation are found (see Gillies and De Meillon, 1968), is puzzling but requires further confirmation.

The results of ELISA showed that *An. gambiae s.s.* and *An. funestus s.s.* are the vectors of *P. falciparum* in the sampled areas. However, the overall sporozoite rates were lower when compared with those recorded from other countries of West Africa (e.g. Boudin *et al.*, 1991; Awolola *et al.*, 2002). The low sporozoite rates observed may be explained by the presence of huge number of young mosquitoes as the samples were collected during the rainy season. All the *An. gambiae s.s.* found infected with malaria parasites were of the M-form. *An. melas* is a malaria vector in others areas of West Africa (Akogbeto and Romano, 1999; Awolola *et al.*, 2002; Bryan, 1983; Diop *et al.*, 2002), but was not found infected in our samples, perhaps due to the very small numbers collected.

In a recent study by Carrara, *et al.* (2002), the *kdr* mutation was absent in mosquitoes collected in two regions of Angola; Namibe and Cacuaco, respectively. However, results from the present study indicate the existence of *kdr* mutations in Angola in both M and S forms. It is interesting to notice that in Cacacuo and Lobito where only M-form occur, only the *kdr* susceptible allele was found, in contrast to Benguela where the two forms co-exist and the *kdr* resistance allele was detected in both. Resistant M-form has been found in Benin (Fanello *et al.*, 2000; Weill *at al.*, 2000), Burkina Faso (Diabate *et al*, 2003) and in Ghana (A. Egyir-Yawson, personal communication). It is worth mentioning that the *kdr* allele has been previously detected in the M form in Benin in the presence of several susceptible sympatric S populations (Fanello *et al.*, 2000).

Finding pyrethroid resistance in localities of Angola raises more questions than answers. It as been suggested in Benin that the agricultural use of insecticides is involved in the selection of resistance to pyrethroids by mosquito malaria vectors (Diabate *et al.*,

2002). Huambo was once considered "The Granary of Angola", but for 30 years as the region was at the epicentre of the civil war, and the agricultural system disintegrated. Less affected by the war was the costal area of Benguela but with economy largely based on fishing industry. A programme to deploy pyrethroid impregnated bed nets was launched in Lobito just as these mosquitoes were collected. In contrast, in Cacuoco where the programme was introduced much earlier, no *kdr* resistance allele was found (Carrara *et al*, 2002). The authors found also no *kdr* alleles in Namibe, southern Angola. Hence there is little evidence to indicate how a pyrethroid resistance allele could have been selected.

The existence of pyrethroid resistance in *An. gambiae s.s.* raises fears that such compounds might have a limited effective usage in ongoing programmes of promotion and scaling up of the use of ITNs. However, it was shown, surprisingly, in Côte d'Ivoire that pyrethroid treated bed nets are still effective as a prophylactic measure against malaria even in areas where *An. gambiae s.s.* is resistant to pyrethroids (Darriet *et al.*, 2000). Nevertheless, the results stress the need to monitor pyrethroid resistance and the presence of *kdr* alleles preferably before implementation of ITN programmes. In the few areas of Angola where the programme has been launched, it will be important to monitor insecticide resistance on a continuing basis. This is indispensable for the implementation of rational and effective malaria control tools. Furthermore, the monitoring and assessing of *kdr* resistance at the level of molecular forms within *An. gambiae* may improve our comprehension of the relationship between and within populations of *An. gambiae s.s*, including the process of speciation (eg. Wondji *et a.t*, 2002, Fannelo *et al.*, 2003).

7.4.1 Conclusions

1   The *An. gambiae* complex in Angola is composed of *An. gambiae s.s.*, *An. arabiensis* and *An. melas*. *An. arabiensis* is reported for the first time in the country. The population of *An. gambiae s.s.* comprises both M and S molecular forms, with each form having *kdr*-resistance alleles in some populations.

2   The M-form is the most common in Angola, although the S form has been found in both coastal and inland sites. The results of the present study together with that of others authors, suggests a wide distribution of the M molecular form, rather than it being confined to West Africa.

3   The *kdr* resistance allele is reported for the first time from Angola and represents the most southerly distribution known in Africa.

4   *An. funestus s.s.* was the only member of the *An. funestus* complex found indoors in human dwellings. However, the existence of other members of complex cannot be ruled out due to the limited nature of sampling in this study.

CHAPTER VIII

The population genetic structure of *Anopheles funestus* s.s. in Mozambique and Angola

Abstract

An. funestus is one of the most important vectors of malaria and filariasis in Africa, but the population structure of this species has previously only been studied using cytological-based techniques. Microsatellite primers designed specifically for *An. funestus s.s.* were tested in several geographical populations of *An. funestus s.s.*, collected in Mozambique and Angola, to address questions of population structure and the pattern of gene flow.

A high level of genetic diversity was observed but deviations from Hardy-Weinberg expectations were significant at most loci used. Although many factors may be involved, problems in resolving alleles may have led to underestimate of heterozygosity in some gels. Null alleles may also be a cause of heterozygote deficit

Significant differentiation was found between populations from Mozambique and Angola 2500 km apart (pair-wise F_{ST} range: 0.0238-0.0777). *An. funestus* populations only several hundreds km apart, in Mozambique were also differentiated (pair-wise F_{ST} range: 0.0126-0.1202). Results were consistent across loci reflecting a genome-wide pattern. Nevertheless, caution is necessary in interpreting these results due to the heterozygosity deficits observed in many loci.

8.1 Introduction

An. funestus s.s. is one of at least eight species comprising the *An. funestus* complex, which is difficult to distinguish on the basis of morphological criteria (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987). *An. funestus* is widely distributed throughout tropical Africa and together with *An. gambiae* is the major malaria vector in the continent. In some localities its role as a malaria vector is greater than that of *An. gambiae s.s.* (review in section 2.3.2). In southern localities of Mozambique, *An. funestus* and *An. arabiensis* have been found to play an equal role in malaria transmission (Mendis *et al.*, 2000).

Despite the long-term prospect of vaccines, the only appropriate prophylactic method against malaria relies on the use of insecticides for residual indoor house spraying and/or impregnated bed nets. However, there is hope for control of malaria transmission by the release of genetically engineered mosquitoes refractory to malaria. The use of genetically modified mosquitoes requires a profound knowledge of population structure as well as the pattern of gene flow within a target species. Knowledge of gene flow is useful as an indicator of direction and rates of dispersal among populations (Slatkin, 1987), and may be useful in predicting the spread of genes for insecticide resistance (Kamau *et al*, 2002). Resistance to pyrethroid insecticides has already been found in *An. funestus* populations of southern Mozambique (Brooke *et al.*, 2001)

Studies based on chromosomal inversion polymorphisms suggested genetic heterogeneities in *An. funestus* populations (e.g. Baccolini *et al.* 1994, Lochouarn, 1998; Costantini *et al*, 1999; Dia *et al.* 2000, Kamau *et al*, 2002), but mtDNA and rDNA failed to support these evidence (Mukabayire *et al.*, 1999). Microsatellites have proven to be very useful as genetic markers (review in 2.7) and have been successfully used in the studies of population genetics in the *An. gambiae* and *An. arabiensis* (Lanzaro *et al.*, 1995; Lehmann

et al, 1996, Lehmann *et al.*, 1997, Donnelly *et al.* 1999, Donnelly *et al.*, 2001). However, the population genetic structure and pattern of gene flow of *An. funestus* populations remains to be determined. The primary objective of this work was to test microsatellite DNA primers in several geographical populations, and use this marker to provide information on population genetic structure and of gene flow between these populations.

8.2   Material and methods

8.2.1   Origin of biological material

Samples for the present studies were collected from localities of Mozambique and Angola. In Mozambique the collections sites are numbered and showed in Table 3.1 and Figure 3.5 of Chapter III. Adult *An. funestus* mosquitoes were collected resting in houses from March to April 2001 in Catembe (site no.3), Massinga (site no. 16), Guara-Guara (site no. 20), Pemba (site no. 40) and Metangula (site no. 47). In Angola specimens of *An. funestus* were also collected resting indoors during April 2001 in Funda, a locality of Cacacuo municipality about 30 km from Luanda, capital of Angola. The details of collection sites are presented in Table 7.1. and the location in Figure 7.1 of Chapter VII.

In each locality the samples consisted of specimens collected in a area with a radius less than 2 km. Previous studies in the *An. gambiae* complex revealed no population subdivision within and among adjacent villages separated by 10-50 km. Mosquitoes within a house were considered to represent a random sample of the population (Petrarca and Beier 1992; Besansky *et al.* 1997; Lehmann *et al.* 1997, 1998; Donnelly *et al.* 1999). In *An. funestus*, using chromosomal inversions, the results of Kamau *et al.* (2002), suggested that the deme may be bigger than 2 km in radius.

8.2.2 DNA extraction, species identifications and primers

DNA from individual mosquitoes was prepared following the phenol extraction of Ballinger-Crabtree (1992). The method is fully described in section 3.6.3. Only those mosquitoes that gave one clearly identifiable band corresponding to *An. funestus* (Koekemoer *et al.*, 2002) were used in the study.

Twelve primers (Table 8.1) were selected from among those published by Sinkins *et al.* (2000) taking into account their heterezygosity level and the expected number of alleles. Ten selected loci were di-nucleotide repeats (AF1, AF2, AF6, AF8, AF12, AF17, AF18, AF19, AF20 and AF21), whilst two (AF3 and AF11) were tri-nucleotide repeats. The forward primer of each locus was synthesised incorporating a fluorescent label, FAM, TET or HEX, which allowed products to be a multiplexed on the ABI377 DNA automatic sequencer.

The loci amplified by each primer have not yet been localized relative to the known chromosomal inversions. However, there is a strong evidences that locus AF12 is X-linked (males being hemizygous at X-linked single copy loci), and the male heterozygosity observed at all of the other loci indicates that they are autosomal (Sinkins, *et al.*, 2000).

8.2.3 PCR amplification and allele scoring

The PCR amplifications were carried out in 1/400 of total DNA template in 15 μL reaction volume using 0.5 mL Eppendorf tubes with a mineral oil overlay. The reaction master for a single locus included 7.5 pmol of each primer, 2.0 mM Mg Cl$_2$, 0.2 mM dNTPs and 1 x reaction buffer and 0.5 U of *Taq* DNA polymerase (Sigma and Qiagen).

PCR cycling were carried out in a Hybaid Omnigene thermal cycler using the following amplification programme: 94 °C denaturation for 5 min, followed by 35 cycles

of 94 °C for 25s, 55 °C for 28s and 72 °C for 30s, with a final step 72 °C extension of 5 min.

Products were first visualized in 2% agarose-Tris-borate-EDTA gel containing ethidium bromide. Those samples with no product were re-amplified in second attempt. Amplified products were run on a Perkin-Elmer ABI PRISM 377 DNA Automatic Sequencer using the default settings. Data were automatically collected and analysed using GENESCAN and GENOTYPER software (Applied Biosystems). The allele were sized using a local southern option, which estimates the alleles sizes by using the three molecular weight markers closest in size to the product to determine a best fit estimate. In order to avoid scoring artefacts and to ensure accuracy in allele scoring, gel images were called on GENESCAN and allele sizes compared with those provided automatically.

TABLE 8.1. Microsatellite loci studied in *An. funestus*. The loci numbers, size range number of alleles and the heterozygosity estimates are those of Sinkins *et al.*, (2000).

Locus	Repeat Motif	Primers sequences	Size range (bp)	No. alleles	Ho
AF1	GA (11)	CGG TTG A AA ATT AAG GACCA CAA AAG ACA CCA AAC ACC AG	210-230	13	0.44
AF2	GT (9)	ATA AAC CCG TCC ATT CCC TT CCT ATG ATT CGC TCC TGA CA	131-151	12	0.69
AF3	GGT(5+4)	ACG ACT GTA ACC ACA ACA CC TAG TAG CGA AGG CGA AAG A	171-195	6	0.53
AF7	GT (8)	TGC ATC ATT CGA CTC GGA AG AAC GGC ACT ACC GTT CAC TG	70-84	9	0.83
AF8	GT (7+2)	ACC ATT TGC ATC CTA GAA C1C CAT GAA CTA TTG TTA ACC ACC C	117-136	13	0.78
AF11	GCT (5)	TTT CAA CTA TTT CCG TAA CGA G GAT CAT CAC CAC TAC CAG GC	125-134	4	0.42
AF12	GA (6)	GTT CTC CAT CGC TGT TCT ACT C TAT AAC GTT TCG TAC ACA CGC C	87-107	11	0.44
AF17	GT (8)	AAA ACGCCA CAA AGA GCA C CGG GTC AAA TTC TAC CGT AAG	129-157	14	0.56
AF18	GA (10)	AAA GCG CAC TTT ATG AAC G AAA ACA AAC ACA GGA AGG C	210-231	14	0.33
AF19	GT (12)	CAG AAC CAC TTC GAT TCA AC CCT GCA CTC AGA AAC ACA C	172-205	12	0.75
AF20	GT(7+2)	AGA ACC ACA TTA GGG AAC AG TTT ACA ACC AGC AGC ACA C	123-139	9	0.69
A21	GA (9)	CCG CAC ACC AAC TTA CAC TC TGG CGT GGG ATT AAA TAG G	96-104	4	0.3

8.2.4 Statistical analyses

8.2.4.1 . Allelic variability at each locus

This was based on estimation of the observed (H_o) and expected heterozygosity (H_{exp}), calculated using the Microsatellite Analyser (MSA) software developed by Dieringer and Schlötterer (2003). Variability included the number and frequency of alleles per locus for each locality.

Deviation from Hardy-Weinberg Equilibrium (HWE) expectations was calculated at each locus using the GENEPOP V3.3 program (Raymond & Rousset, 1995), which performs significance tests using Markov chain procedures. The null hypothesis is that of random union of gametes and the alternative hypothesis of heterozygote deficiency.

8.2.4.2 Population differentiation

The estimate of F_{ST} among populations, which is the variance in allele frequencies standardized by the mean allele frequencies among populations, was calculated according to Weir and Cockerham (1984) using MSA software (Dieringer and Schlötterer (2003). Measure of population differentiation using F_{ST} is based on the assumption of an infinite allele model (IAM- each mutation creates a novel allele at a given rate).

For interpretation, it has been suggested that a value lying in the range 0-0.05 indicates little genetic differentiation; a value between 0.05 and 0.15, moderate differentiation; a value between 0.15 and 0.25, great differentiation; and values above 0.25, very great genetic differentiation (Wright, 1978, Hartl and Clark, 1997). F_{ST}

significance was based on genotypic permutations tests (in which genotypes are shuffled among subpopulations a great number of times, say, 10,000 times).

8.3 Results and discussion

8.3.1 Genetic variability

Initially 12 loci were used for this work. AF12, which is sex-linked, was monomorphic for all localities of Mozambique and Funda in Angola. The only allele was 96 bp. This result is discussed below. AF19 produced inconsistent amplification in all localities. These loci were not included in the further analysis.

The sample size, the number of alleles and the values of heterozygosity in populations of *An. funestus* are shown in Table 8.2. The level of polymorphism found in *An. funestus* varied from moderate to high, but was relatively lower for trinucleotide loci (AF3 and AF11). This difference is most likely attributable to lower mutation rates for trinucleotide than for dinucleotide loci as suggested in Scotti *et al.* (2002).

In total, the average number of alleles per locus was 8.7 ranging from 2 (in locus AF3) to 19 (in locus AF6). The average heterozygosity (unbiased) was 0.7 ranging from 0.067 (locus AF11 in Massinga) to 0.893 (locus AF2 in Guara-Guara). Funda was the only locality outside Mozambique included in the study. The highest mean number of alleles was found in this locality (10.6) followed by Guara-Guara (10.4). In general the genetic diversity measured by number of alleles and average heterozygosity suggested differences in effective population sizes between localities. The genetic variability estimates presented in Sinkins et al. (*loc. cit*) were calculated based on samples sizes of 38 mosquitoes collected in Tanzania, Kenya and Burkina Faso. Compared with the work of Sinkins (*loc. cit*), the present results indicate: a) the

average number of observed alleles were lower in 8 loci, similar in one locus and only higher in one locus, b) the level of observed heterozygozity were higher in half of the selected loci but c) the allele ranges were wider in all loci. The sample size of the present study was relatively small compared with the previous authors. It varied from 25 mosquitoes at locus AF18 to 29 mosquitoes at loci AF2 and AF21. However, sample sizes alone cannot explain these discrepancies. Theoretically, it was expected that there would be much more variability in the present work because samples were collected from more localities. Multiple factors may be explain these results, such as for example the presence of null alleles (see section 8.3.2), although problems in resolving alleles may have led to an underestimate of the genetic variability in some gels. The lack of polymorphism in the sex-linked locus AF12 is difficult to explain, but the difficulties in amplifying and scoring this locus in populations available for this study may have been the key factor. In contrast, in the study of Sinkins *et al.* (2000) this locus had 11 alleles and an observed heterozygosity of 0.44.

The allele frequency distribution in localities for each locus (Figure 8.1a-j) showed that all loci have rare alleles (frequency <0.05), but no discernible shift in alleles sizes was observed between localities. The alleles were not uniformly distributed within a given locus across the localities. In some cases the most frequent allele in one locality may be present in lower frequency or even absent in others. This may suggest some degree of heterogeneities between populations, an issue that is fully discussed in section 8.3.3.

TABLE 8.2. Sample size (2N), total number of alleles (NA), observed heterozygosity (Ho), expected heterozygosity (HE) and Nei's unbiased heterozygosity (Hnei) by locus for samples of *An. funestus* collected in five localities of Mozambique and one in Angola (Funda). Loci at Hardy-Weinberg equilibrium are shown in bold.

		Catembe	Massinga	Guara-Guara	Pemba	Metangula	Funda
AF1	2N	52	58	60	44	58	64
	NA	9	8	12	7	8	12
	Ho	0.538	0.483	**0.733**	**0.591**	**0.759**	0.625
	HE	0.816	0.779	**0.858**	**0.687**	**0.791**	0.878
	Hnei	0.811	0.775	0.852	0.680	0.784	0.874
AF2	2N	56	60	58	46	60	62
	NA	9	8	15	12	11	11
	Ho	**0.857**	0.867	0.759	0.739	**0.833**	**0.742**
	HE	**0.851**	0.810	0.900	0.896	**0.803**	**0.868**
	Hnei	0.844	0.803	0.893	0.887	0.796	0.862
AF3	2N	56	60	56	36	54	60
	NA	4	3	5	4	2	8
	Ho	**0.464**	**0.333**	0.643	**0.500**	0.407	0.433
	HE	**0.523**	**0.445**	0.708	**0.665**	0.498	0.701
	Hnei	0.519	0.442	0.703	0.658	0.494	0.697
AF6	2N	60	58	60	42	52	62
	NA	11	6	16	8	9	19
	Ho	0.567	0.414	**0.800**	**0.714**	**0.731**	0.742
	HE	0.775	0.713	**0.894**	**0.844**	**0.830**	0.878
	Hnei	0.770	0.710	0.888	0.836	0.822	0.872
AF8	2N	54	60	60	42	52	54
	NA	6	6	11	8	6	11
	Ho	0.593	**0.833**	**0.800**	0.810	**0.846**	0.593
	HE	0.776	**0.694**	**0.834**	0.830	**0.719**	0.871
	Hnei	0.770	0.687	0.827	0.821	0.710	0.866
AF11	2N	58	58	44	44	56	62
	NA	4	2	3	5	5	5
	Ho	**0.448**	**0.069**	**0.591**	0.409	0.357	**0.484**
	HE	**0.413**	**0.068**	**0.489**	0.606	0.419	**0.527**
	Hnei	0.409	0.067	0.483	0.601	0.416	0.523
AF17	2N	60	58	60	48	58	56
	NA	15	11	11	10	11	14
	Ho	0.733	0.690	0.900	**0.917**	**0.793**	**0.857**
	HE	0.875	0.849	0.895	**0.887**	**0.863**	**0.889**
	Hnei	0.869	0.843	0.888	0.878	0.856	0.881

TABLE 8.2. *Continued*

AF18	2N	44	52	56	38	52	54
	NA	12	10	13	11	11	11
	Ho	0.409	0.308	0.429	0.263	0.500	0.296
	HE	0.855	0.793	0.886	0.869	0.812	0.828
	HNei	0.850	0.790	0.882	0.866	0.807	0.825
AF20	2N	60	56	54	48	56	64
	NA	8	5	8	7	7	7
	Ho	0.533	0.464	0.704	**0.917**	0.857	0.469
	HE	0.796	0.683	0.815	**0.781**	0.710	0.610
	HNei	0.792	0.679	0.808	0.771	0.702	0.606
AF21	2N	56	56	60	48	58	64
	NA	7	6	10	8	9	8
	Ho	0.429	0.607	0.833	**0.958**	0.966	0.344
	HE	0.742	0.673	0.822	**0.801**	0.792	0.766
	HNei	0.738	0.667	0.815	0.791	0.783	0.764
X ± SE	NA	8.5±1.11	6.5±0.90	10.4±1.3	8±0.79	7.9±0.94	10.6±1.26
	Ho	0.556±0.05	0.507±0.08	0.719±0.04	0.681±0.07	0.705±0.07	0.560±0.06
	HE	0.742±0.05	0.651±0.07	0.810±0.04	0.787±0.03	0.724±0.05	0.782±0.04
	HNei	0.737±0.05	0.646±0.07	0.804±0.04	0.779±0.03	0.717±0.05	0.777±0.04

FIGURE 8.1a. Allele frequency distribution of *An. funestus* at locus AF1

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FIGURE 8.1b. Allele frequency distribution of *An. funestus* at locus AF2
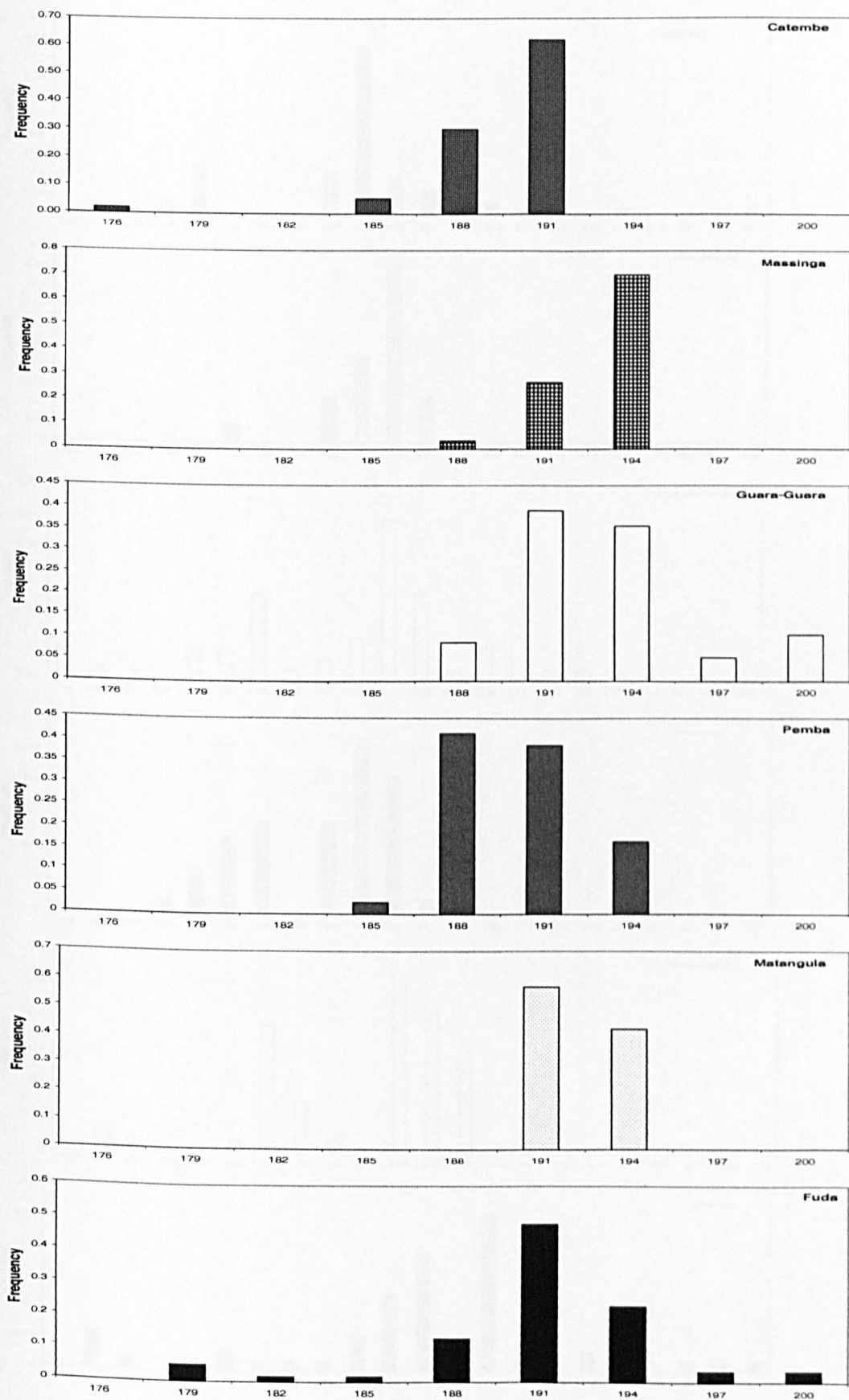
FIGURE 8.1c. Allele frequency distribution of *An. funestus* at locus AF3

FIGURE 8.1d. Allele frequency distribution of *An. funestus* at locus AF6

FIGURE 8.1e. Allele frequency distribution of *An. funestus* at locus AF8

FIGURE 8.1f. Allele frequency distribution of *An. funestus* at locus AF11

FIGURE 8.1g. Allele frequency distribution of *An. funestus* at locus AF17
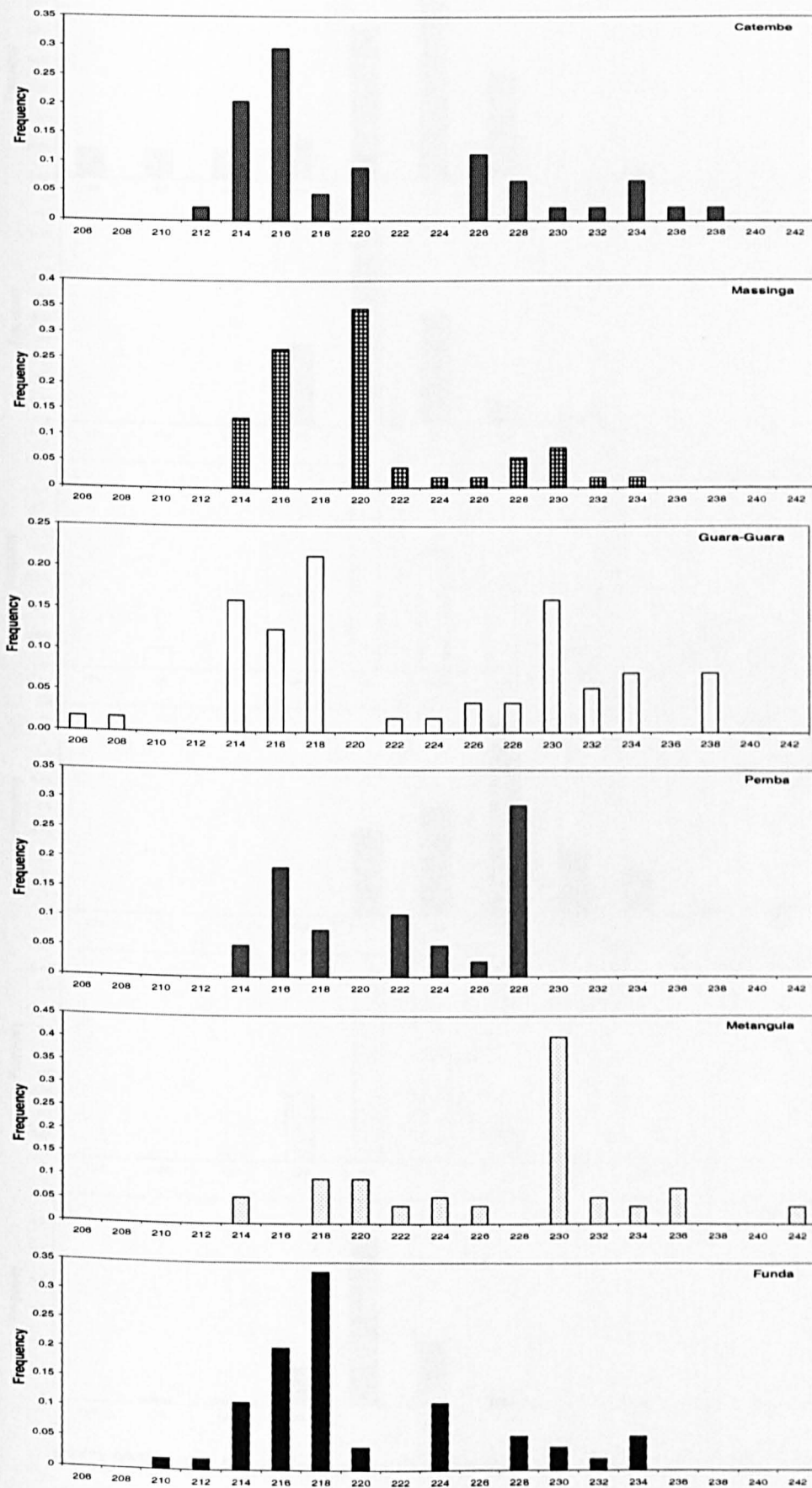
FIGURE 8.1h. Allele frequency distribution of *An. funestus* at locus AF18
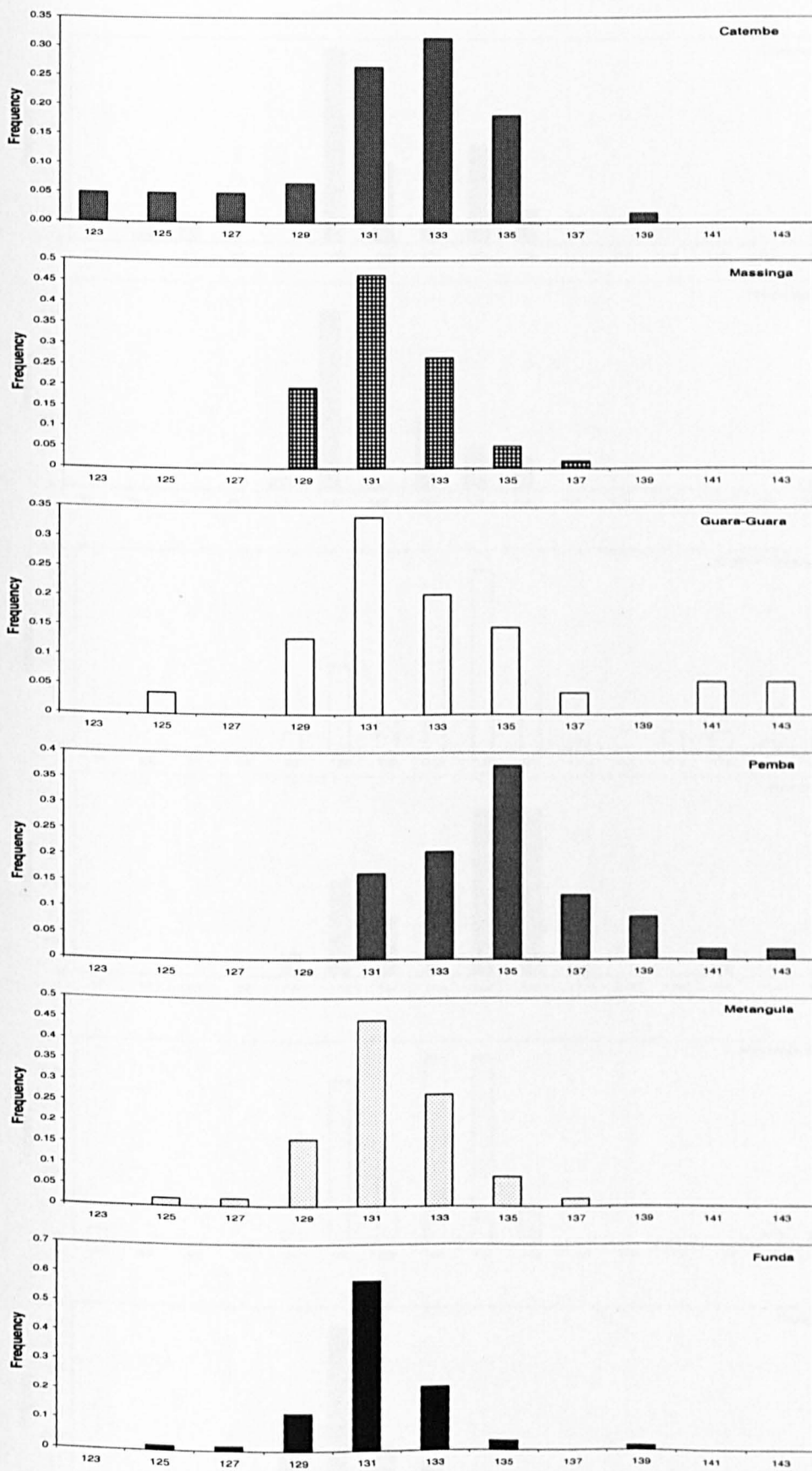
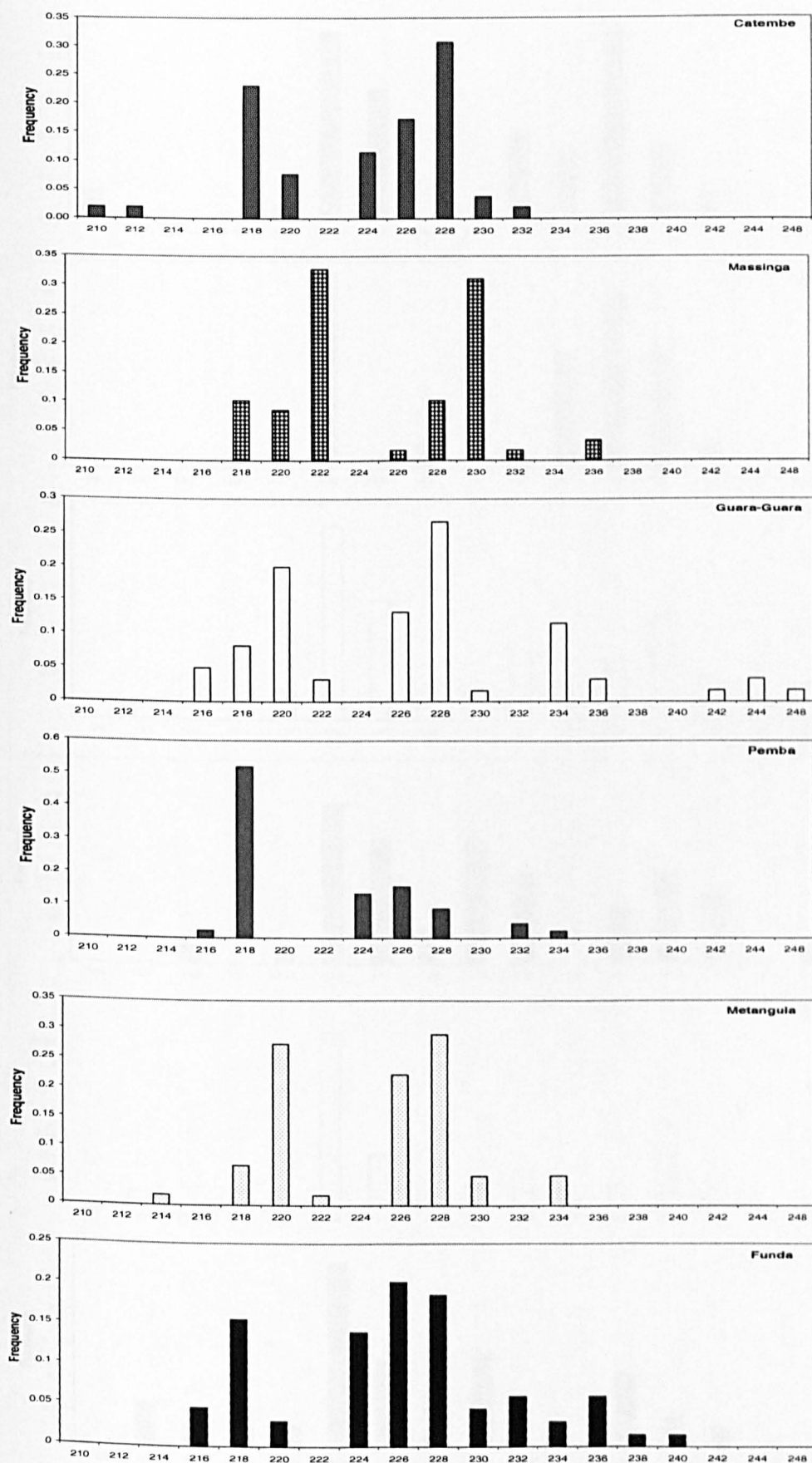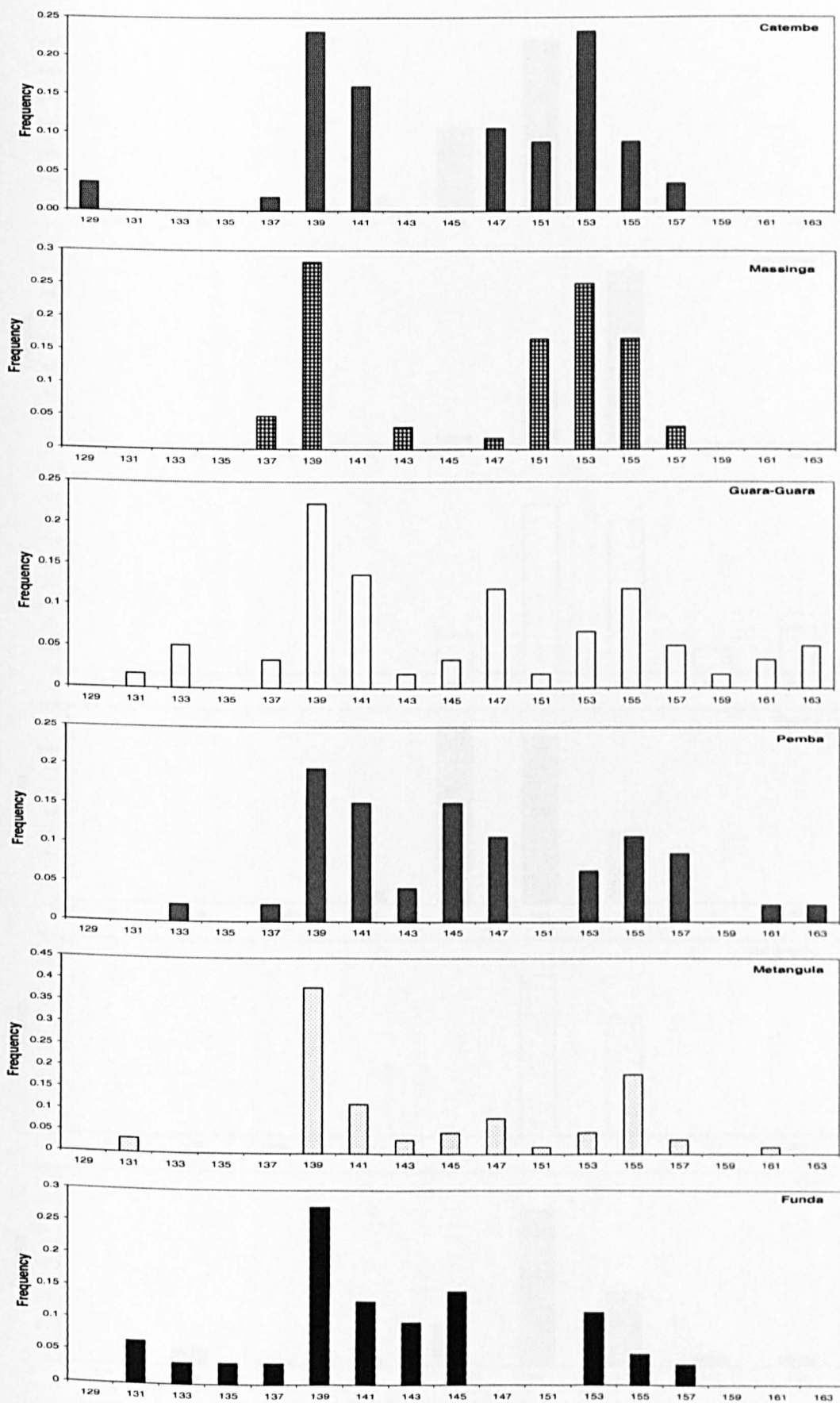**FIGURE 8.1i.** Allele frequency distribution of *An. funestus* at locus AF20.

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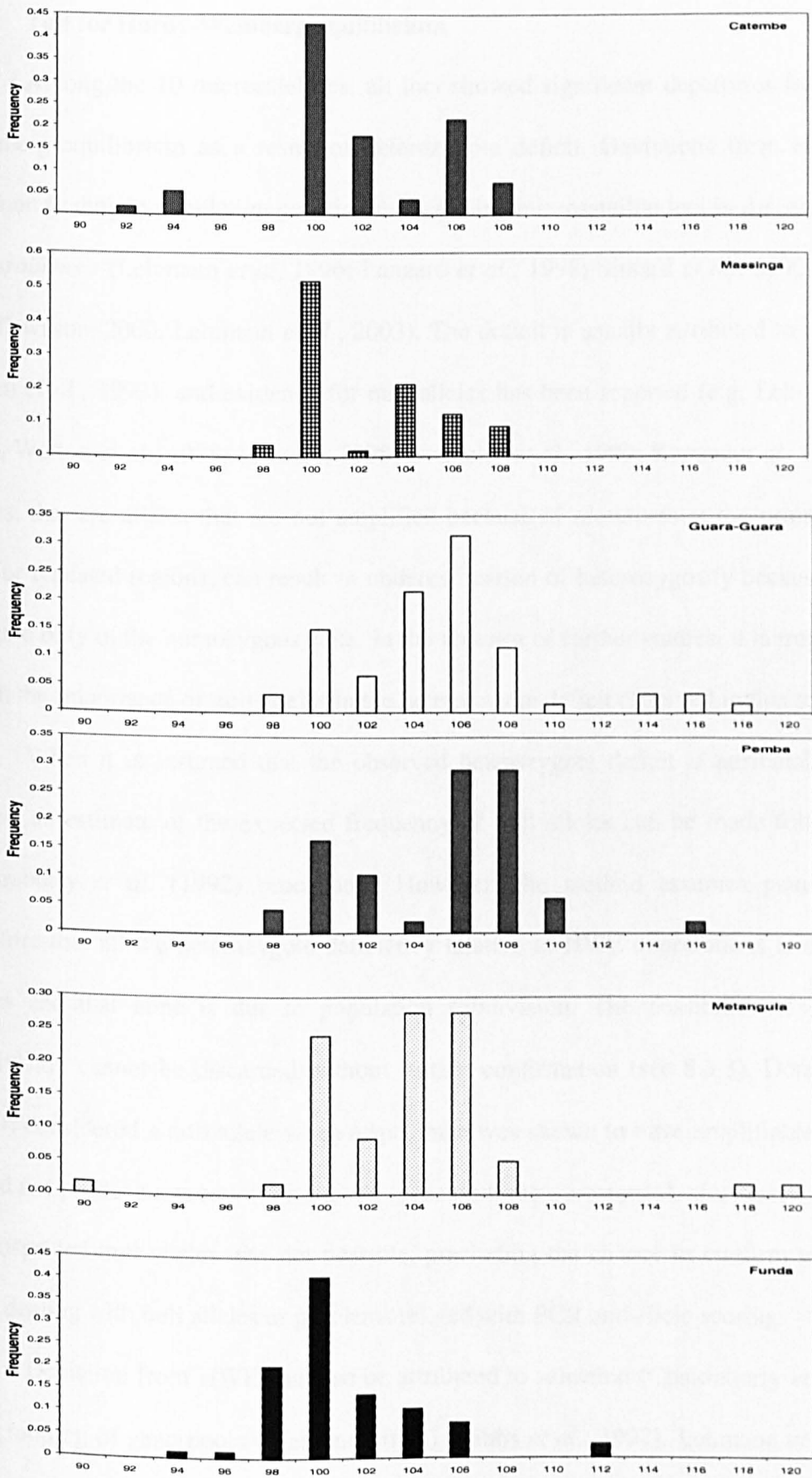
FIGURE 8.1j. Allele frequency distribution of *An. funestus* at locus AF21
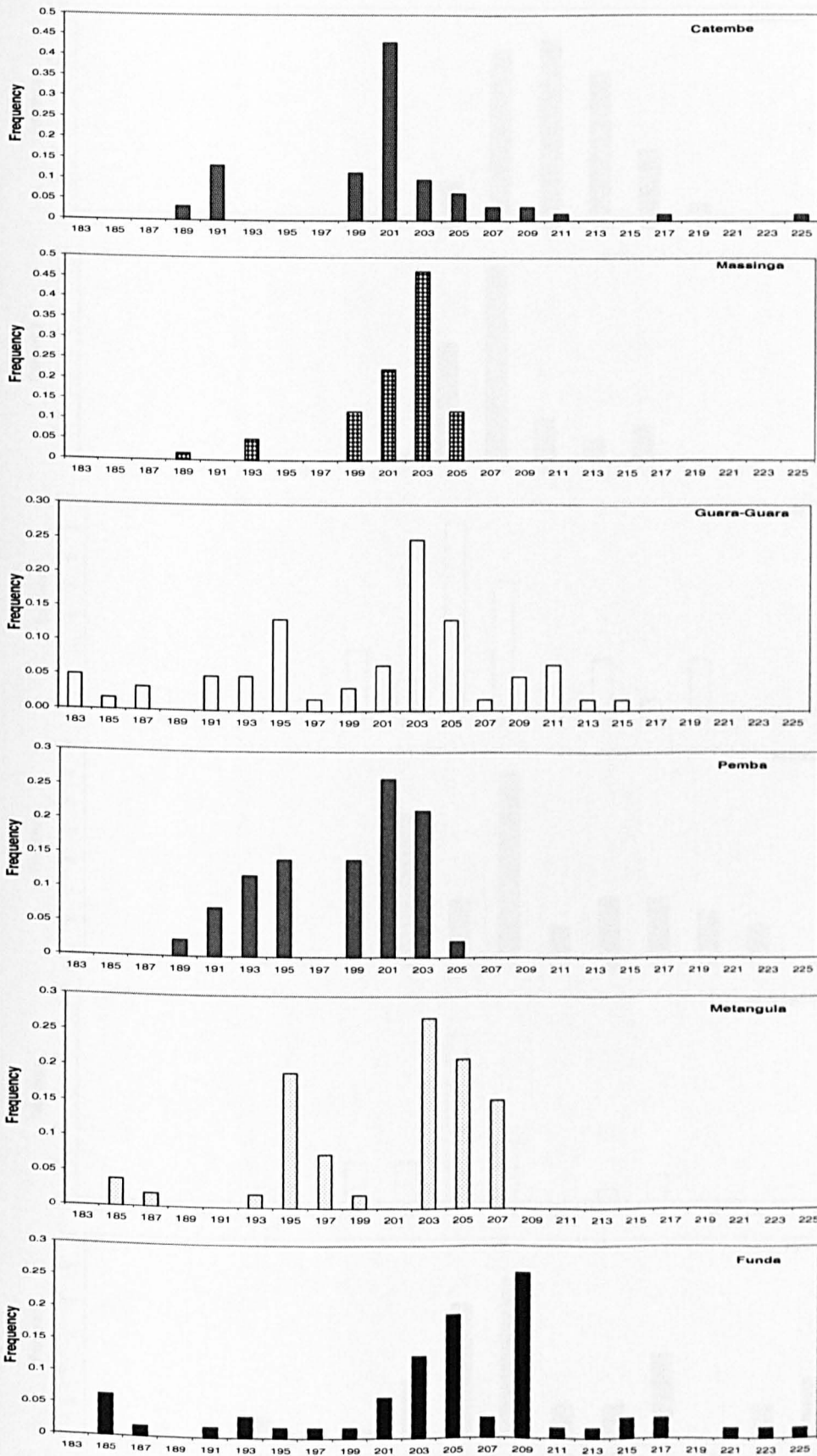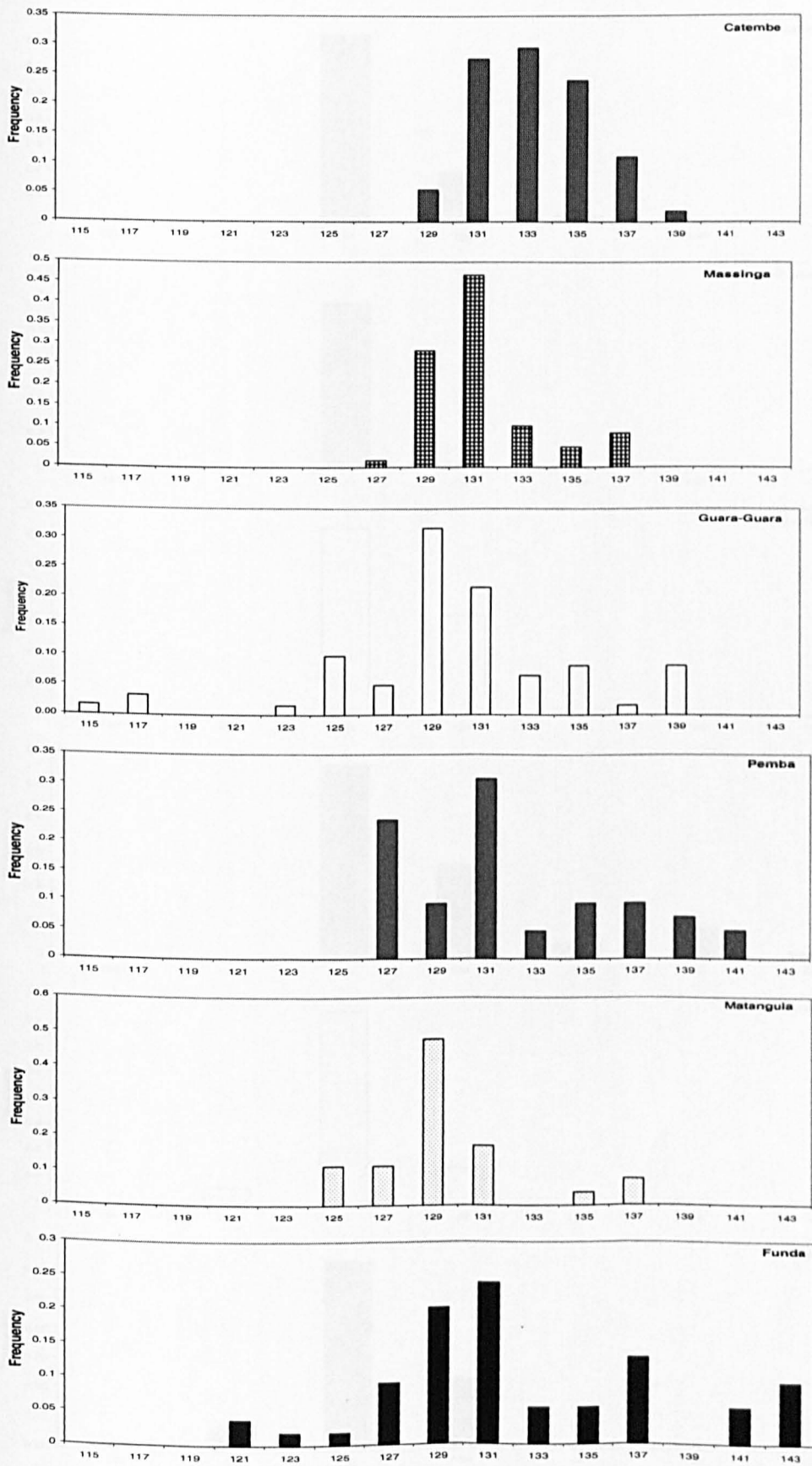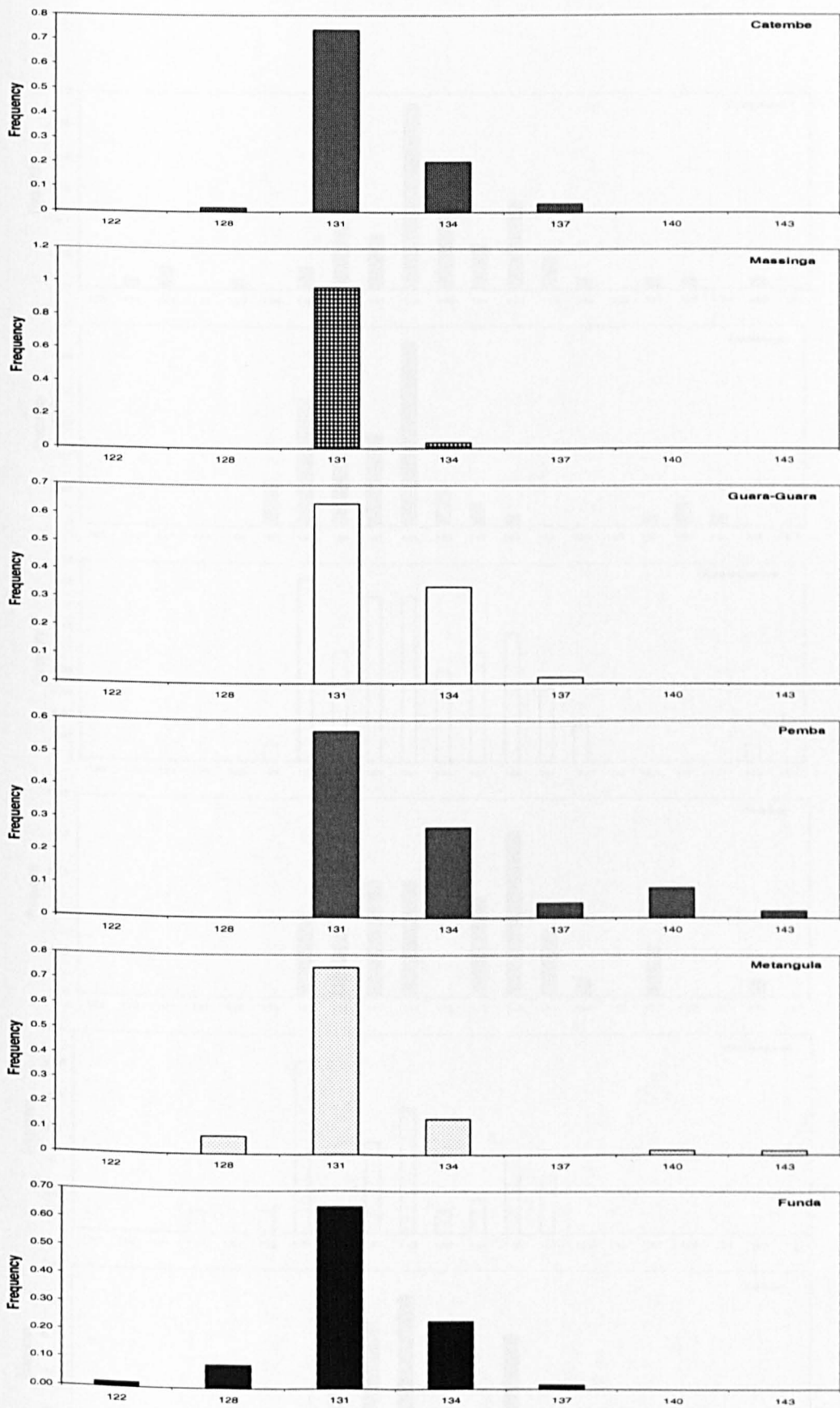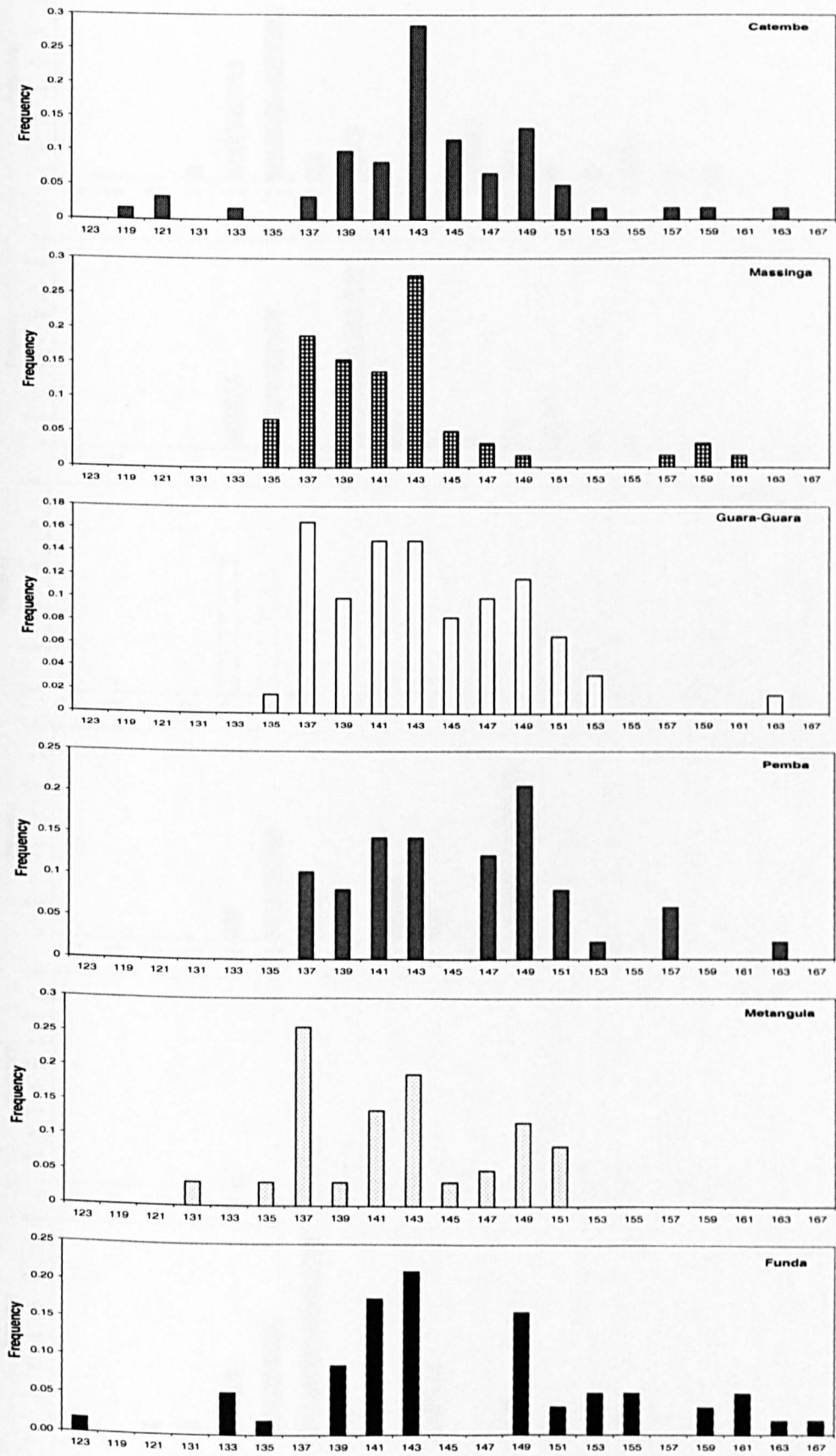
8.3.2 Test for Hardy-Weinberg equilibrium

Among the 10 microsatellites, all loci showed significant departures from Hardy-Weinberg equilibrium as a result of heterozygote deficit. Deviations from HWE are a common finding in population genetics studies using microsatellite loci in *An. gambiae* and *An. arabiensis* (Lehmann *et al*, 1996; Lanzaro *et al*., 1998; Simard *et al*., 2000; Donnelly and Townson, 2000, Lehmann *et al*., 2003). The deficit is usually attributed to null alleles (Calen *et al*., 1993), and evidence for null alleles has been reported (e.g. Lehmann *et al*, 1996, Walton *et al*., 1998; Lanzaro, 1998, Donnelly *et al*., 1999; Kamau *et al*. 1999). Null alleles, that are alleles that are not amplified because of mutations at the primer binding sites or repeated regions, can result in underestimation of heterozygosity because they are detected only in the homozygous state. In the absence of further studies, it is impossible to weigh the importance of null alleles in the heterozygote deficit observed in this study.

When it is assumed that the observed heterozygote deficit is attributable to null alleles, an estimate of the expected frequency of null alleles can be made following the Chakraborty *et al*. (1992) procedures. However, the method assumes panmixia, and therefore that all the heterozygote deficiency relative to HWE expectations is due to null alleles and that none is due to population subdivision. The possibility of population subdivision cannot be discarded without further confirmation (see 8.3.3). Donnelly *et al* (1999) considered a null allele when a specimen was shown to have amplifiable DNA but would not produce a product for a given locus on three occasions. Unfortunately, retesting the suspected null alleles was not possible, precluding the chance to confirm whether we were dealing with null alleles or problems related with PCR and allele scoring.

Deviation from HWE can also be attributed to selection (Chakraborty *et al*., 1992) and grouping of gene pools (Wahlund effect) (Gibbs *et al*., 1997). Lehmann *et al*. (1996) suggested that the deme of *An. gambiae* is at least 50 km diameter. In *An. funestus*, based

on chromosomal studies, Kamau *et al.* (2002), found that the deme of this species is more that 2 km radius. Since the mosquitoes were collected within a radius of 2 km, well know a normal flight range for *An. funestus* the accidental grouping of separate gene pools is unlikely. Inbreeding or non-random mating may also result in heterozygote deficit (Donnelly *et al.*, 1999) and this cannot be discounted without further evidence. In *An. funestus*, the cytological studies by Costantini *et al.* (1999) indicates that in West Africa the taxon *An. funestus*, may consist of more than one biological entities, but this and others similar studies are discussed in detail in section 8.3.3.

Other factors may be involved in the production of an apparent heretozygote deficiency. It seems likely that problems in resolving alleles have led to an underestimate of heterozygosity in some gels. PCR reagents were from recognised companies and were the same used for mosquitoes identification for this study and these reagents were used by other colleagues from the same laboratory, without problems. Nevertheless quality of *Taq* DNA polymerase, in particular, may have some influence on the quality of results (Dr H. Phuc, personal communication), and PCR conditions, principally annealing time and temperature, may be crucial in PCR reactions.

The heterozygote deficit observed in this study may be a result of the combinations of several factors as discussed before, but we argue that null alleles may be the most important cause as seen in previous studies that employed microsatellite loci (eg. Lehmann *et al*, 1996, Walton *et al.*, 1998; Lanzaro, 1998, Donnelly *et al.*, 1999; Kamau *et al.* 1999). Nevertheless, this requires further confirmation.

8.3.3 Genetic differentiation

There is a growing body of evidence that R_{ST} may not be an appropriate estimator of population substructure for all microsatellite data (Donnelly *et al.*, 1999). Forbes *et al.* (1995) found that F_{ST} was more sensitive to differences between allopatric populations and Perez-Lezaun *et al.* (1997) observed that genetic distance measures such as F_{ST}, which do not consider mutational relationships to differentiation by drift, better reflect currently understood patterns of human evolution than do the mutation-based distance measures, such as R_{ST}.

Estimates of F_{ST} varied between loci, ranging from 0.014 at locus AF17 to 0.136 at locus AF3, but were significant for all loci (Table 8.3). Overall there was a significant differentiation between populations (F_{ST}= 0.061, P = 0.0001). All pairwise comparisons also showed a differentiation with the exception of samples from Mozambique between Guara-Guara and Metangula, whose value indicates little differentiation, but this was not statistically significant (Table 8.4). These results suggest some restriction of gene flow between populations involved. Nevertheless, caution is necessary in interpreting these results due to the heterozygosity deficits. The only known study of *An. funestus* using molecular markers is that of Mukabayire *et al.* (1999). Using mDNA the authors did not find evidence of population partioning by distance in mosquitoes sampled across Africa (approximately 6000 km). Elsewhere in Africa chromosome-based studies provided interesting insights. Green and Hunt (1980), observed significant genetic differences between samples from Zimbabwe/Rhodesia, Namibia and Nigeria. In a recent study by Kamau *et al* (2002), they found that overall, the level of differentiation between populations from two different sites (western and coastal Kenya) was significant, suggesting that the two populations are genetically isolated. Genetic heterogeneities have been observed even in sympatric populations of *An. funestus*. In Senegal, West Africa, the general pattern of

karyotype distribution found by Lochouarn (1998) led to the suggestion of the presence of 2 genetically differentiated populations, a similar result was found later in Burkina Faso, by Costantini *et al.* (1999). Most interestingly in this last study, differentiation was found between indoor human-fed samples *versus* corresponding outdoor animal-fed samples.

Its well accepted that geographic distance between populations may lead to genetic differentiation (isolation by distance). Therefore, restriction in gene flow would be expected to be far greater between samples of Mozambique and Funda in Angola due to the distance (isolation by distance), but this was not the case. In *An. gambiae*, Kamau *et al.* (1999) suggested that geographical distance by itself might not be a barrier to gene flow among continental populations. Significant levels of gene flow were postulated between populations of *An. gambiae* from Kenya in East Africa and Senegal in the West Africa (Lehmann *et al.*, 1996). More recently, recent range expansion has been the most commonly accepted reason for genetic similarities between geographically disparate populations (e.g. Donnelly and Townson, 2000; Donnelly *et al.*, 2001; Onyabe and Conn, 2001). However, care, is necessary in interpreting the present results because of the heterozygosity deficit. The heterozygosity deficit may either inflate differences or increase similarities between populations.

This is the first study on *An. funestus* using microsatellite loci. The importance of *An. funestus* as malaria vector obliges requires further studies of behaviour of loci in populations of *An. funestus* across the continent. The numbers of loci that can be studied have now increased with the publications of new microsatellite primers by Cohuet (2002). Such studies will improve our understanding of population structure and the patterns of gene flow in populations of *An. funestus*.

TABLE 8.3. Genetic differentiation between populations of *An. funestus* .

Locus	F_{ST}	P
AF 1	0.083	0.0001
AF 2	0.021	0.0004
AF 3	0.136	0.0001
AF 6	0.072	0.0001
AF 8	0.061	0.0001
AF 11	0.059	0.0001
AF 17	0.014	0.0023
AF 18	0.071	0.0001
AF 20	0.045	0.0001
AF 21	0.06	0.0001
Overall	0.061	0.0001

P refers to the statistical significance of F_{ST} .

Values, determined by 10,000 permutations of genotypes (or alleles) among groups.

TABLE 8.4. Genetic differentiations estimates (F_{ST}) for each sample pair of the *An. funestus* populations. Value in bold indicates non significant estimate.

	Catembe	Massinga	Guara-Guara	Pemba	Matangula
Catembe					
Massinga	0.0956				
Guara-Guara	0.0470	0.0699			
Pemba	0.0383	0.1202	0.0402		
Matengula	0.0845	0.0778	**0.0126**	0.091	
Funda	0.0411	0.0777	0.0238	0.0557	0.0457

8.3.4 Conclusions:

1   There was departure from HWE equilibrium in all loci studied due to the heterozygote deficit. Although many factors may have been involved, null alleles and problems in resolving alleles in some gels may have been the main cause of heterozygote deficit. Nevertheless, population subdivision cannot be ruled out without further studies.

2   Restricted gene flow was observed between populations separated by a few hundreds kilometres in Mozambique. The pattern was similar between population from Mozambique and the population of Funda, which are separated by at least 2500 km.

Resistance to pyrethroid insecticides has already been found in *An. funestus* populations of Beluluane, a locality of southern Mozambique (Brooke *et al.*, 2001). The limited gene flow, as suggest by the results means that there is a hope that resistance is localized in these southern populations of *An. funestus*. This provides some hope and time to control the spread of resistance to another populations. However, because of deviation from HWE in most loci, the estimates of F_{ST} must be interpreted with caution.

CHAPTER IX

General discussion, conclusions and recommendations

Although Mozambique is located at the southern edge of the distribution of malaria in continental Africa, the disease is the most important parasitic disease in the country. It is the principal cause of morbidity and mortality, accounting for more than 40% of the attendance in the public health clinics. The disease is responsible for 42% of hospitalisation in paediatric services with a fatality rate of about 5.3%. Chloroquine-resistant *P. falciparum* has been reported since early 1983 (Franco *et al.*, 1984; Schwalcach and Maza 1985) and is now widespread throughout the country. Nevertheless, the drug is still the first-line drug for the treatment of uncomplicated malaria.

The main malaria control activity in Mozambique relies on the use of indoor residual insecticide spraying. Lately, this has been performed exclusively in sub-urban areas of large cities of the country. Early documentation indicates that control activities with insecticides had been initiated in the 1950's (Soeiro, 1956; Soeiro *et al*, 1956). Despite this relatively long history of the use of insecticides in malaria control, documentation of the impact of these activities is scarce or non-existent. In addition, the few studies concerned with the bionomics and role of malaria vectors in disease transmission were concentrated in an area around Maputo, the capital of Mozambique. This is attributed to several factors, which include the lack of expertise and the war hostilities that started in the middle 1960s (the liberation war) and internal conflicts that ended in 1992. Hence, the vector control has been based on the assumption that mosquitoes of the *An. gambiae* complex are the primary malaria vectors during the wet season, with *An. funestus* maintaining transmission in the dry season, albeit at a lower level

This thesis describes studies on the bionomics, population genetic structure and the roles in transmission of malaria vectors with samples collected from several localities of Mozambique. The study was extended to some localities of Angola. As with Mozambique, Angola is now rebuilding its malaria control programme after the several years of civil war. Basic information on vector bionomics and disease transmission is sorely needed in both countries for rational planning and evaluation of malaria control activities.

In Mozambique, Petrarca *et al.* (1984), showed the presence of four species: *An. arabiensis*, *An. gambiae*, *An. merus* and *An. quadriannulatus*. Results from this study extend our knowledge of the distribution of members of *An. gambiae* complex in Mozambique as it involved more sample sites and a larger sample size per locality. Firstly, the results confirm the existence of four members of *An. gambiae* complex. Secondly, this study identifies the ecological parameters that play a key role in the distribution of species of the *An. gambiae* complex. The distribution of *An. arabiensis* and *An. gambiae s.s.* S form is associated with the pattern of rainfall; *An. arabiensis* although widely distributed, appears to predominate in areas of reduced rainfall whereas *An. gambiae* is uncommon in southern areas where the rainfall is less than 600 mm per annum. The vegetation transition from *Deciduous Miombo* to *Open Forest* at 24° S seems to limit the distribution of *An. gambiae s.s.* although other ecological factors are not excluded. This is the southernmost record of this species and may represent its southernmost limit in continental Africa.

An. quadriannulatus is confined to the dry areas south of 24° S but it was not possible to explain this restricted distribution. More recently *An. quadriannulatus* has been found in a dry area of Malawi (above latitude 16° S), in both adult and larvae collections (A. Spiers, personal communication). It is unclear whether the species is adapted only to the dry areas and found in indoor collections when outdoor resting sites are less suitable or also exists in wet areas and is not found indoors as the resting sites outdoors are

permissive. Adaptation to dry areas seems to be the most plausible explanation as the species has been collected only from dry areas of southern Africa localities and in Ethiopia.

An. merus is found as far as 350 km inland as well as along the coast. This species showed an ability to colonise both brackish and fresh water used by other members of the *An. gambiae* complex. *An. merus* larvae were found together with *An. arabiensis* and *An. quadriannulatus* even during the dry season suggesting that these three species breed throughout the year. This is also true for *An. gambiae s.s.* The persistence of members of the *An. gambiae* complex during the dry season in many of the localities surveyed is due to their ability to exploit the human-made breeding sites. However, breeding in sandy or rocky pools left in riverbeds seems to be most important during the dry season.

Within the *An. funestus* complex, *An. funestus s.s.* (98%) was the most common and the exophilic and zoophagic sibling species *An. rivulorum* and *An. leesoni* were occasionally found in human dwellings in Mozambique. Surprisingly, *An. leesoni* was collected on human biting collection indoors in a region located in the central area of Mozambique. In the same region another two specimens of *An. leesoni* were found resting in human dwellings. Taking into account the known behaviour of this species one would not be surprised to find a higher biting activity outdoors. More collections are needed to test this assumption. The existence of rapid PCR will facilitate observations on the behaviour of this species and perhaps its role in diseases transmission. In Angola only *An. funestus s.s.* was collected in Funda. It was impossible to provide a reasonable explanation for the apparent absence of this species in all other localities of Angola where ecological conditions appeared permissive for this species. The results suggested that it is of less importance, in operational studies or vector control activities, to identify members of *An. funestus* complex to species, as *An. funestus s.s.* is almost exclusively the species in indoors resting collections, and in indoor and outdoors human bait catches.

The few larval collections undertaken provided interesting insights. In places in Mozambique where both larva and adult collections were identified, the species compositions were divergent. For instance, identification of adult mosquitoes from Homoine showed the presence of *An. gambiae* and *An. arabiensis*, but in a small sample of larvae, *An. merus* was found. A similar situation was observed in Combumune where only *An. arabiensis* was found in mosquitoes collected resting in houses, but the larvae collections revealed the presence of another two species: *An. merus* and *An. quadriannulatus*. This indicates that the breeding requirements of at least fresh water species are similar but also underlines differences in adult behaviour. Together these observations stress that in studying species composition or distribution, various collection techniques must be applied in order to involve species with different breeding, resting and biting behaviour.

The non-uniform distribution of members of the *An. gambiae* complex throughout Mozambique suggests different epidemiological malaria patterns and therefore different strategies of malaria control may need to be sought according to the ecology and behaviour of vector species in a specific area. For example where *An. funestus* is the predominant species ITNs may provide higher protections again malaria. In contrast, where *An. arabiensis* and/or *An. gambiae s.s.* are the predominant malaria vectors others methods may be necessary to achieve better protection against the disease.

In Mozambique, the few previous published papers on malaria transmission were concentrated in the Maputo area. The present study involved for the first time samples from other localities of the country and include *An. gambiae s.s.* The sporozoite rates indicated that *An. gambiae s.s*, followed by *An. funestus* and *An. arabiensis* are the primary malaria vectors in Mozambique. The importance of these vectors in disease transmission is significantly greater during the wet season with sporozoite rates ranging from 9.32 % in

An. gambiae s.s. (n= 676), 5.59 % in *An. funestus* (n= 1573) and 3.99% in *An. arabiensis* (n= 1177). During the dry season the three species maintain disease transmission equally. As mentioned before, the vector control activities in Mozambique has been based on the assumption that mosquitoes of the *An. gambiae* complex are the primary malaria vectors during the wet season, with *An. funestus* maintaining transmission in the dry season, albeit at a lower level. The findings of this study rejected the hypothesis on which indoor residual spray has been made in Mozambique, but supported one round of residual spray per year just before the rainy season, considering the present economic context of the country. Additionally, the findings underline the need for the correct identification of members of the species complexes in epidemiology and control activities in Mozambique rather than conduct control activities based on assumptions. Basic expertise and human resources for malaria vector studies are now becoming available in Mozambique, which will hopefully change the nature of vector control activities. Whereas the present study presented information on the individual role of malaria vectors in disease transmission, locally directed studies will be needed to define the importance of any species individual role in a specific locality. The fact that *An. merus* was found in the present study to be an important malaria vector in Boane emphasises this need.

In Angola, a single specimen of *An. arabiensis* was found in a suburban area of Luanda. This is the first record in the country. *An. melas* was found only in a coastal area of Funda in PSC collection. In both cases no other collection methods were used. *An. arabiensis* is known for it's versatile resting and biting behaviour, although breeding requirements are similar to other fresh water species within the *An. gambiae* complex. There is no obvious ecological barrier that may preclude the flourishing of *An. arabiensis* in all sampled sites and *An. melas* in all coastal areas where sampling was performed. It is unclear whether the absence of *An. melas* in our samples in the coastal areas of both

Benguela and Lobito is due to mainly sampling in urban areas or its largely exophilic habits or the seasonality of this species, as observed elsewhere in West Africa. The limited sampling and number of sites sampled together with the limited number of collections techniques employed may have missed some species. Further studies involving over seasons and several collection methods may bring a clear picture on species composition and distribution of members of *An. gambiae* complex in Angola.

Based on samples from two coastal localities (Namibe and Cacuaco), *An. gambiae s.s.* has been shown to consist of the M molecular form with no allele that confers resistance to pyrethroids (Carrara *et al.*, 2002). In the present studies sampling was performed in Cacuaco and extended to Benguela, Lobito (coastal areas) and to the high plateau of Huambo. The S molecular form is also found, albeit at a lower frequency, in both coastal Benguela (3.8%, n= 132) and in the high plateau of Huambo (19%, n= 79). The results of the present study together with those of above cited author, suggest that the M form must be the predominant species in Angola.

The *kdr* alleles conferring resistance to pyrethroid are reported for the first time in Angola and were present in both forms of *An. gambiae s.s.* This represents the southernmost known distribution of the *kdr* in *An. gambiae*, suggesting a much wider distribution than hitherto recorded. The pyrethroid resistance in Angola raises fears that such compounds might have a limited effective usage in ongoing programmes of promotion and scaling up of the use of ITNs. This finding underlines the need to monitor pyrethroid resistance and the presence of *kdr* alleles preferably before implementation of ITN programmes. In localities where pyrethroid-impregnated bed nets are in use, it will be important to monitor insecticide resistance on a continuing basis. This is indispensable for the implementation of rational and effective malaria control tools.

An. gambiae s.s. (SP ≈ 2%, n=583) and *An. funestus s.s.* (SP ≈ 1%, n= 140) are the vectors of *P. falciparum* in Angola. *An. melas* was not found infected but is assumed to be malaria vector based on epidemiological evidence from elsewhere in West Africa. Only M form was found positive for malaria infection. The role of the two molecular forms in disease transmission is still not known. Huambo, where both S and M form may be found in considerable numbers, may be a good area to elucidate the individual role of these forms in disease transmission.

A separate study on vector behaviour and malaria transmission was carried out on the *An. gambiae* complex between 1998-2000 in Boane, about 25 km from Maputo city. Larvae studies have been discussed elsewhere in this report. Comparable delayed oocyst rates between *An. merus* (14%, n=72) *An. arabiensis* (11%, n=83) and *An. funestus* (7%, n=761) (including infective mosquitoes with sporozoites of *P. falciparum* in *An. merus*) were found in Boane. It is therefore concluded that *An. merus* is an important malaria vector in localities where the species is abundant. Another locality where *An. merus* is abundant is Salamanga, southern Mozambique. The partial exophilic of *An. merus*, together with its pronounced tendency to feed outdoors suggests that house spraying alone would not be able to control this species.

A dramatic change in species composition was observed after the heavy rain and floods of 2000 in Boane. The magnitude of change was that *An. arabiensis*, which occurred in insignificant numbers during 1998 (0.1 mosquito/house) and 1999 (0.4 mosquito/house), its densities (2.0 mosquito/house) became similar to those of *An. merus* after the heavy rain. This is without doubt a direct impact of the rain, which modified primarily the breeding characteristic of mosquitoes, favouring *An. arabiensis* while restricting *An. merus*. A marked increasing in densities of *An. funestus* from 1999 (1.4 mosquito/house) to 2000 (15.7 mosquito/house) is also attributed to the e rainfall/floods.

The main collection for this thesis have been planned to start at the beginning of 2000. Unfortunately in early 2000, parts of Southern Mozambique received exceptionally high rainfall, the highest observed over a period of 50 years, resulting in flooding in most of the river basins and low land areas. During the flood situation I had to be involved in humanitarian relief work. In early 2001 the floods hit the central and northern areas of the countries. The floods destroyed large stretches of road and many bridge making it virtually impossible to travel to some of localities initially selected for sampling. The floods affected also the frequencies of sampling in many areas. In the absence of data before flooding occurred it is not clear whether this phenomena impacted upon the species composition as observed in Boane.

The biting activity and disease transmission of the most important malaria vectors (*An. arabiensis*, *An. gambiae* and *An. funestus*) were studied together for first time in Mozambique. As expected, *An. funestus* had a more endophagic tendency. *An. arabiensis* and *An. merus* were more exophagic and unexpectedly *An. gambiae* fed equally indoors and out. Both vectors were found infected in indoor and outdoor collections. Furthermore, the results indicated that the risk of being infected outdoors, mainly by the members of the *An. gambiae s.s.* cannot be ignored. However, the majority of infective bites occurred after 21 h both indoors and outdoors, when most children and pregnant women are in bed, suggesting that the use of bed nets may protect this high-risk group. However, Mozambique is one of poorest countries in the world, and in the poor rural communities of the country, affordability of bed nets might pose a serious obstacle. The use of insecticide treated screens and curtains, which are sometimes more effective than impregnated bed nets in reducing parasite rates (Sexton *et al.*, 1990) may represent one cheaper alternative. This has been experimented in southern area of Mozambique with significant impact in reduction of *P. falciparum* parasitaemia in children (Crook and Baptista, 1995) and this method is now

being revived in Morrrumbene district some 500 km north of Maputo (D. Charlwood, personal communication).

Human biting collections were based on a limited number of collections, eleven human biting catches in Boane and eight in other localities. There is no doubt that few collections are more prone to introduce bias, resulting for example in different attractiveness of collectors and meteorological conditions, perhaps the most common bias in human bait catches. Weather is an important limiting factor affecting the activities and biology of adult mosquitoes (Service, 1978). It is difficult to weigh the impact of reduced number of collections in the results obtained. However, it is clear that the study provided valuable information on biting behaviour and disease transmission that can contribute to the rational use of the available tools for malaria vector control in Mozambique.

During the 1980s, vector control activities relied on DDT and were restricted to Maputo suburban areas (Martinenko et al., 1989) and by 1985, An. funestus was reported to be predominant over An. arabiensis (Zharov, 1992). This situation still prevails in the Maputo area where despite almost annual spraying with pyrethroids, there have been outbreaks of malaria. There is a need, however, to confirm whether this upsurge of An. funestus is a consequence of resistance to pyrethroid insecticides, already observed in southern areas of Mozambique (Brooke et al., 2001) and the South Africa areas bordering Mozambique (Hargreves et al., 2000) or is due to other biological or ecological phenomena, including behavioural aspects of this vector, such as for example exophily and possible exophagy, but some other explanations are also possible. This is also true for An. arabiensis, the other vector of malaria in the region. The biting behaviour of these species is discussed elsewhere in this chapter. Insecticide susceptibility studies are ongoing in some localities of the country (S. Casimiro, personal communication), but vector behavioural studies are sorely

needed. This will help to use available tools in more rational manner for malaria control in Mozambique.

The population structure of *An. funestus s.s.* was assessed on the basis of twelve microsatellite loci. Results indicate a high level of genetic diversity but deviations from Hardy-Weinberg expectations were significant at most loci used. The null alleles were among the main causes of heterozygote deficit, although problems in resolving alleles may have led to an underestimation of heterozygosity in some gels. The results were consistent with the hypothesis that the limited flight range of *An. funestus* may determine genetic distinctiveness between geographical populations of this species. The patchy distribution characteristic of this species (Gillies & De Meillon, 1968) also favours some genetic differentiation. Significant differentiations were found in samples collected in Mozambique (pair-wise F_{ST} range: 0.0126-0.1202) as well as when these were compared with a sample from Angola 2500 km apart (pair-wise F_{ST} range: 0.0238-0.0777). Results were consistent across loci reflecting a genome-wide pattern. This provides some hope for management of the spread of resistance in *An. funestus*, but may pose additional difficulty for those who contemplate the use of transgenic mosquitoes as a mean of controlling malaria. Nevertheless, caution is necessary in interpreting these results due to the heterozygosity deficits observed in many loci.

In some localities the role of *An. funestus* as a malaria vector is greater that that of *An. gambiae s.s.* (see review in section 2.2.2). However, much of the research in vector control had been targeted *An. gambiae s.s.* Eliminating transmission by *An. gambiae* without affecting transmission by *An. funestus* is likely to have little long-term effect on malaria. This is the first study on *An. funestus* employing microsatellite loci to study population genetic structure. Therefore the importance of *An. funestus* as a malaria vector justifies further studies with enlarged sample sizes and loci numbers. Such studies will

improve our understanding of population structure in *An. funestus* and are of direct importance in the manipulation of the spread of insecticide resistance and in control of malaria through genetically engineered mosquitoes.

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Appendix 1

The numbers of mosquitoes tested, the numbers found to be carrying *Plasmodium falciparum* circumsporozoite antigen and the corresponding percentage rates for *An. arabiensis*, *An. gambiae* and *An. funestus* collected in localities of Mozambique during the wet seasons of 2000 to 2002. The total numbers caught per locality are presented in Table 3.1 (Chapter 3) and the numbers of mosquitoes identified to species level are shown in Table 5.1 (Chapter 5).

Site Number	Locality	Month/year	*An. arabiensis*			*An. gambiae*			*An. funestus*		
			No. tested	positive	%	No. tested	positive	%	No.tested	Positive	%
1	Catuane	02/00	-	-	-	-	-	-	62	-	-
5	Maragra	11/00	15	-	-	-	-	-	66	1	1.5
6	Chihaquelane	04/00	635	8	1.3	-	-	-	231	18	7.8
8	Massingir	02/02	40	1	2.5	-	-	-	133	2	1.5
12	Nhamba	03/01	33	4	12.1	49	5	10.2	154	12	7.8
13	Macomane	03/01	-	-	-	42	3	8.5	-	-	-
14	Chindjiguire	03/01	29	-	-	70	1	1.4	184	5	2.7
14	Chindjiguire	04/01	29	1	3.4	53	2	3.8	64	1	1.6
14	Chindjiguire	02/02	72	6	8.3	198	19	9.1	156	13	8.3
17	Pambarra	02/02	6	1	16.7	65	13	20.1	161	9	5.6
18	Mabote	02/02	29	8	27.6	-	-	-	-	-	-
20	Guara-Guara	04/00	256	16	5.9	112	3	2.7	195	7	3.6
20	Guara-Guara	12/00	15	-	-	5	-	-	46	-3	-
23	Machipanda	04/00	-	-	-	-	-	-	107	13	12.1
25	Guro	12/00	14	2	13.3	65	16	25	-	-	-
33	Mocuba	11/00	4	-	-	17	1	5.9	14	4	28.6
Total			1177	47	3.99	676	63	9.32	1573	88	5.59

Appendix 2

The numbers of mosquitoes tested, the numbers found to be carrying *Plasmodium falciparum* circumsporozoite antigen and the corresponding percentage rates for *An. arabiensis*, *An. gambiae* and *An. funestus* collected in localities of Mozambique during the dry seasons of 2000 to 2002. The total numbers caught per locality are presented in Table 3.1 (Chapter 3) and the numbers of mosquitoes identified to species level are shown in Table 5.1 (Chapter 5).

Site Number	Locality	Month/year	An. arabiensis			An. gambiae			An. funestus		
			No. tested	positive	%	No. tested	positive	%	No. tested	positive	%
4	Boane	06/00	9	-		-	-	-		-	-
6	Chihaquelane	05/00	258	9	3.5	-	-	-	244	3	1.2
9	Combomune	07/01	58	4	6.9	-	-	-	-	-	-
14	Chindjiguire	07/00	32	-		28	1	3.6	59	1	1.7
14	Chindjiguire	07/01	27	-		49	1	2.1	152	4	2.6
8	Massingir	07/01	18	-		-	-	-	103	-	-
18	Mabote	07/01	64	1	1.6	-	-	-	-	-	-
17	Pambarra	06/01	31	-		182	1	0.6	279	5	1.8
19	Machanga	06/01	27	-		-	-	-	-	-	-
20	Guara-Guara	07/01	87	2	2.3	34	-	-	442	6	1.2
22	Caia	07/00	-	-		-	-	-	58	8	13.8
30	Angonia	08/00	-	-		-	-	-	17	-	
28	Moatize	06/01	-	-		-	-	-	47	5	10.6
32	Macuse	07/00	3	-		21	-	-	-		

Appendix 2: *Cont.*

Site Number	Locality	Month/year	An. arabiensis			An. gambiae			An. funestus		
			No. tested	positive	%	No. tested	positive	%	No. tested	positive	%
35	Chapale	06/01	8	-	-	22	3	13.6			
38	Mossoril	08/00	-	-	-	9	1	11.1	97	-	
39	Erati	08/00	-	-	-	44	2	3.6	74	9	12.2
36	Ribawe	07/00	-	-	-	21	2	9.5	54	2	3.7
41	Montepuez	08/00	-	-	-	33	1	3	30	2	6.7
40	P.Miazi	05/00	5	1	20	15	1	6.7	26	2	7.69
42	M. Praia	06/01	-	-	-	162	6	3.7	69	7	10.1
45	Maua	06/01	-	-	-	20	2	10	120	4	3.3
46	Marrupa	06/01	-	-	-	-	-		77	5	6.5
47	Metangula	06/01	11	1	9.1	24	2	8.3	19	-	
Total			638	18	2.82	664	23	3.46	1967	63	3.2