BIOLOGY AND TRANSMISSION POTENTIAL OF MALARIA VECTOR MOSQUITOES IN ELABERED SUB-ZONE, ERITREA

By

Yohannes Bein Okbaldet

Thesis submitted in fulfilment of the requirements for the degree of

MAGISTER SCIENTAE

In the

Department of Zoology and Entomology

(Entomology Division)

Faculty of Natural and Agricultural Sciences

University of the Free State

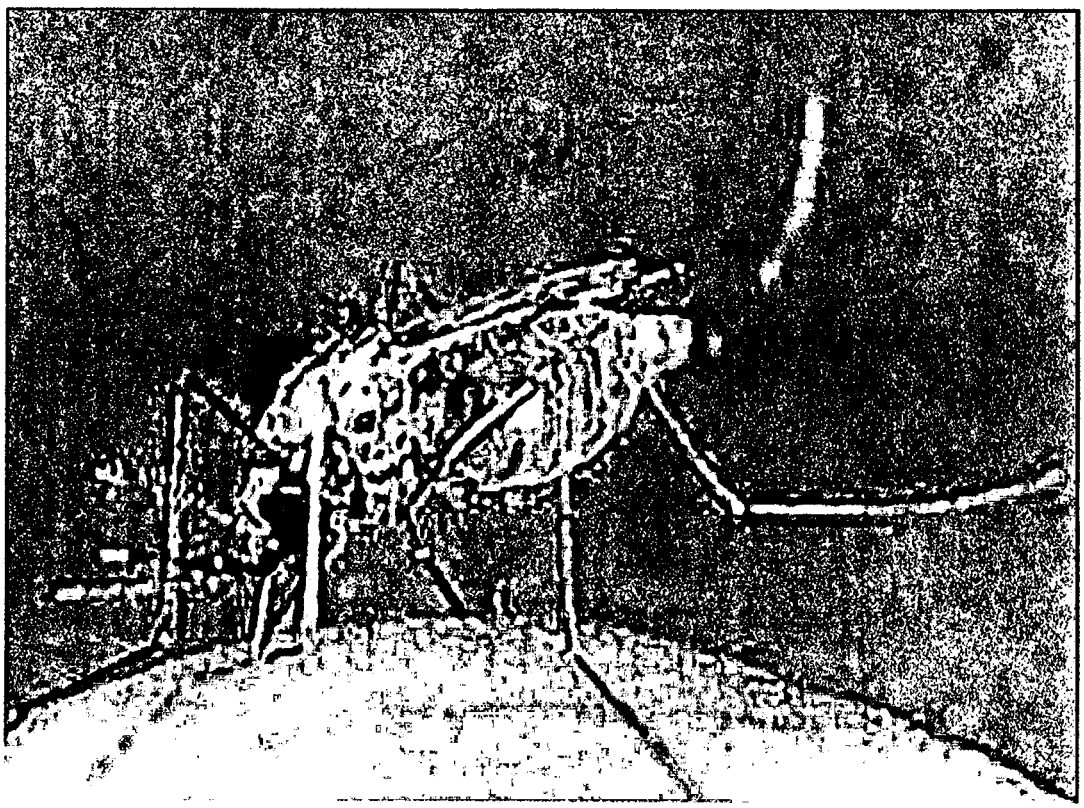
Bloemfontein

November 2001

Supervisor: Prof. M. Coetzee

Co-supervisors: Prof. T.C. van der Linde

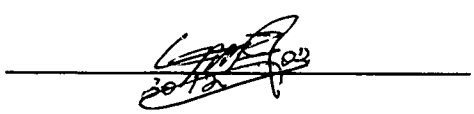
Prof. R. H. Hunt



FEEDING FRENZY: Female *Anopheles gambiae* taking a blood meal (Photograph from the scientist, October 1997).

DECLARATION

I declare that the thesis hereby submitted by me for the Masters Degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I further more cede copyright of the thesis in favour of the University of the Free State.



30th **Day of** November **2001**

DEDICATION

This thesis is dedicated to my dear parents, may God rest their soul!

ABSTRACT

This study was intended to assess the biology and transmission potential of the malaria vector mosquitoes in Elabered sub-zone, Eritrea. Field collected *Anopheles* mosquitoes sampled during the malaria transmission season, from September to November 2000, were identified morphologically in the field. Morphological identification revealed that members of the *An. gambiae* complex were the most abundant and the only malaria vector species present during the study period. All the members of the *An. gambiae* complex were subjected to polymerase chain reaction (PCR) assay and results showed *An. arabiensis* was the only member of the *An. gambiae* complex found in this area.

Blood meal ELISA tests showed that 16.9% and 66.9% of 266 *An. arabiensis* were human and bovine fed, respectively. The percentage of mixed feeds, on both on human and bovine was only 3.8%. A total of 12.4% of the samples failed to react either to human or bovine anti-sera. None of the non-vector anophelines tested positive for human blood. *Anopheles arabiensis* in this particular area preferred to feed and rest outdoors rather than indoors, and biting was more intensive in the first half than in the second half of the night.

Plasmodium falciparum infection was also determined using ELISA for 589 *An. arabiensis* specimens. There was no apparent monthly variation in infection rate, 0.5%, 0.8% and 0.7% for September, October and November, respectively. Based on the entomological inoculation rate derived from the hourly night bait catches

carried out, during the whole transmission season, a villager could be exposed to 0.08 infective bites/night.

Anopheles arabiensis in this particular area is susceptible to deltamethrin, lambda-cyhalothrin, propoxur and DDT. However, permethrin resistant strains might be present and more tests at field level are required to verify the result and monitor the situation.

Key words: Malaria, *Anopheles gambiae*, *Anopheles arabiensis*, *Plasmodium falciparum*, vector biology, transmission, infection rate, entomological inoculation rate, polymerase chain reaction, enzyme linked immuno-sorbent assay, insecticide susceptibility, Eritrea,

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Prof. Maureen Coetzee (Head of the Department of Medical Entomology, National Health Laboratory Services-NHLS {formerly South African Institute for Medical Research (SAIMR}) for her untiring guidance, tolerance, criticism and encouragement throughout this study.

I am also indebted to Prof. Theunis, van der Linde (Department of Entomology, University of the Free State) and Dr. Richard Hunt (Department of Animal, Plant and Environmental Sciences, University of the Witwatersrand). I would like to thank Prof. Theunis van der Linde for his advice, comments and suggestions and to Prof. Richard Hunt for his invaluable theoretical and practical support in fieldwork and rearing of the mosquitoes brought from the field.

My gratitude also goes to Dr. Bsrat Hagos, head of the Department of Research and Human Resource Development, Dr. Andom Okbamariam, head of the CDC and Dr. Tewolde Ghebremeskel, head of the National Malaria Control Program, Eritrea. I thank to Dr. Bsrat Hagos for her great effort in getting me sponsors and facilitating it; Dr. Andom Okbamariam for nominating me as a candidate for continuing my study and providing me permission letter whenever I need during my field work and Dr. Tewolde Ghebremeskel for allowing me to use the field wark equipment from the department.

I also owe thanks to all staff members of the Department of Medical Entomology, NHLS, for their great help. Special thanks go to the following persons: Dr. Emanuel Temu for teaching me the PCR and ELISA techniques and also for critical reading of parts of the manuscript. Dr. Basil Brooke also for his valuable critical reading of part of the manuscript and teaching me the susceptibility tests and biochemical assays. Dr. Lizette Koekemoer, for her valuable help during PCR and ELISA tests. Mrs. Joyce Segerman, for her help in proof reading of my thesis.

Many thanks to the staff of the Elabered Health Centre and Adi Bosqual villagers for their outstanding cooperation.

This project was made possible by funding from the USAID. I would like to thank Mss. Rahel Adamu for overseeing my progress and administering the USAID grant.

Last, but not least, I thank NHLS for allowing me access to their facilities.

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CHAPTER ONE

INTRODUCTION

1.1: The malaria burden

Malaria is getting away from us, escaping our control. Resistance to both the drugs and insecticides is growing and some of the old strategies are past their sell-by date. The much-vaunted vaccine is apparently not imminent

(Foster & Phillips, 1998)

Malaria remains a major health problem in many tropical areas but the main impact of the infection is felt in sub-Saharan Africa. Today, it is by far the most widespread tropical parasitic disease, threatening at least four out of every ten people in the world. Ninety percent (90%) of the worlds' cases occur in sub-Saharan Africa and two thirds of the rest occur in Asia and Latin America. Almost the entire African population is at risk with 300-500 million clinical cases and 1.5-2.7 million deaths from malaria every year. It kills one person, often a child under five, every 12 seconds (Butler, 1997).

The economic consequences of malaria related diseases are high. It is estimated that US$ 1.8 billion is spent annually on direct and indirect costs. Approximately, US$ 150 million or about 15% of all disability adjusted life years are lost to malaria (Foster & Phillips, 1998).

Human malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by *Anopheles* mosquitoes. Out of the four *Plasmodium* species (*Plasmodium falciparum, P. vivax, P. ovale* and *P. malariae*), which affect humans, *P. falciparum* is the most virulent and prevalent species in Africa.

There are about 420 species of *Anopheles* mosquitoes throughout the world with about 68 of them being important as transmitters of malaria. In Africa there are over 120 species of *Anopheles*, yet only three of them (*Anopheles gambiae* Giles, *An. arabiensis* Patton and *An. funestus* Giles) are major vectors of malaria (Gillies & Coetzee, 1987). The very high malaria transmission in this continent is mainly attributed to the high efficiency of these vectors in transmitting the parasites.

1.2: Malaria in Eritrea

Eritrea is situated in the North East of Africa and forms part of the region known as the Horn of Africa. The eastern border, about 1,200 kilometres long, is the coastal line of the Red Sea, while Sudan, Ethiopia and Djibouti are neighbouring countries (Fig.1.2). It is a relatively small country of 124,000 square kilometres and a population of about 3.7 million people. It has a wide variety of climatic conditions ranging from temperate highlands to very hot and very dry arid coastal plains and lowlands (Ministry of Health, 1999).

Malaria is the most important health problem in Eritrea affecting over 67% of the population. The disease is the main cause of morbidity and mortality particularly

in children under five (18%) and pregnant women (22%). The case fatality rate due to malaria in children in hospitals is about 7.4% and among children admitted to health facilities, 19.6%. It accounts for 31.5% of all out patients seen in health facilities and 28.4% of all patients admitted to the health facilities. It is the first cause of deaths in adults and the third commonest cause of deaths in children under five (National Malaria Control Programme, 1998).

Comparing the past five years, 1998 was the year when a high malaria morbidity and mortality was recorded in the country (Fig.1.1). This was mainly due to the heavy rainfall in this year. According to meteorological reports, it was the highest rainfall recorded in the last two decades.

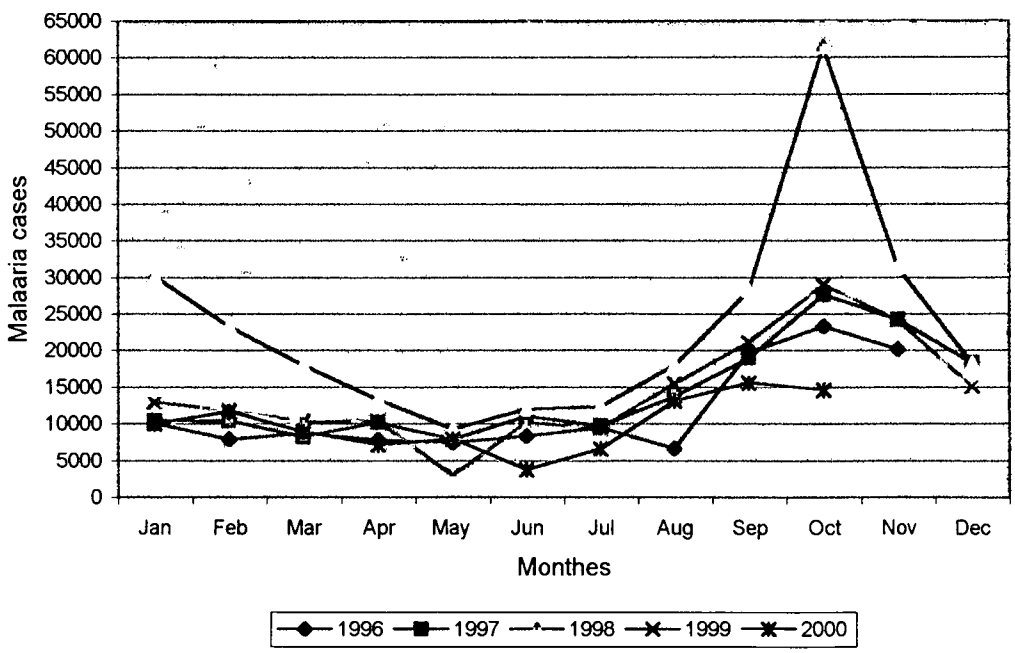


Fig.1.1: Malaria cases reported in health facilities by month (January 1996 to October 2000)

Epidemiologically, the country is divided into four strata: the highlands with elevation about 2,000 meters above sea level, the southern and northern coastal lowlands (0-1,000 metres above sea level) and the western lowlands (700-1,500 metres above sea level). A map showing the distribution of stable malaria in Eritrea is given below (Craig, *et al.*, 1999). The high land includes the central, areas found around the capital city, Asmara, and some areas extending towards the south and the northern highlands. Most parts of the highlands are malaria free except in some area, around Adi Ugria, where transmission is occasionally recorded and are becoming prone to epidemic. The southern and northern coastal lowlands extend from the north to the south along the red sea coast. In those lowlands, transmission of malaria is strongly seasonal. The south western and north western lowlands are included with the western lowlands. In most parts of those lowlands, malaria transmission extends from three to six months, although in some parts, especially in those where there are dams and irrigation projects, transmission is perennial.

There are two transmission seasons in Eritrea: September to December for the southern and western lowlands and some parts of the highlands, and January to March for the northern and southern coastal lowlands (National Malaria Control Programme, 1998).

Plasmodium falciparum is the most common malaria parasite found in the country. As reports from health facilities indicate, it accounts for more than 94.4% of the total malaria cases recorded. *Plasmodium vivax* is the second most

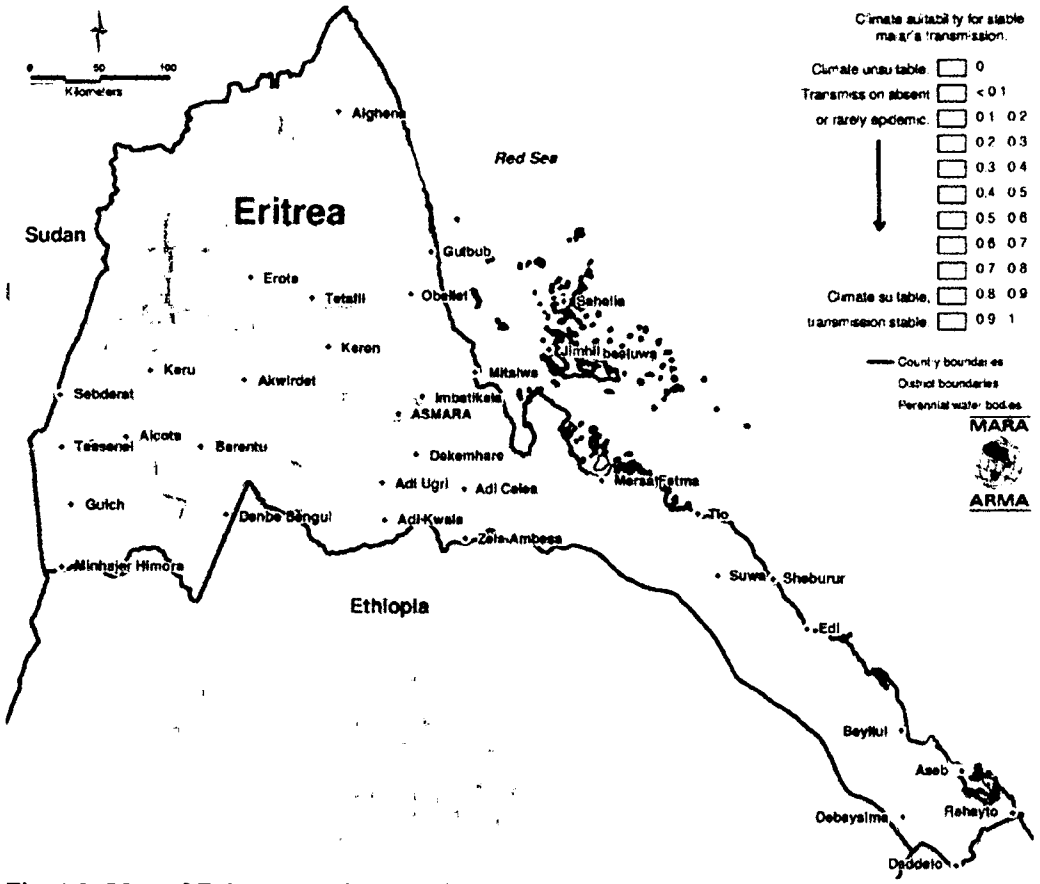


Fig. 1.2: Map of Eritrea showing the distribution of stable malaria transmission

abundant species found in the country, with *P. malariae* also occasionally found (National Malaria Control Programme, 1998).

1.3: East African malaria vectors

This geographical region consists of Eritrea, Ethiopia, Sudan, Djibouti, Somalia, Kenya, Tanzania and Uganda. The main malaria vectors are *An. gambiae* and *An. arabiensis* of the *An. gambiae* complex and *An. funestus*. *Anopheles gambiae* and *An. arabiensis* coexist in the southern part of this region (Kenya, Uganda and Tanzania) but *An. gambiae* does not extend its distribution to the north (Zahar, 1985) with *An. arabiensis* being the main vector in the rest of the region. A recent

study has revealed the presence of *An. gambiae* in addition *An. arabiensis* in Juba and Wau, Southern Sudan (Petrarca *et al.*, 2000).

Anopheles quadriannulatus Theobald has been recorded in East Africa only from Ethiopia and Zanzibar/Pemba islands. In Ethiopia, this species, although willingly biting man, has been regarded as zoophilic. While it mainly rests outdoors, occasionally it also rests indoors in animal shelters and mixed dwellings. It was found with an extremely low human blood index and negligible sporozoite rate (White, 1974). Because of all these characteristics, the role of *An. quadriannulatus* in malaria transmission in Ethiopia was discounted (Zahar 1985). Recently, Hunt *et al.* (1998) carried out a study in Ethiopia with the aim of defining the relationship that exists between the South African and Ethiopian populations of *An. quadriannulatus*. Although *An. quadriannulatus* from Ethiopia is similar to *An. quadriannulatus* from South Africa, having homosequential chromosomal banding patterns and identical PCR products, cross-mating studies between these two populations showed: (i) resultant males were sterile, (ii) there was marked sex ratio distortion, and (iii) there was extensive asynapsis of the ovarian polytene chromosomes. As a result, they concluded that the Ethiopian population is a different species from that of South Africa and designated it as *An. quadriannulatus* species B, *An. quadriannulatus* species A being that found in South Africa.

Anopheles funestus is primarily a Savannah species and it extends to areas of high altitude: 1,800 meters in Kenya and Uganda and 2,150 meters in Ethiopia. It is

also found in most parts of the Sudan, attaining its highest density in the southern and southwestern regions, where it is a major malaria vector (Gillies & De Meillon, 1968; Zahar, 1985; Gillies & Coetzee, 1987).

1.4: *Anopheles* fauna in the State of Eritrea

Beginning with the Italian and British colonization of the country, some studies were carried out to determine the *Anopheles* fauna of Eritrea. *Anopheles dancalicus* Corradetti was reported from the upper reaches of the Danakil depression. Larvae breed in small saline puddles encrusted with salt in a closed basin some 200 kilometres from the sea at an altitude of about 250 meters. Although damaged, the specimen collected from Arafaile, housed in the British Museum, appears to be *An. salbaii* Maffi and Coluzzi and uncertainty exists on its identification (Gillies & De Meillon, 1968).

De Burka & Shah (1943) reported collecting a single larva of *Anopheles erythraeus* Corradetti in Ghinda but failed to find further specimens. Although these authors considered that *An. erythraeus* was a variant of *An. dthali* Patton, Gillies and De Meillon (1968) examined the specimen (loaned by Professor Corradetti) and considered that it represented the larva of a distinct species.

Melville *et al.* (1945) found *An. dthali* along the Red Sea Coast in Ghinda, the only species found at a place where malaria was endemic and they strongly

suspected it to be a vector. *Anopheles culicifacies adenensis* Christophers was also recorded from Assab port (O' Connor, 1967; Gillies & De Meillon, 1968).

Mara (1950) studied the conditions that favour the breeding of *An. gambiae s.l.* created by the vast cotton irrigation system in Tesseney, western low lands of the country, and the possibility of epidemics developing among agricultural labourers coming from other areas. During his study, he has recorded several anopheline species (Zahar, 1985).

Verrone (1962) in his key to *Anopheles* species in Ethiopia and O'Connor (1967) in his study of the distribution of the anopheline mosquitoes in Ethiopia have recorded about 20 *Anopheles* species in what was then known as the Northern Region (now Eritrea). All these authors considered *An. gambiae s.l.* as the main malaria vector species because it was responsible for malaria epidemics and was adaptable to a variety of ecological conditions. *Anopheles funestus* and *An. pharoensis* Theobald were considered to be secondary vectors (Zahar, 1985).

In 1998, a preliminary study was carried out in the malarious areas of Eritrea to determine the distribution of the malaria vectors. *Anopheles gambiae s.l.* was found to be the dominant species and main vector comprising 99.7 % of the total mosquitoes collected (Seulu F., unpublished data). Lyimo (1998), in her draft report of entomological support to the national malaria control program in Eritrea, reported that *An. gambiae s.l.* as the main vector with *An. funestus* and *An. pharoensis* as secondary vectors. *Anopheles culicifacies adenensis,* another

possible secondary vector, has been recorded only from the Red Sea coast of Assab port and its surrounding (O'Connor, 1967).

1.5: The *Anopheles gambiae* complex

Early entomological studies based on the classic morphological approach lead to the identification of two main components in the vector system, *An. funestus* and *An. gambiae*. Although this classification was useful in elucidating the fundamental patterns of malaria transmission in Africa, the relatively simple *funestus-gambiae* model soon showed its limitations. Epidemiologically significant bionomical heterogeneities were revealed in each of the two taxa, suggesting higher levels of complexity in the system (White, 1974; Coluzzi, 1984).

Earlier studies indicated that populations of *An. gambiae* seemed to vary in their breeding places, ranging from temporary fresh water to marshes and saline water (Evans, 1938). Adult females were also observed to differ in their feeding preference and their resting behaviour (De Meillon, 1947). Ribbands (1944a,b) presented detailed morphological and physiological differences between the salt water and fresh water populations and he concluded that the two populations were distinct species with *An. gambiae* breeding in fresh water and *An. melas* Theobald breeding in saline water. Muirhead-Thomson (1948) carried out crosses between these two populations and detected sterility in the F_1 males. He also noted the existence of morphological differences between the eggs of *An. melas* and *An.*

gambiae. From this evidence, he concluded that *An. melas* is a distinct species, thus supporting Evans' (1938). Bruce-Chwatt (1950) made crossing experiments between the salt water and fresh water populations and reported that his work contradicted Muirhead-Thomson's, because, although the eggs did not hatch, his F_1 females oviposited. As a result of this, Muirhead-Thomson's findings were ignored until his work was confirmed by the elucidation of the *An. gambiae* complex in the early 1960s.

Paterson (1962) did crossings between the East African salt water and fresh water breeders and concluded that they were separate species. Davidson and Jackson (1962) made crossings between 15 different fresh water strains and concluded that two groups (forms A and B) existed which, when mated within group, produce fertile male offspring and, between groups, sterile males. From this evidence, Paterson (1964) argued that there were four distinct species within *An. gambiae* and later added another fresh water form, form C, from South Africa (Paterson *et al.*, 1963). Davidson & Hunt (1973) presented evidence for a new sixth species, species D, from hot mineral springs in Bwamba, Uganda. Recently, a new species, *An. quadriannulatus* B, was added from Ethiopia (Hunt *et al.*, 1998).

It is now clear that at least seven morphologically indistinguishable yet genetically and behaviourally distinct species are combined under the name *Anopheles gambiae* (Hunt *et al.*, 1998). Today we recognise four fresh water breeders (*An. gambiae, An. quadriannulatus* A and B and *An. arabiensis*), two

salt-water breeders (*An. merus* from East Africa and *An. melas* from West Africa) and one mineral water breeder (*An. bwambae* White).

1.5.1: Biology of the *An. gambiae* complex

Anopheles gambiae and *An. arabiensis* are the two most anthropophilic members of the complex. Although differences exist in their behaviour, seasonal prevalence and level of vectorial efficiency, both species are of primary medical importance in Africa (White, 1974; Coluzzi, 1984; Gillies & Coetzee, 1987). In West Africa, the situation is even more complicated with at least two "molecular forms" and five "chromosomal forms" being recognized within *An. gambiae*. Coluzzi *et al.* (1985) and Toure *et al.* (1998) described five distinct groups based on chromosomal inversion differences and referred to them as "incipient" species because of overlapping variation. Recently, two molecular forms, M (Mopti) and S (Savannah), have been described which sometimes, but not always coincide with particular chromosomal inversion (della Torre *et al.*, 2001).

Anopheles gambiae is predominantly endophilic (preferring to rest indoors) and anthropophilic (preferring to feed on human) with the female spending most of its gonotrophic cycle resting in houses. *Anopheles arabiensis* shows partial or complete endophilic behaviour with zoophilic and anthropophilic feeding habits (White, 1974). Under natural circumstances, where the majority of the hosts are domestic and indoors at night, most of the *An. arabiensis* feed indoors and rest there for one to two days. Marked zoophily has been documented in *An.*

quadriannulatus in South Africa and Zanzibar, whereas at high altitudes in Ethiopia female *An. quadriannulatus* (species B) tend to be endophilic in stable and mixed dwellings. It is considered to be of little medical importance (White, 1974).

There are no major differences in the larval habitat between the fresh water species (Gillies & Coetzee, 1987). Larvae breed in open sunlit pools which range from borrow-pits, drains, brick-pits, car-tracks and hoof-prints around ponds and water-holes, to those resulting from overflow of rivers, pools left by receding rivers, backwaters and rainwater collected in natural depressions (Gillies & De Meillon, 1968; Gillies & Coetzee, 1987).

Adult females of *An. melas* and *An. merus* bite humans particularly in the absence of an alternative host and their vectorial efficiency for the transmission of malaria is considered to be lower than that of *An. gambiae* and *An. arabiensis* (White, 1974). *Anopheles melas* breeds in patches of salt grass in tidal swamps and in pools, ponds, lagoons flooded by spring tides and mangrove swamps. *Anopheles merus* breeds in brackish lagoons, ponds, swamps, pools and puddles that are flooded at spring tides and subsequently diluted by rainfall or seepage from the land and saline thermal springs inland (Gillies & De Meillon, 1968; Coetzee *et al.*, 1993).

Anopheles bwambae females are markedly anthropophilic and display strong endophilic and endophagic tendencies. Despite its low sporozoite rate, its

abundance around Buranga hot springs makes it the principal vector in this part of Uganda. Breading is confined to mineral water swamps, vegetated principally with *Cyperus loevigatus* formed by geothermal activity in the Rift Valley (White, 1985). Larvae prefer sunlit pools, especially animal footprints among the marsh sedges and are not found in fresh water streams and pools nearby (White, 1973).

1.5.2: Distribution of the *An. gambiae* complex

Anopheles gambiae and *An. arabiensis* have the widest distribution and can occur together over extensive areas of Africa. *Anopheles gambiae* predominates in zones of forest and humid situations whereas *An. arabiensis* is more successful in arid Savanna and steppes. Accordingly, *An. gambiae* is unknown from the horn of Africa and Southern Arabia where its spread appears to have been blocked by belts of Savanna and steppes across northern Kenya and Sudan. On the other hand, *An. arabiensis* is absent from many of the humid areas in the rain forest belts of West Africa, the Congo basin and parts of East Africa. West African populations of *An. gambiae* penetrate arid Shale Sahelian habitats in Mauritania and Mali to an extent more typical of *An. arabiensis*. Mixed populations of *An. gambiae* and *An. arabiensis* are present in Madagascar and Mauritius but only *An. arabiensis*, frequently with *An. merus,* is found in other Indian Ocean islands. *Anopheles gambiae* with *An. melas* occurs in Fernandopo (Gillies & De Meillon, 1968; White, 1974; Coluzzi *et al.*, 1979; Gillies & Coetzee, 1987).

Anopheles quadriannulatus appears to have a very disjunctive distribution having been recorded from Ethiopia, Pemba/Zanzibar islands, Zimbabwe, Mozambique, Swaziland and South Africa (White, 1974; Coluzzi *et al.,* 1979; Gillies & Coetzee, 1987). Both *An. quadriannulatus* species A and B occur sympatrically with *An. arabiensis*, species A in Southern Africa and species B in Ethiopia. Little is known about the Pemba/Zanzibar population of *An. quadriannulatus* and its presence in this area needs to be confirmed (Hunt *et al.,* 1998). *Anopheles melas and An. merus* occur on the West and East African coasts respectively, with *An. melas* being confined to coastal areas (Gillies & De Meillon, 1968; White, 1974). *Anopheles merus* is not confined to the coast and can be found far inland at distances of over 120 kilometres in South Africa (Paterson *et. al.,* 1964; Coetzee *et al.,* 1993) and 50 kilometres in Tanzania (White, 1974). *Anopheles bwambae* is found only in the hot mineral water springs of the Semliki forest, Uganda (White, 1973).

1.5.3: Identification of the *An. gambiae* complex

1.5.3.1: Morphological identification

Despite the intensive studies carried out (Ribbands 1944a,b, Muirhead-Thomson, 1945, 1951; Coluzzi, 1964; Green, 1971; White & Muniss, 1972; Coetzee, 1986, 1989; le Sueur & Sharp, 1991; Lounibos *et al.,* 1999), no reliable and consistent morphological differences have been found between the members of the *An. gambiae* complex. Although variation occurs in the means and ranges of certain characters in every stage of the life cycle of the mosquito, affording little scope

for identification purposes, especially under field conditions, some of the morphological studies carried out on each of the life stages of these mosquitoes will be discussed briefly.

Eggs: As early as 1945, Muirhead -Thomson (1945) was able to distinguish eggs of *An. melas* from those of *An. gambiae* by measurement of the deck width. In East Africa, Kuhlow (1962) reported that the eggs of *An. merus* were slightly larger than those of the three fresh water species. These findings were later verified and supported by Paterson (1963), Coluzzi (1964) and Lounibos *et al.* (1999). However, no difference between eggs of the fresh water species exists as yet.

Larvae: Ribbands (1944b) was able to distinguish *An. melas* from the West African fresh water species based on larval pectin. Green (1971) used the number of branches on the inner shoulder hair to distinguish *An. gambiae* and *An. arabiensis* in areas where *An quadriannulatus* was absent.

Pupae: Various setal count and the shape of the male genital lobe give partial separation in *An. gambiae* and *An. arabiensis* (Reid 1975a,b). Although Coluzzi (1964) found pupal setae of some use in his investigation, Coetzee (1989) found it of little use for these characters in distinguishing between members of the *An. gambiae* complex in South Africa. Therefore, there is no distinct character that can be used for distinguishing the pupae of the *An. gambiae* complex.

Adults: The palpal ratio, length of the 4th and 5th segments to the 3rd segment of the female palp, was the most reliable character used to distinguish species of *An. melas* and *An. merus* from the fresh water *An. gambiae* and *An. arabiensis* (Coluzzi 1964). Bryan (1980) found that palpal ratio could be utilized to identify 96.20% of *An. melas* and 91.95% of *An. gambiae* when they occur together in the absence of the other members of the complex. Coluzzi (1964) used the antennal sensillae to separate *An. gambiae* from *An. merus*. Hind leg banding patterns were used by Coetzee *et al.* (1982) to separate field-collected specimens of Southern African populations of *An. arabiensis* and *An. gambiae* from *An. quadriannulatus* and *An. merus*. Sharp *et al.* (1989), however, found overlap in this character between *An. arabiensis* and *An. quadriannulatus* in kwazulu/Natal, South Africa.

1.5.3.2: Salinity tolerance tests

Ribbands (1944b) was able to distinguish the fresh water *An. gambiae* from the salt-water form *An. melas* by placing individual egg batches in distilled water. After hatching out, the first instar larvae were transferred into a solution that contained 75% seawater (23.5gm NaCl/lit). Larvae that survived for two hours were considered as *An. melas*. Muirhead-Thomson (1951) then went on to differentiate the fresh water *An. gambiae* and *An. merus* from East Africa. This test required live first instar larvae and only those larvae that prefer a saline habitat (*An. melas* or *An. merus)* survived.

1.5.3.3: Cross-mating experiments

Muirhead-Thomsom (1951) was the first to demonstrate reproductive incompatibility in crosses between *An. gambiae* and *An. melas* by the production of sterile male hybrids. Davidson & Jackson (1962) identified two groups, group A and B, in crosses they made between the West African fresh-water populations. Sterile males were produced in the cross between *An. gambiae* and *An. merus* from East Africa (Paterson 1962). Paterson *et al.* (1963) crossed species A and B with species C. Similarly, crossing experiments were made by Davidson & White (1972) and Davidson & Hunt (1973) to determine the status of *An. bwambae* from East Africa. Recently, Hunt *et al.* (1998) have done crosses between South African and Ethiopian *An. quadriannulatus* populations and named the Ethiopian population as *An. quadriannulatus* species B.

Originally crosses were made between biologically different populations and between fresh-water and salt-water breeders. Subsequently this method was used to cross unknown specimens with reference strains. If the offspring are from intra-specific crosses, fertile hybrids are produced and if they are from inter-specific crosses, F_1 males are always sterile. Hybrids can be confirmed by the presence of atrophied and non-functional male testes and asynapsis of polytene chromosomes from the salivary glands of fourth instar larvae and female ovarian nurse cells. The technique is time consuming and laborious and requires well-established colony material.

1.5.3.4: Isoenzyme electrophoresis

This technique is used to separate isoenzymes by electric field histochemical staining and depends on the relative mobility of diagnostic allozymes in the study population. It was first applied by Mahon *et al.* (1976) who were able to separate *An. gambiae, An. arabiensis, An. quadriannulatus* and *An. merus* based on the distribution of allele frequencies of three isoenzyme loci. Miles (1978) separated the six members of the complex using species-specific isoenzyme patterns. Since the gene frequency of diagnostic allozymes may vary geographically, it has been recommended that results should be checked either chromosomally or by their crossing characteristics with known members of the group (Hunt & Coetzee, 1986).

The advantages of isoenzyme electrophoresis are: (i) it can be carried out on crude extracts, (ii) large samples can be processed in a relatively short time, and (iii) it is simple to perform and interpret. Its disadvantages are that specimens need to be kept alive or stored in liquid-nitrogen and sophisticated and expensive laboratory equipment is required. Moreover, there is an overlap in the diagnostic enzymes.

1.5.3.5: Cytogenetic analysis

This technique for identifying the sibling species in the *Anopheles gambiae* complex uses giant polytene chromosomes with distinct banding patterns found in the salivary glands of fourth instar larvae and in the ovarian nurse cells of half-

gravid females. These banding patterns are species-specific due to fixed paracentric chromosomal inversions. The diploid number of *Anopheles* mosquitoes is 2n=6, with two autosomal pairs and one pair of sex chromosomes (Coluzzi & Sabatini, 1967, 1968a,b, 1969; Green, 1972; Hunt, 1973).

The distinct pattern of the X chromosome of *An. arabiensis* separates it from the other members of the complex. *Anopheles gambiae* and *An. merus* share the same X chromosome banding pattern but can be separated by fixed inversions on arm 2R of the autosomes. *Anopheles bwambae, An. melas* and *An. quadriannulatus* also have identical X chromosomes but can be identified by various fixed differences in their autosomal chromosomes.

Some of the disadvantages of this technique are that only half-gravid females and fourth instar larvae can be used and a high level of expertise is required to interpret the banding patterns. However, it has the advantage of being cheap and accurate and samples can be stored in carnoy's fixative for later identification.

1.5.3.6: The polymerase chain reaction (PCR)

Most of the above-described methods, including the cytogenetic analysis, have some limitations that preclude the extensive use required for epidemiological studies of transmission or in support of vector control programmes. Advances made in the field of molecular biology resulted in the development of the polymerase chain reaction assay which amplifies species-specific DNA fragments

using a thermostable DNA polymerase and primers derived from sequences flanking the target fragment. It is now possible to use this technique for the identification of large numbers of insect vectors of disease, such as the *An. gambiae* complex.

The ribosomal RNA genes (rDNA) were selected as the basis of this diagnostic method for three reasons. First, they are present in hundreds of tandem copies per cell nucleus in most multicellular organisms, more than 500 copies per diploid genome for *An. gambiae.* So a very small amount of nuclear DNA obtained from a small part of a single individual provides sufficient template for PCR amplification. Second, these genes, which are highly conserved among multicellular eukaryotes, are known to contain spacer regions with evolutionarily labile sequences that might be expected to differ between very closely related species. Third, genes such as those for rDNA are molecularly homogenized in ways that single copy genes are not. Thus, intraspecific variation in a rDNA sequence is potentially less of a complicating problem than it would be for a single copy locus (Scott *et al.*, 1993).

This technique was first applied by Paskewitz & Collins (1990) for identifying mosquitoes. They produced three primers derived from rDNA sequences that separated *An. arabiensis* and *An. gambiae.* This method utilizes a universal plus-strand derived from the conserved region at the 3^1 end of the 28S rDNA coding region and two species-specific minus stranded primers derived from the intergenic spacer. The universal primer reacts differently with the species specific

primers to produce a 1.3kb DNA fragment when *An. gambiae* is used as a template and 0.5kb DNA fragment when *An. arabiensis* DNA is used. Products can then easily be separated on an agarose gel.

Scott *et al.* (1993) extended this work and published a protocol using oligonucleotide primers to identify five members of the complex. The primers consist of one universal primer that is complimentary to all five species and four species-specific primers for *An. gambiae, An. arabiensis, An. quadriannulatus* and *An. merus/An. melas* combination. Townson & Onapa (1994) produced a rDNA-PCR for *An. bwambae.*

The advantages of this method are that it can be applied to any life stage or sex of the mosquitoes, a very small portion of the mosquito DNA can be amplified leaving the rest for additional analysis, dried or alcohol preserved specimens can be used, and it is fairly simple and easy to interpret. Use of expensive laboratory equipment and chemicals, use of ethidium bromide (a mutagen) and the need to maintain the sterility of the reagents are some shortcomings of this technique.

1.6. The *Anopheles funestus* group

Anopheles funestus is one of the three major malaria vectors in Africa, together with *An. gambiae* and *An. arabiensis.* However, in some areas, it is more important than the other vectors. The existence of distinct "varieties" of *An. funestus* was first established by Evans & Leeson (1935, 1937). They

demonstrated by larval morphology that *An. funestus* is a group of closely related species. Except for *An. funestus*, which is anthropophilic, the other members of the group appear to be zoophilic although they also readily bite human in the absence of other hosts (Gillies & De Meillon, 1968). Only *An. funestus* is considered to be a vector. Although *An. vaneedeni* Gillies and Coetzee was not found naturally infected, laboratory tests showed that it is fully susceptible to *P. falciparum* (De Meillon *et al.*, 1977; Gillies & Coetzee, 1987). Moreover, Wilkes *et al.* (1996) recently showed by salivary gland dissection that *An. rivulorum* Leeson from Tanzania was infected with *P. falciparum.*

1.6.1: Biology and Distribution of the *An. funestus* group

Anopheles funestus is one of the most anthropophilic mosquitoes known, in many areas attacking man, even in the presence of abundant alternative hosts such as sheep and cattle (Gillies & De Meillon, 1968). It feeds both indoors and outdoors, and after feeding rests mainly indoors. It is widespread in distribution and abundant over the whole sub-Saharan region, wherever there is sufficient permanent water and no intensive use of residual insecticides. The northern-most records are from the Niger River and in the south it extends as far as the northern part of Namibia and Kwazulu Natal, South Africa (Gillies & De Meillon, 1968). The normal breeding places of *An. funestus* are those of more or less permanent nature, especially with vegetation, such as swamps, edges of lakes and ponds, pools in river banks and small streams as well as rice fields (Gillies & De Meillon, 1968).

Anopheles rivulorum is mainly found in western and eastern Africa. Larvae are found in gently flowing water or in vegetation along the side of rivers, occasionally along margins of large expanses of open water. It is an exophilic and zoophilic mosquito, only occasionally found in houses (Gillies & De Meillon, 1968).

Anopheles vaneedeni is essentially an outdoor biting species, frequently caught biting man outside houses in the early hours of the night (De Meillon *et al.*, 1977; Smith *et al.*, 1977). This species has been recorded only from South Africa. The larval habitat of *An. vaneedeni* is not apparently different from that of *An. funestus* (Gillies & Coetzee, 1987).

Anopheles confusus Evans and Leeson is confined to the plateau area of eastern Africa from Kenya and Ethiopia to South Africa. Although occasionally found indoors, very little is known about the adult biology and it is presumed to be zoophilic and exophilic. Larvae are usually found in slowly flowing water (Gillies & De Meillon, 1968).

Anopheles leesoni Evans is a widespread species but localized in the savannah region of eastern and western Africa. Although occasionally collected in houses (Evans 1931), it is usually collected in natural outdoor resting sites and is presumed to be zoophillic (De Meillon, 1933, 1936; Leeson, 1937). Larvae are mostly found at the edges of slowly flowing streams (Gillies & De Meillon, 1968).

Anopheles brucei Service is known from Nigeria. Nothing is known about the egg or adult biology. Larvae have been found in shady forest streams and partially dried river- beds (Gillies & De Meillon, 1968).

Anopheles parensis Gillies is found in eastern Africa, mainly in the lowlands, known at present from the Kenya coast, northeast Tanzania, Pemba Island, Swaziland and Kwazulu Natal, South Africa. It was first recognized in Tanzania after residual house spraying had led to the elimination of *An. funestus*. It has an exophilic resting habit, although it has been found indoors in South Africa in certain formerly sprayed houses. Larvae are found in permanent swamps and ponds among reeds and emergent vegetation. It appears scarce or absent in streams and moving water (Gillies & De Meillon, 1968).

Anopheles aruni Sobti is known at present from the type locality of Zanzibar. Little is known about the adult biology beyond the fact that the females attack man outside at night and adults of both sexes can be caught resting by day in shaded banks and tree bases (Gillies & De Meillon, 1968).

Anopheles fuscivenosus Leeson is known from Zimbabwe. Nothing is known beyond the fact that specimens have been collected in outdoor resting sites (Gillies & De Meillon, 1968).

1.6.2: Identification of the *An. funestus* group

1.6.2.1: Morphological identification

Adult stage

Anopheles aruni can be distinguished from *An. funestus* by having more broadly banded palps and paler wings (Gillies & De Meillon, 1968). Gillies & Coetzee (1987) were able to distinguish female *An. aruni* from all the other members of this group, except *An. vaneedeni,* by plotting the wing-spot ratio against palpal band ratio. They were also able to separate all males of *An. aruni* from the other members of the *funestus* group by the presence of a fairly broad patch of pale scales at the base of the palpal club.

Anopheles brucei resembles *An. rivulorum,* but the female can be separated from it by distinguishing features on the palps, pharynx, mesonotum and wings and palps of the male (Gillies & De Meillon, 1968).

Although *An. confusus* is indistinguishable from *An. funestus* on external characters, the post pharyngeal ridges of the female which are about equal in length to the width of the ridge, the presence of a pale patch of scales at the base of the club of the males in about half of the specimens can be used to separate it from *An. funestus*. Moreover, the length of the external accessory seta on the genitalia, in East Africa, can be used to distinguish it from *An. funestus,* but these resemble *An. leesoni* (Gillies & De Meillon, 1968).

Anopheles fuscivenosus resembles *An. funestus.* However, it can be distinguished from all the other members of the group in having very dark wings, absence of a costal sector pale band, but not always, and pre-accessory dark spots on the first vein broader than the accessory sector pale spots (Gillies & De Meillon, 1968).

Anopheles leesoni differs from all the other members of the group, except *An. brucei*, by the presence of a small patch of pale scales at the apex of the sixth vein in 2/3-3/4 of specimens and a pale fringe spot present opposite the sixth vein in about ¼ of the specimens. Specimens without these two characters, about 25-30% of the total, are inseparable from *An. funestus* (Gillies & De Meillon, 1968).

Anopheles vaneedeni resembles *An. aruni,* but the darker wings and breadth of the pale bands on the palps can be used to separate it from *An. aruni* (Gillies & De Meillon, 1968). Although De Meillon *et al.* (1977) were able to distinguish *An. vaneedeni* from *An. funestus* in the Transvaal, South Africa, by plotting the wing-spot ratios against the palpal ratios, Gillies & Coetzee (1987) recorded that the degree of overlap in populations from other areas of Africa were quite considerable. De Meillon *et al.* (1977) were also able to distinguish *An. vaneedeni* from *An. funestus* using pre-sector pale spots on the costa, a pale spot in the middle of vein 3 and a pale spot on the tarsal joints.

Anopheles rivulorum is quite distinct from the other members of the group in having an orange-brown scutum that is characteristic of this species (Gillies & De

Meillon, 1968). However, it is not always present and it can be confused with the other members of the group.

Pupal stage

The pupae of *An. aruni*, *An. vaneedeni* and *An. parensis* are inseparable from that of *An. funestus*. The pupa of *An. fuscivenosus* is unknown. Pupae of *An. confusus* can be distinguished from that of *An. funestus* in having seta one with fewer branches. *Anopheles leesoni* differs from all the other members of the group on hair 9 in segment VII, seta 1 in segment III-VII and with the accessory paddle seta with 3-4 branches. *Anopheles rivulorum* differs from all the other members of the group except *An. brucei* in that the paddle fringe does not extend along the posterior border beyond the apical seta (Gillies & De Meillon, 1968).

Larval stages

Anopheles confusus can be distinguished from the *An. funestus* subgroup by having shallower abdominal plates. *Anopheles leesoni* differs from other African members of the *funestus* group in the presence of a pair of small metathoracic plates. The ventral surface of its abdomen is also without belts of spicules, thus differing from *An. confusus* and the *funestus* subgroups. *Anopheles brucei* resembles *An. rivulorum* in the larval stage, but it can be separated on the length of the clypeal hairs and by the accessory plates. *Anopheles vaneedeni, An. parensis* and *An. aruni* are indistinguishable from *An. funestus*. The larva of *An. fuscivenosus* is unknown (Gillies & De Meillon, 1968).

Eggs

Anopheles leesoni eggs are the only ones that are distinguishable from the other members of the group. *Anopheles confusus* and *An. rivulorum* differ from *An. funestus* in the smaller size of the bosses on the exochorion, but it is not an easily appreciated character. Eggs of *An. parensis* and *An. vaneedeni* are like *An. funestus*. The eggs of *An. brucei, An. aruni* and *An. fuscivenosus* are unknown (Gillies & De Meillon, 1968, Gillies & Coetzee, 1987).

1.6.2.2: Cytogenetic analysis

The polytene chromosomes from ovarian nurse cells of half-gravid females of *An. funestus* were used as a standard and compared with those of *An. parensis* and *An. vaneedeni* (Green & Hunt, 1980). This method was used by Green (1982) to identify *An. funestus/vaneedeni, An. parensis, An. rivulorum, An. leesoni, An. fuscivenosus* and *An. confusus. Anopheles vaneedeni* is homosequential with the *An. funestus* arrangement, differing only in the presence of a polymorphic inversion on arm 2 (Green & Hunt, 1980).

1.6.2.3: Single strand confirmation polymorphism (SSCP)

The single strand confirmation polymorphism (SSCP) analysis is based on the principle that electrophoretic mobility of a single-strand DNA molecule in a non-denaturing gel depends upon both its size and shape (Hiss *et al.*, 1994). Koekemoer *et al.* (1999) used this technique for identifying four members of the *An. funestus* group: *An. funestus, An. vaneedeni, An. rivulorum,* and *An. leesoni.*

Its advantages are that (i) it does not require construction of species-specific primers, (ii) it is rapid, and (iii) it is simple to perform and interpret results with straightforward staining methods. Its disadvantages are that equipment used in vertical polyacrylamide gel electrophoresis (PAGE) are more expensive, a long time is spent in performing the electrophoresis and the silver staining method is laborious.

1.7: Objectives of the study

Given the very complex nature of the malaria vectors, it is advisable that before any vector control programme is planned or implemented, there should be sufficient information on the heterogeneity that exists between and within species, vector behaviour, dynamics of transmission and resistance to insecticides.

This study is therefore intended to assess the biology and transmission potential of malaria vector mosquitoes, with reference to the malaria transmission season, in Elabered subzone, Eritrea. No previous entomological studies have been carried out in this area and the information will be of value to the malaria control programme.

CHAPTER TWO

MATERIALS AND METHODS

2.1: Study area

This study was carried out in Elabered, one of the eleven administrative sub-zones of Anseba zone, Eritrea. There is no an available electronic map that shows the study village in particular. However, a map of Eritrea showing the administrative subzones of Eritrea with reference to the study sub-zone (Elabered) is given in Fig. 2.1. The study village is situated approximately 64 kms, 38°17' E and 15°42'N, north west of the capital city, Asmara. Altitudes range from 1400-1450 meters above sea level. It has a hilly and rugged topography. As the result of this, most of the villages are found at the base of the hills. About 24,000 people (67.98%) of the total population (34,759) are exposed to malaria and out of 69 villages 51 of them are malarious with most of the malaria free villages being found on the upper part of the mountains (Anseba Zone Malaria Control Programme, 1998).

Most of the people belong to three tribes: Belien, Tigrigna, and Tighre. They are either Christians or Muslim with the majority being Christians. About 97% of the villagers are farmers living on subsistence farming and grow sorghum, maize and pear millet. Some of them grow horticultural plants such as onions, potatoes, tomatoes, carrots, oranges, mandarins, papaya and lemons (Anseba Zone Malaria Control Programme, 1998). The commonest domestic animals found in the village

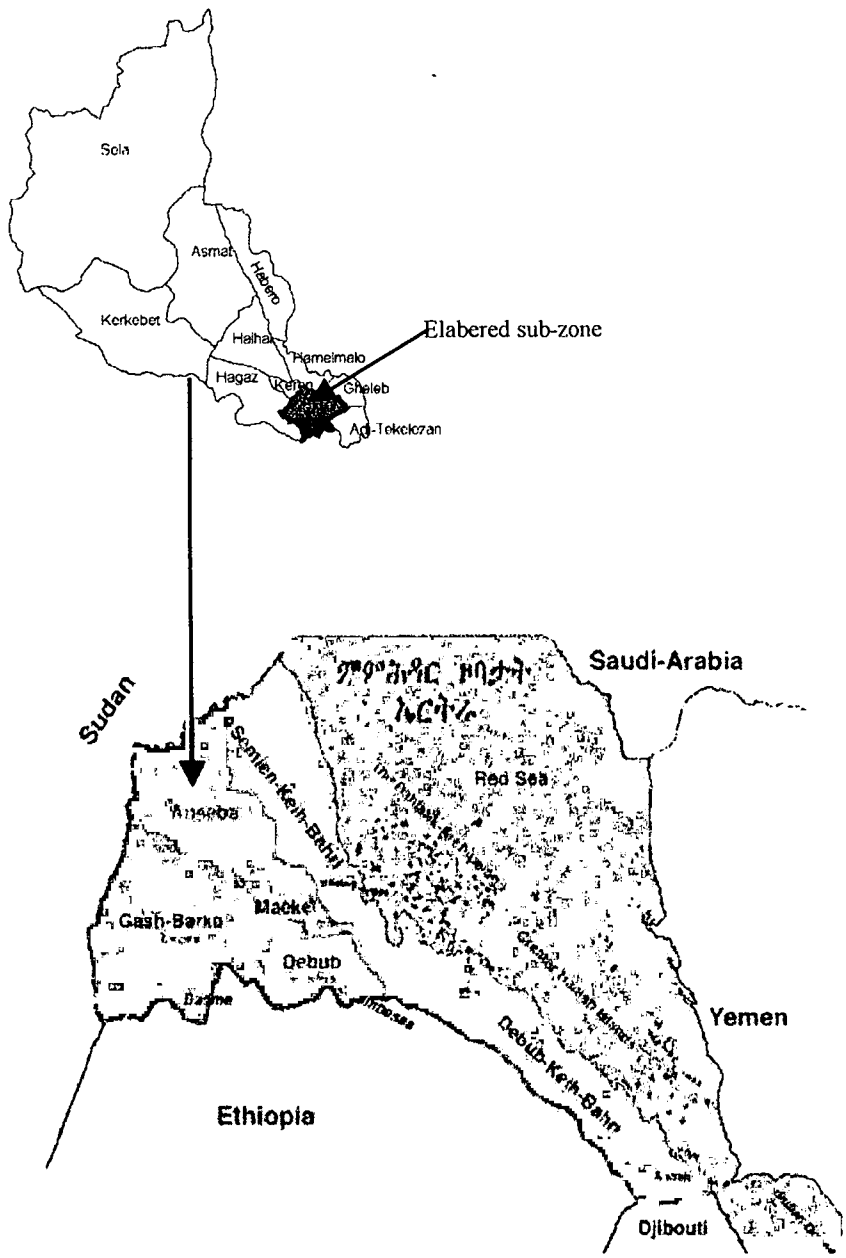


Fig. 2.1: Administrative zones of Eritrean with reference to the study subzone

perimeter are cattle, sheep, goats and chickens. Some also keep donkeys, cats and dogs.

Most of the dwellings, except those of Eden, which has European-style houses

with main electricity and flush toilets, are rectangular or circular and have mud walls with corrugated or thatched roofs. Some of the villagers, especially those from the Tigrigna tribe, own additional rectangular and cemented houses. In some cases the walls of the rectangular dwellings are stalked with millet and plastered with mud on the inside. Most of the rooms are furnished at most with simple bedsteads. On average, there are two bed nets per household and they are in good condition. However, most of the bed-nets are not re-treated with insecticides.

One of the key features of the Elabered sub-zone is the presence of a farming estate that was established in 1958 by an Italian entrepreneur named De Nadai. It is a complex and integrated farming unit with fruits, crops, livestock and diary products as its main products. The estate covers about 1,200 hectares. Within this estate, there are seven dams and twelve ground water wells. Moreover, along the Balwa stream and Anseba River, there are numerous hand dug water wells owned by the villagers, used for irrigation. The area is prone to malaria which is the major health problem.

The Elabered sub-zone has a moderate temperature and the climate is characterized by a cool-dry season (December to February), followed by a hot-dry season (March to May) and a warm-humid season (June to November). The mean annual temperature and mean annual relative humidity are 23 °C and 64%, respectively (Data from Elabered farming estate). Rainfall extends from June to August (Fig. 2.2) and varies annually: 562.4 mm in 1997, 858.2 mm in 1998, 285.5 mm in 1999 and 378.2 mm in 2000.

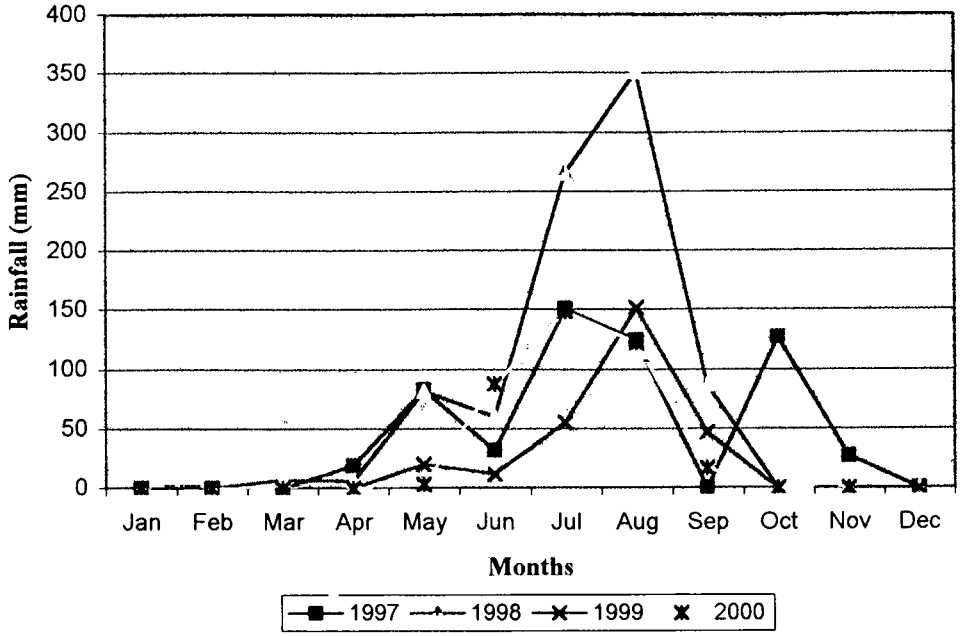


Fig. 2.2: Average monthly rainfall (mm) recorded at Elabered sub-zone for the period 1997-2000

As in most parts of the country, major transmission of malaria extends from September to November with peak transmission in October (Fig. 2.3). However, as is it is shown in the figure, transmission occures at a very low level during the dry season of the year. Since the monthly malaria cases reported here are based on slide confirmed cases, it is very hard to postulate that the whole year round transmission observed resulted from clinical misdiagnosis. Although malaria transmission is generally regarded as seasonal, the presence of dams and ground-wells found in the Elabered farming estate and its surrounding, which can serve as potenial larval breeding sites during the dry season, could be the main contributors for the low transmission observed through out the year.

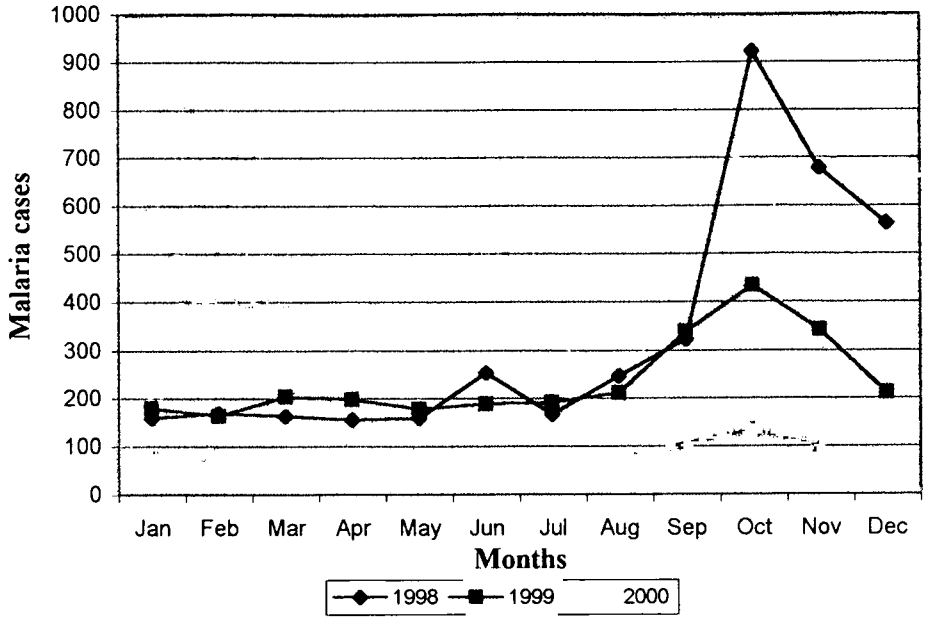


Fig. 2.3: Monthly malaria cases reported at Elabered health centre for the period January 1998 to November 2000

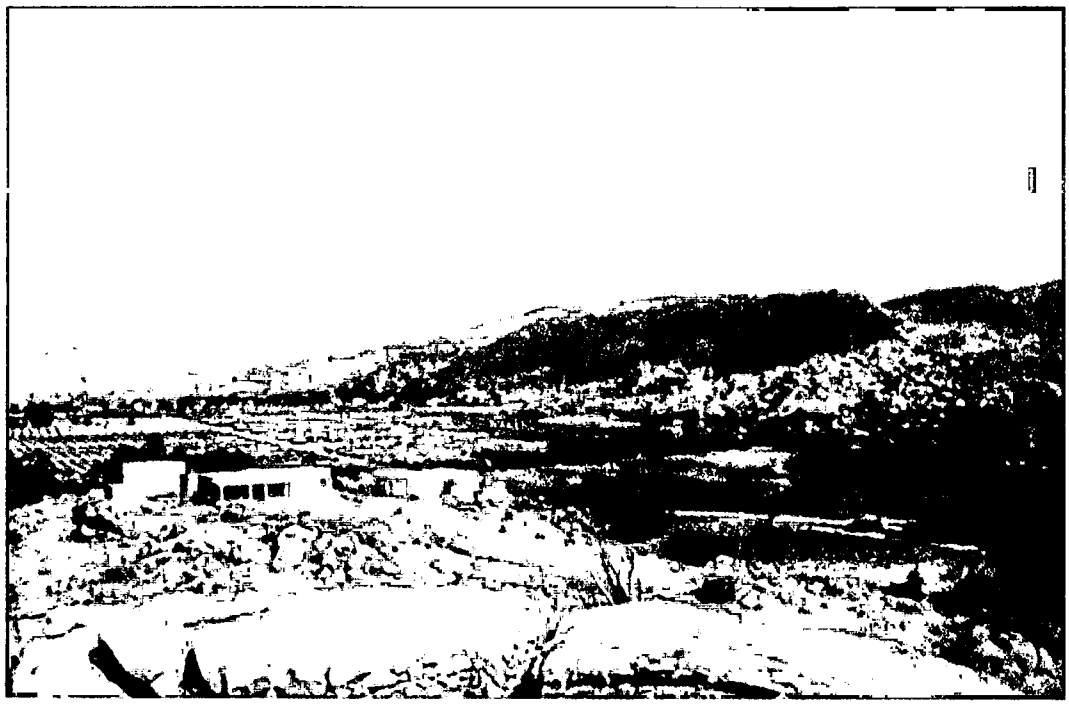
Overview of the Balwa stream, the main potential larval breeding site, overview of the study village and Elabered farming estate are shown on Fig. 2.4, 2.5 and 2.6 A & B, respectively.

2.2: Mosquito collections

Adult mosquitoes were collected from September to November 2000. Collection was done only from one village, Adi-bosqual. Some of the reasons why collection was done only from this village are: 1) It has been selected as a pilot spot for entomological studies by the National Malaria Control Program, 2) Most of the houses are small with thatched roofs and plastered walls which makes them suitable for entomological surveys 3) It is situated close to the Elabered farming

Fig. 2.4: The Balwa stream: a potential breeding site for *Anopheles* larvae

Fig. 2.5: Overview of the study village



A

B

Figs. 2.6 A & B: Overview of the Elabered farming Estate

estate were extensive use of insecticides for agricultural purposes are underway 4) It is easily accessible for transportation. Three collection methods were used to sample adult mosquito populations.

2.2.1: Human bait collections

A total of six night-biting catches on human bait were carried out during the whole study period. Three of these collections were done throughout the night, 18.00 to 06.00. The other three were done from 18.00 to 22.30. Two human 'baits' were seated, one indoor and one outdoor, and hungry female mosquitoes coming to bite were caught using an aspirator with the help of a flashlight. The human baits were replaced by other men at mid night. The collected mosquitoes were kept in humidified paper cups until they were identified morphologically and then kept on desiccant (silica) until processed in the laboratory. Whenever possible, mosquitoes were caught before biting the human bait. In practice, however, it was impossible to collect all adults before they have bitten and collectors were therefore given a prophylactic anti-malarial drug.

2.2.2: Pyrethrum spray catches

Four houses were selected for pyrethrum spray catches (PSC) and mosquitoes were collected twice a month for three months (September to November). All occupants, animals and easily removable objects such as chairs, tables, exposed food and drinking water were first removed out of the houses that were to be

sprayed. White spray sheets, small (2 x1 m) and large (2 x 2 m), were laid over the entire floor, the beds and other furniture and miscellaneous objects that could not be removed. After all potential escape routes were covered with surplus sheets, the door was closed and the room sprayed with 0.2% pyrethrum in kerosene. After 10 minutes the dead mosquitoes were collected from the spray sheets and placed in petri dishes.

2.2.3: Day resting collections

Mosquitoes were collected, using an aspirator, from artificially made pits from outdoor resting places. Collections were also made from under bridges and in animal dwellings. Some of those collected from the pit shelters were transported to Johannesburg live to be used for insecticide susceptibility tests.

All mosquitoes collected from the field were transported to the temporary laboratory in Elabered health centre and were identified to species group using Gillies & De Meillon (1968) and Gillies & Coetzee (1987) keys. After identification, each mosquito was placed in a labelled vial with a desiccant. Information on the physiological status (unfed, fed, half gravid, gravid), collection technique used, date and place of collection for each mosquito was recorded in a record book with reference to the number given to each mosquito in the labelled vials. Processed samples were transported to the Department of Medical Entomology, South African Institute for Medical Research (SAIMR), Johannesburg, South Africa, for further processing/testing.

2.3: *Anopheles gambiae* complex identification by PCR

Legs from mosquitoes collected in the field and morphologically identified as *An. gambiae* complex were placed in a polypropylene micro-centrifuge PCR tubes. The protocol developed by Scott *et al.* (1993) was used in this particular study except that one leg from each mosquito was placed in a 1.0ml Eppendorf tube to which 12.5µl of the PCR master mix containing 10x PCR buffer (Tris-HCL, EDTA, DTT, Tween20, Nonidet P-40, and Glycerol); 2.5mM of each dNTP; 25mM $MgCl_2$; 3.3pmol of each primer, 4.9µl of deionized distilled water and 0.5 unit of thermostable DNA polymerase was added. No DNA extraction was done. The reaction mix was centrifuged for two minutes at 16,000 revolutions per minute in a microcentrifuge in order to release the template DNA from the tissues. Then the reaction mix was overlaid with one drop of mineral oil and placed in a Hybaid thermal cycler for 30 cycles, consisting of 94°C denaturing for 30 seconds, 50°C annealing temperature for 30 seconds, and 72°C extension for 30 seconds with an additional auto-extension step of 72°C for 10 minutes. The resulting amplified DNA was run on a 2.5% agarose gel, stained with ethidium bromide, submerged in 1x TAE buffer and electrophoresed until the bromophenol blue migrated about 3cm. Four control specimens from insectary colonies (*An. gambiae, An. arabiensis, An. quadriannulatus* and *An. merus*) as well as a negative control amplified along with the specimens were also loaded in each gel. Finally, the gel was viewed under an ultraviolet trans-illuminator and photographed on polaroid film. The *Anopheles gambiae* complex species-diagnostic primers (Scott *et al.* 1993) used in this study are given in table 2.1.

Table 2.1: *Anopheles gambiae* **complex ribosomal DNA (rDNA) intergenic spacer species diagnostic primers**

Primer name*	Primer sequence (5' to 3')	Sequence base pair
UN	GTG TGC CCC TTC CTC GAT GT	
ME	TGA CCA ACC CAC TCC CTT GA	464
GA	CTG GTT TGG TCG GCA CGT TT	390
AR	AAG TGT CCT TCT CCA TCC TA	315
QD	CAG ACC AAG ATG GTT AGT AT	153

*The UN anneals to the same position of the rDNA of all five species, GA anneals specifically to *An. gambiae*, ME anneals to both *An. merus* and *An. melas*, AR anneals to *An. arabiensis* and QD anneals to *An. quadriannulatus*

2.4. Host blood meal identification using ELISA

2.4.1: Introduction

Knowledge of the feeding behavior of arthropod vectors of disease to human and domestic animals is essential in understanding the relationship that exists between the vector and host and their roles in disease transmission cycle (Tempelis, 1975). Several serological techniques have been used to detect host-specific blood meals: e.g. the haemoglobin crystallization tests (Washino & Else, 1972), the fluorescent antibody technique (Gentry *et al.*, 1967; McKinney *et al.*, 1972), the passive haemagglutination inhibition tests (Tempelis & Rodrick, 1972), the latex agglutination test (Boorman *et al.*, 1977) and the precipitin test (Tempelis & Lofy, 1963).

The most commonly used serological test in identifying the source of arthropod blood meals has been the precipitin test. The precipitin test, although it requires

little equipment, reagents are easy to prepare and its execution and interpretation is not difficult (Washino & Tempelis, 1983), it lacks sensitivity and specificity and can be somewhat time consuming unless an automated dispenser is used (Service *et al.*, 1986). The passive haemagglutination test offers greater specificity and sensitivity than the precipitin test but it is variable, time consuming and difficult to use routinely. The latex agglutination test, although much easier to perform, cannot distinguish between closely related hosts and is less sensitive than the precipitin test (Washino & Tempelis, 1983; Service *et al.*, 1986). The fluorescent antibody technique requires sophisticated laboratory equipment and technology, and has not been used in identifying meals for field-collected arthropods (Washino & Tempelis, 1983).

None of these methods described above satisfies the requirements of a simple yet sensitive and specific test, which can be considered as an alternative to the precipitin test. However, the enzyme-linked immunosorbent assay (ELISA) that has been developed for blood meal identification has been proven useful for field studies (Service *et al.*, 1986).

There are two basic ELISA procedures available for blood-meal identification: the direct and indirect ELISA. In the indirect ELISA, also referred as sandwich technique, host specific antisera are incubated in microtiter plates. Homologous immunoglobulins from the blood meal sample are captured by anti-IgG on a coated plate. In the direct ELISA, the blood meal sample is incubated directly in the microtiter plate well. It uses a host specific antibody-enzyme conjugate to

detect homologous IgG in the blood meal samples. The main difference between the two procedures is that the indirect ELISA uses an antiserum to capture a specific IgG, and the direct ELISA uses an antibody-enzyme conjugate alone to bind host specific IgG in the blood meal (Beier *et al.*, 1988).

The indirect ELISA is technically more difficult, because an antiserum must be produced for each host to be tested. However, it is very sensitive and specific. It is therefore most appropriate in providing information on feeding preference of mosquitoes on a wide range of wild hosts. On the other hand, the direct ELISA is more useful to investigators desiring information on the rate of human feeding (Beier *et al.*, 1988). The direct ELISA developed by Beier *et al.* (1988) was used in the current study.

2.4.2: Blood meal ELISA procedure

Blood-fed and half-gravid female anophelines collected by day-resting collections and pyrethrum spray catches (PSC) were cut transversely between the thorax and abdomen and the posterior portion containing the blood meal were placed individually in labeled vials. Each mosquito was ground in 100µl 0.01M phosphate buffered saline (1xPBS), pH 7.4, with 400µl 1xPBS added after grinding. Blood meals were identified by direct ELISA using anti-host (IgG) conjugates against human and bovine (peroxidase conjugated anti-human IgG[1] and phosphatase conjugated goat anti-bovine IgG[1]). 50µl of the sample was added

[1] Kirgegaard and Perry laboratories, Inc., Gaithersburg, Md, USA.

to wells of polyvinyl chloride, U-shaped, 96-well microtitre plates[1] which were covered and incubated for three hours at room temperature. Each well was then washed twice with 1xPBS containing 0.5% Tween20 (PBS-Tw20). This was followed by the addition of 50µl host-specific conjugates (anti-host[2]) diluted (1:2000 for human and 1:250 for bovine) in boiled casein containing 0.5% Tween20. After one hour of incubation, wells were washed three times with PBS-Tw20 and 100µl of ABTS (2,2'-azino-di-[3-ehtyl benzthiazoline sulfonate]) peroxidase substrate[2] was added to each well. Absorbance at 405nm was determined with an ELISA plate reader 30 minutes after the addition of substrate. After reading absorbance at 414nm, the wells were washed three times with PBS-Tw20 and 100µl phosphate substrate was added to each well. Plates were read after one hour to determine positive bovine reactions. Samples were considered positive when the absorbance value exceeds the mean plus three times the standard deviation of four negative controls.

2.5: Circumsporozoite (CS) protein identification by ELISA

2.5.1: Introduction

The sporozoite rate, the prevalence of female mosquitoes with sporozoite in their salivary glands, is the most sensitive and powerful parameter for describing the epidemiology of malaria in a particular area. This measure is required for determining the entomological inoculation rate (EIR), the product of the mosquito biting-rate and the proportion of mosquitoes carrying sporozoites in their salivary

[1] Dynatech laboratories, Inc., Alexandria, Va, USA.
[2] Kirgegaard and Perry laboratories, Inc., Gaithersburg, Md, USA

glands. It can be used to establish both vector identity and differences in transmission intensity over space and time (Collins *et al.*, 1984).

Microscopic examination is the classic method used for detection of malaria sporozoite in salivary glands of natural populations of mosquitoes. Although more accurate and representative of transmission potential, examination of the salivary glands of individual mosquitoes, using light microscopy, is labor-intensive and requires trained and dedicated personnel. Furthermore, it can only be performed on freshly captured mosquitoes and is useless in routine surveys of vector populations with sporozoite rates much less than one percent (Collins *et al.*, 1984).

The availability of monoclonal antibodies specific to the major surface antigens of the sporozoites of a number of different malaria species has enabled the development of immunological techniques for detecting sporozoites in infected mosquitoes. Some of the alternative methods that use species-specific monoclonal antibodies (MAbs) raised against circumsporozoite (CS) proteins are: Immunoradiometric assay (Zavala *et al.*, 1982), Immunofluorescent assay (Ramsey *et al.*, 1983) and enzyme linked immunosorbent assay for *Plasmodium falciparum* (Burkot *et al.* 1984), *P. vivax* (Wirtz *et. al.*, 1985), *P. ovale* and *P. malariae* (Collins *et al.*, 1988).

The immunoradiometric assay (IRMA) provides a rapid species-specific determination of both the presence and number of sporozoites in infected mosquitoes, and it can be performed on fresh caught or dried mosquitoes. The

disadvantage of this assay is that it requires the use of short-lived radioactive reagents that have inherent drawbacks associated with transportation and disposal after use. Moreover, it requires the use of equipment and power supplies, which are difficult to transport and maintain in the field (Zavala *et al.*, 1982). For fieldwork, ELISA has a distinct advantage over immunoradiometric and immunofluorescent assays. It is stable with easily transportable reagents that avoid the disposal problems associated with radioisotopes; and the results can be obtained visually, thereby facilitating routine use of the method in laboratories that have no ϒ-counters or fluorescent microscopes (Wirtz *et al.*, 1987). The ELISA is, therefore, the most attractive alternative to microscopy. It has an advantage in that large samples can be examined compared to the laborious technique of salivary gland dissections. Unlike the dissection method, ELISA can be used to distinguish between human and non-human malaria and also identifies the human malaria species. Moreover, it can be carried out on fresh, frozen or dried specimens (Stoffels *et al.*, 1995).

The test detects CS proteins that can be present in the developing oocysts, dissolved haemolymph and on sporozoites present in the haemocoel or salivary glands (Beier *et al.*, 1990). It only implies that the vector is infected and not necessarily infective and a positive ELISA on the mosquito does not establish that species as a vector (Robert *et al.*, 1988). Hence, ELISA results are not synonymous with salivary gland sporozoite rates.

2.5.2: Sporozoite ELISA procedure

The heads and thoraces of the mosquitoes collected by the different collection methods described in section 2.2 were placed individually in labeled vials and each mosquito was ground in 50µl of blocking buffer (BB) with 0.5% Nonidet P-40, a non ionic detergent (BB:Np-40), pH 7.0-7.4, in a 1.5ml polypropylene micro-centrifuge tube. After grinding, 150µl of BB was added, to bring the total volume of the triturate to 200µl. In each well, 50µl of this mosquito extract was used.

The basic procedure used in this study follows that of Wirtz *et al.* (1987). Each well of flexible polyvinyl chloride (PVC), U-shaped, 96-well micro-titration plate[1] was coated with 50µl of 1xPBS solution containing the *P. falciparum* and *P. vivax* capture antibodies (5ml PBS/40µl Pf2A10 and 5ml/10µl Pv210[2]), covered and incubated overnight at 4 °C. Separate plates were used for each species. The next morning, the contents of the plates were aspirated, each well filled with blocking buffer and incubated at room temperature for one hour. Subsequently, the blocking buffer was aspirated and 50µl of the mosquito triturate was added to the appropriate wells. After incubation for two hours, the mosquito triturates were transferred to other plates coated with *P. vivax* capture Mab. The plates were washed twice with PBS-Tw20 solution. Then, 50µl of horseradish phosphatase labeled Pf2A10 antibody[2], diluted in blocking buffer, was added to each well. The plates were covered and incubated in a dark place for one hour.

[1] Dynatech laboratories, Inc., Alexandria, Va, USA.
[2] Kirgegaard and Perry laboratories, Inc., Gaithersburg, Md, USA

The plates were then washed with PBS-Tw20 three times and 100µl of peroxidase substrate[1] was added to each well. Finally, absorbance was read at 405nm using an ELISA plate reader after half an hour of incubation. Each positive sample was retested for confirmation. Samples were considered positive if absorbance exceeds twice the mean of seven negative controls.

N.B: The same procedure was used for detecting *P. vivax* CS proteins except that unlabeled Pv210[1] and peroxidase labeled Pv210[1]were used as capture and conjugate Mabs, respectively.

[1] Kirgegaard and Perry laboratories, Inc., Gaithersburg,.Md, USA

CHAPTER THREE

VECTOR ABUNDANCE AND BEHAVIOUR

3.1: Introduction

Since there is not an effective vaccine available for the control of malaria and the parasites are increasingly becoming resistant to most of the available drugs, control of the vectors has become even more important (Newsame, 1999). For implementing an effective vector control, the major vectors of malaria must be known, and their susceptibility to insecticides, their resting, feeding and biting habits clarified and related to disease transmission. If this is not done, efforts to control the vector populations will be fruitless. In the area where this study was carried out, a number of control measures, such as environmental management, use of mosquito nets and larvicides, are in use to control the vector. However, no study has been carried out to determine the vector responsible for the transmission of malaria, its behaviour, vectorial efficiency and response to insecticides. This chapter will, therefore, deal with the vector abundance, behaviour and transmission potential of the malaria vector mosquitoes. The issue regarding the response of the vector to insecticides will be discussed in the next chapter.

3.2: Results

3.2.1: Mosquito identification and vector abundance

A total of 698 anopheline mosquitoes belonging to five species were collected during the three months study period (Table 3.1). The most abundant species

collected was *An. gambiae s.l.*, comprising 84.4% of the total anophelines collected. It is also the only vector species collected during the study period.

Other anopheline species collected during the study period were *An. cinereus, An. demeilloni, An. pretoriensis* and *An. dthali,* all of which have never been confirmed as vector species. In all collection methods employed, a high percentage of *An. arabiensis* were collected (Table 3.1).

Table 3.1: Species composition of adult anopheline mosquitoes collected in the study area

Anopheles Species	Method of collection						
	Pyrethrum spray catch	Human bait catches	Animal Dwellings	Out door collection		Total	%
				Pit shelter	Under a Bridge		
An. gambiae s.l.	12	277	25	274	1	**589**	**84.4**
*An. cinereus**	0	6	4	66	3	**79**	**11.3**
*An. demeilloni**	0	1	2	10	0	**13**	**1.9**
*An. pretoriensis**	0	3	0	4	0	**7**	**1.0**
*An. dthali**	0	1	1	7	1	**10**	**1.4**
Total	**12**	**288**	**32**	**361**	**5**	**698**	

*Non-vector species

All those morphologically identified as members of the *An. gambiae* complex were subjected to PCR for sibling species identification. Based on the PCR result (Table 3.2), about 91.0% of the members of the *An. gambiae* complex were identified as *An. arabiensis.* The remaining 9.0% of the samples did not amplify the DNA and therefore could not be identified to species level (Table 3.2). The most probable cause for the negative PCR results could be due to morphological mis-identification of the specimens or degeneration of the DNA poor preservation in the field.

Table 3.2: PCR result for the members of the *Anopheles gambiae* complex

Collection methods		No. Tested	PCR results			
			An. arabiensis	%	Negative	%
Pyrethrum spray catches		12	11	91.7	1	8.3
Human bait catches		277	250	90.3	27	9.7
Hand catches	*Pit shelter*	274	250	91.2	24	8.8
	Under a bridge	1	1	100	0	0
	Animal dwellings	25	24	96.0	1	4.0
Total		**589**	**536**	**91.0**	**53**	**9.0**

3.2.2: *Anopheles arabiensis* behaviour

3.2.2.1: Resting habits

Two methods were used in order to determine the resting behaviour of *An. arabiensis.*

A: Fed to gravid ratio: Out of 274 *An. arabiensis* collected resting outdoors in pit shelters, the proportions of unfed, blood-fed, half-gravid and gravid were 4.7%, 68.6%, 16.1% and 10.6%, respectively (Table 3.3). Unlike the outdoor resting places, 83.3% and 16.7% of those collected from human dwellings were fed and half gravid, respectively (Table 3.3). The percentage of fed was also high for those collected from animal dwellings (Table 3.3). The fed to gravid ratio for *An. arabiensis* was therefore, 6.5:1 (189:29) for outdoor resting samples and 9.3:1 (28:3) for indoor resting samples.

B: Comparison of indoor and outdoor resting collections: Most of the *Anopheles arabiensis* were collected from outdoor resting places (pit shelters). Therefore, it was difficult to compare with those collected from indoor resting

Table 3.3: Gonotrophic state (in percentage) of *Anopheles arabiensis* collected by the different collection methods

Collection method		Total collected	Blood status (%)			
			Un-fed*	Fed*	Half-gravid*	Gravid*
Pyrethrum spray catch		12	0	83.3 (10)	16.7 (2)	0
Hand catch	*Pit shelter*	274	4.7 (13)	68.6 (188)	16.1 (44)	10.6 (29)
	Under a bridge	1	0	100 (1)	0	0
	Animal dwellings	25	4.0 (1)	72.0 (18)	12.0 (3)	12.0 (3)

*Values in parenthesis are total numbers collected for each blood status

places. However, the comparison may give some indication of the relative resting habits of *An. arabiensis*. The density of *An. arabiensis* was 22.8x greater in pit shelters than in human dwellings and 11.0x greater than in animal dwellings. When the data for the human and animal dwelling was combined, the density from pit shelters was still 7.4x greater. In general, 88.1% of the *An. arabiensis* were collected from outdoor resting-places, but only 11.9% from human and animal dwellings.

3.2.2.2: Biting pattern

The biting pattern reported here is based on the human bait catches done through out the night. The hourly nocturnal biting cycle for in and out separately is given in Figures 3.1 and 3.2. The data for in and out was statistically analyzed to see if there is any difference between in and out catches. However, no statistical difference was found between these catches (x^2=0.49; p=0. 05, df= 2). Therefore, the data for in and out were treated together to find out the peak biting time. Based on the mean nocturnal biting cycle (Figure 3.3), *An. arabiensis* were

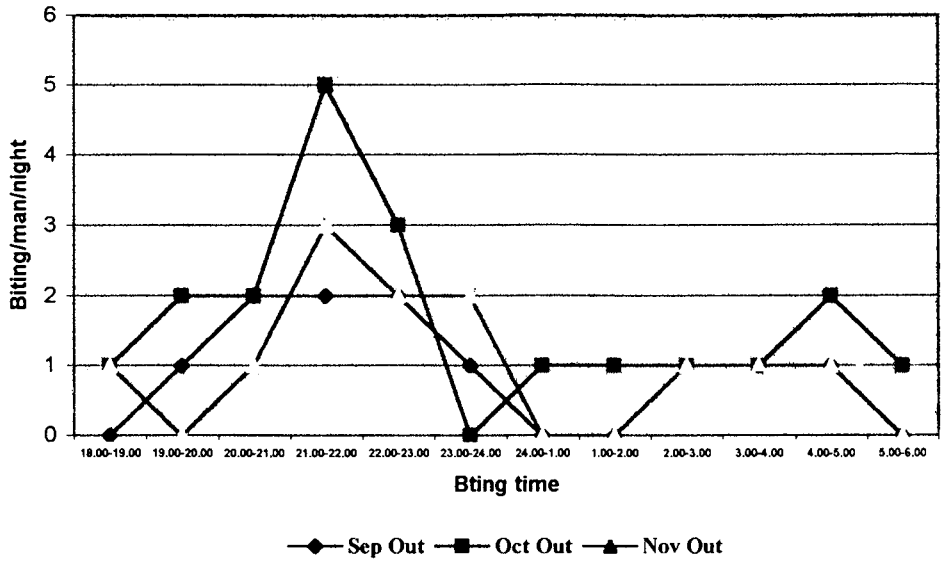


Fig. 3.1: Hourly nocturnal biting cycle for *Anopheles arabiensis* collected from outside house

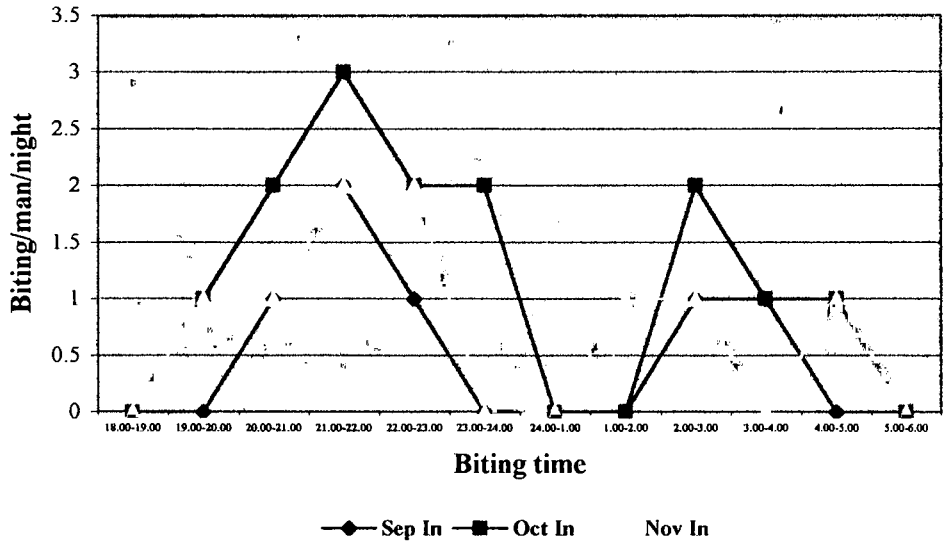


Fig. 3.2: Hourly nocturnal biting cycle for *Anopheles arabiensis* collected from inside house

observed biting throughout the night with the majority (69.2%) caught while they were attempting to bite in the first half of the night for all three months. Peak biting time was between 21:00 and 22:00 with a smaller peak in the early hours of the morning from 02:00-03:00, and 04:00-05:00 (Fig. 3.3)

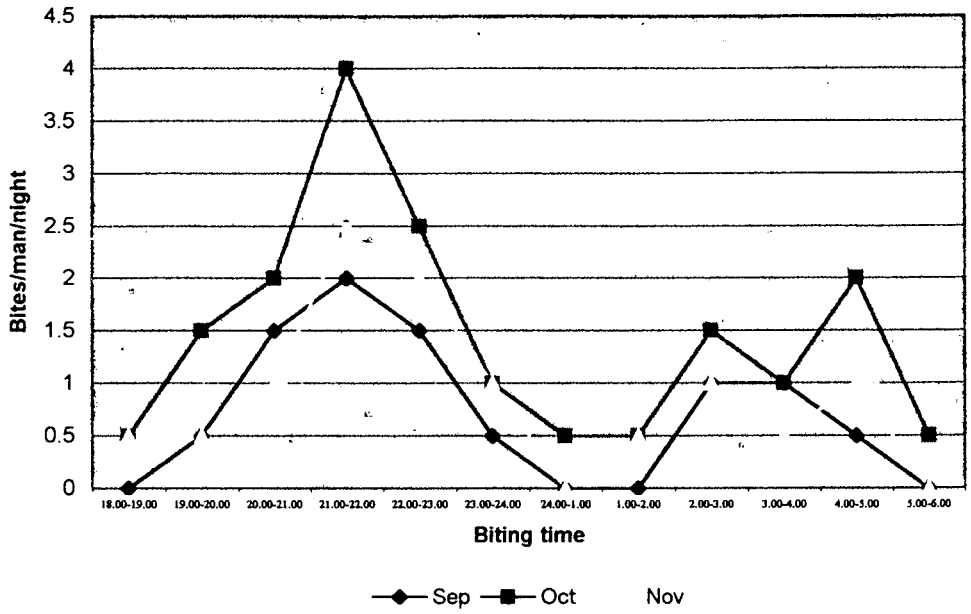


Fig. 3.3: Mean nocturnal biting cycle for *Anopheles arabiensis*

3.2.2.3: Man-biting behaviour

An estimation of the degree of endophagy (preferring to feed indoors) and exophagy (preferring to feed outdoors) has been estimated by comparing the relative proportion of the vector species caught biting indoors and outdoors (Table 3.4). About 62.8% of the *An. arabiensis* were caught while they were attempting to feed outdoors, as compared to 37.2% for those caught indoors. This difference between the number of *An. arabiensis* caught in outdoor and indoor was significant (x^2=7.63, p=0. 05, df= 2).

The mean man-biting rate (MBR = the average number of bites per person per night) was determined from the human bait catches. This was determined by dividing the total *An. arabiensis* caught during the whole night by the number of

baits. On average, during the transmission season, a villager was receiving 12.2 bites per night.

Table 3.4: *Anopheles arabiensis* **females caught on human bait outdoors and indoors from September to November 2000**

Month	*Total caught**	*Outdoor*		*Indoor*	
		Total	*Percentage*	*Total*	*Percentage*
September	51	39	76.5	12	23.5
October	119	77	64.7	42	35.3
November	107	58	54.2	49	45.8
Total	277	174	62.8	103	37.2

*Night bait catches done from 19:00 to 22:30 was also included in the total

3.2.2.4: Host selection

A total of 266 female *An. arabiensis* from different types of collections were tested for human and bovine IgGs (Table 3.5). The highest percentage (75%) of human fed females was obtained in pyrethrum spray catch (PSC) from human dwellings. From the 232 *Anopheles arabiensis* collected in pit shelters, 15.1% were fed on human and 69.4% on bovine blood with 3.4% on both human and bovine. Amongst 21 *An. arabiensis* collected from animal dwellings, one (4.0%) was fed only on human blood, one was positive for both human and bovine and the rest had fed on non-human hosts. The one *An. arabiensis* collected from under a bridge had also fed on bovine. A total of 12.4% of the blood samples failed to react to the human and bovine IgGs suggesting that blood meals had been taken from other hosts, such as goats, sheep or chicken. An investigation was also made to determine the blood preference of the non-vector anopheline species collected

Table 3.5: *Anopheles arabiensis* **females tested for human and bovine blood meals**

Collection method		*Total tested*	*Host*			
			Human	*Bovine*	*Positive for H & B***	*Negative for H & B***
Pyrethrum spray catch		12	9 (75.0)*	2 (16.7)*	1 (8.3)*	0 (0)*
Hand catch	**Pit shelter**	232	35 (15.1)	161 (69.4)	8 (3.4)	28 (12.1)
	Under a bridge	1	0 (0)	1 (100)	0 (0)	0 (0)
	Animal dwellings	21	1 (4.8)	14 (66.7)	1 (4.8)	5 (23.8)
Total		**266**	**45** (16.9)	**178** (66.9)	**10** (3.8)	**33** (12.4)

*Values in parenthesis are percentages.
**H and B represent human and bovine, respectively.

and none fed on humans (Table 3. 6). Small number of the specimens were negative to both human and bovine indicating that blood meals were taken from other hosts.

Table 3.6: Other (than *An. arabiensis)* **anopheline females tested for human and bovine blood meals**

Anopheles **species**	**Total tested**	*Host*		
		Human	*Bovine*	*Negative for H & B***
An. Cinereus	55	0	46 (83.6)	9 (16.4)
An. Demeilloni	8	0	5 (62.5)	3 (37.5)
An. Pretoriensis	2	0	2 (100)	0
An. dthali	5	0	4 (80.0)	1 (20.0)

*Values in parenthesis are percentages.
**H and B represent human and bovine, respectively.

The human blood index (HBI= proportion of *An. arabiensis* found to be positive for human IgG) and the rate of multiple blood feeding (proportion of *An. arabiensis* with mixed blood meals) were 16.9% and 3.8%, respectively (Table 3.5).

3.2.3: Infection and inoculation rates

The sporozoite rates (the proportion of *An. arabiensis* testing positive for circumsporozoite (CS) proteins per unit time) was based on ELISA testing of the *An. arabiensis* females obtained through different collection methods. Thus, 589 *An. arabiensis* were tested for *P. falciparum* and *P. vivax* sporozoite antigens. The monthly *P. falciparum* sporozoite rates were 0.47%, 0.83% and 0.73% for September, October and November, respectively, and there is no significant monthly variation in sporozoite rate. The mean *P. falciparum* sporozoite rate for *An. arabiensis* collected during the three months of the study period (September to November 2000) was 0.68% (Table 3.7). All the positive specimens were collected from pit shelters and human bait catches. None of the specimens were positive for *P. vivax*. The non-vector species were also tested and none was positive (Table 3.8).

Table 3.7: Monthly and seasonal sporozoite rate and entomological inoculation rates for *Plasmodium falciparum*

Months	*Total tested*	*MBR*[1]	*No Positives*[2]	*Malaria indices*		
				SR (%)[3]	*EIR*[4]	*EIR*[5]
September	211	8.5	1 (5)	0.47	0.04	1.2
October	241	17.5	2(5)	0.83	0.15	4.65
November	137	10.5	1 (4)	0.73	0.08	2.4
Mean		**12.16**	**4 (14)**	**0.68**	**0.08**	**7.28**[6]

[1]**MBR- man-biting rate (bites/person/night):** Calculated by dividing the total caught (in and out) during the whole night by two for each month and by three for the mean MBR

[2]Values in parenthesis are positives before reconfirmation test

[3]**SR- sporozoite rate:** Total tested divided by number of positives for each month and by total positives for the mean SR

[4]**EIR- entomological inoculation rate** (infective bites/person/night): The product of the SR and MBR for each month and the mean SR and mean MBR for the mean EIR

[5]**Infective bites/person/month (EIR per month):** The EIR (per night) multiplied by the number of days in each month

[6]**Infective bites/person/season (EIR per season):** The EIR (per night) multiplied by 91

Table 3.8: Sporozoite ELISA test result for the non vector species collected during the study period

Anopheles species	Total tested	*Negative for P. falciparum & P. vivax*	*Positives for P. falciparum & P. vivax*
An. cinereus	79	79	0
An. demeilloni	13	13	0
An. pretoriensis	7	7	0
An. dthali	10	10	0
Total	109	109	0

The entomological inoculation rate (EIR), the standard measure of transmission intensity, which is expressed as the average number of infective bites per person per unit time, was calculated as the product of the mosquito-biting rate and the proportion of mosquitoes carrying sporozoites in their salivary glands. The mean inoculation rate based on the human bait collections from September to November 2000 was 0.08 infective bites per person per night (Table 3.7). The mean number of potentially infective bites that can be received from *An. arabiensis* bites during the three months study period (the transmission season) was therefore 7.28 infective bites per person (0.08 x 91 days).

3.3: Discussion

3.3.1: Mosquito identification and vector abundance

In Africa, malaria transmission is complicated by the number of vector species involved. Studies carried out on the African continent indicate that at least two of the main malaria vector species are often involved in transmission of the disease at many localities. Most of these vector species are members of cryptic or sibling species complexes that include both vector and non-vector species. In Ethiopia,

for example, both *An. arabiensis* and *An. funestus* are main vectors with *An. nili* and *An. pharoensis* being incidental vectors (Zahar, 1985). Moreover, *An. quadriannulatus*, a non-vector member of the *An. gambiae* complex, is found in sympatry with *An. arabiensis* (Zahar, 1985; Coetzee *et al.*, 1993; Hunt *et al.*, 1998). These sympatric species exhibit behavioural and ecological differences that influence their role as vectors and the success of methods used for control. Understanding which anopheline mosquito species are present in a specific area and their respective role in malaria transmission is, therefore, the first and most important step in implementing an effective vector control measure.

Although the *An. gambiae* complex, most probably *An. arabiensis*, is generally regarded as the main malaria vector in Eritrea (Zahar, 1985), no study has been carried out with regards to the heterogeneity that exists between species and within species and their role in malaria transmission. Since not all the species within the *An. gambiae* complex are involved equally in transmission of malaria (Hunt *et al.*, 1998), proper identification of each species is necessary for implementing an appropriate vector control measure.

No previous records exist with regards to the distribution and bionomics of the anophelines mosquitoes in the Elabered sub-zone of Eritrea. This study will therefore provide good baseline data for defining some aspects of the vectorial role of the species in transmission. At least five anopheline species were found in the Elabered sub-zone. *Anopheles arabiensis* was the only member of the *An. gambiae* complex identified during this study and was by far the most abundant

anopheline present. Transmission in the current study area of Eritrea probably only takes place through this single species (*An. arabiensis*). Therefore, control efforts may not be as difficult as in most other parts of the continent where malaria transmission is copmlicated by the presence of two or more of the malaria vector species.

According to Coetzee *et al.* (1993), *An. arabiensis* is the only member of the sibling species of the *An. gambiae* complex which is responsible for the transmission of malaria in Ethiopia and the Sudan. However, a resent study has revealed the presence of *An. gambiae* beside *An. arabiensis* in Juba and Wau, Southern Sudan (Petrarca *et al.*, 2000).

3.3.2: *Anopheles arabiensis* behaviour

3.3.2.1: Resting habits

Although data on the endophilic behaviour of *An. arabiensis* are limited, the high proportion of fed compared to gravid females caught indoors indicated that most of them left the room before they became fully gravid. The high proportion of *An. arabiensis* caught from outdoor resting places compared to indoor resting places was also another indication for outdoor resting preference.

In a study carried out by Sharp, *et al.* (1990), in South Africa, *An. arabiensis.* were observed to leave the huts irrespective of the presence or absence of DDT. Moreover, they recorded that, a high percentage of blood fed *An. arabiensis* were caught leaving the huts, although the percentage of freshly fed leaving the huts was higher in the control huts as compared to the DDT sprayed huts. In the Sudan,

Haridi (1972) carried out a study in the eastern part of the country to find out the resting preference by *An. arabiensis*. He collected a high proportion of fed *An. arabiensis* from outdoor and indoor resting places. This outdoor resting preference coincided with the large exodus observed from sprayed houses as demonstrated by the window trap. He, therefore, concluded that the outdoor resting preference by this species could be partly due to its natural behavioural pattern or due to the irritant effect of DDT. Akiyama (1973) did a study on the same place and reported a natural exophily by *An. arabiensis*. His report was based on his collection of a high proportion of freshly fed females in outside resting sites in unsprayed areas. In Ethiopia, Krafsur (1977) determined the post-feeding behaviour of those collected from indoor resting sites on the basis of the relative proportion of fed and gravid. He found that the expected and observed pre-gravid females made up 16.11% and 4.45%, respectively. Thus, he concluded that observed proportion was much less than the expected, suggesting that the nulliparous deficit among indoor resting populations is principally the result of exophilic behaviour displayed by the pre-gravid group. In a study carried out by Service *et al.* (1978) and Highton *et al.* (1979) in the Kisumu area of Kenya, in an unsprayed zone, the tendency of *An. arabiensis* to occur outdoors was 2.2 times as great as *An. gambiae*.

3.3.2.2: Biting pattern

Anopheles arabiensis readily bites indoors and outdoors throughout the night in Elabered sub-zone. But, biting is more intensive in the first half of the night than

in the second half. This behaviour might be attributed to the lower temperature experienced during the second half of the night.

The biting cycle of *An. arabiensis* observed in the present study is different to some extent from most of the previous observations made in East Africa. In Ethiopia, Krafsur (1977) noted that the biting activity of *An. arabiensis* increased steadily throughout the night until it reached a single peak between 05:00 and 06:00. In the study carried out by Ameneshewa & Service (1997) three peaks were observed at 21:00-22:00, 03:00-04:00 and 05:00-06:00. The result obtained in the current study showed a major peak at 21:00-22:00 and two minor peaks (02:00-03:00 & 04:00-05:00) somewhat resembling the results of Ameneshewa & Service (1997). In the Sudan (Dukeen & Omer, 1986) and in South Africa (Braack *et al.*, 1994), the number of *An. arabiensis* females attacking man were observed to increase from sunset until a peak was reached between 04:00 and 05:00, thereafter dropping by sunrise. In another study in the Sudan, biting was observed to increase steadily until a peak was reached around mid-night and then gradually decreasing until it reach a low level between 04:00 and 06.00 (Zahar, 1985).

3.3.2.3: Man-biting behaviour

Human landing catches indicated that *An. arabiensis* in the study area was mainly exophagic, preferring to feed outdoors. The outdoor biting on humans depended on the availability of people outside their homes in the evening. Most of the

villagers spent the night inside their rooms and went to bed at about 22:00 and children about an hour earlier. However, the cattle spend the whole night outside in an open fenced camp and the goats and sheep go to their dwellings at around 20:30. It appears that in the study area, although *An. arabiensis* readily bites outdoors, most man-vector contact will take place indoors, because none of the villagers sleep outdoors.

In Ethiopia, 73.6% exophagy in *An. gambiae s.l.* (most probably *An. arabiensis*) was observed by Rishikesh (1966). In contrast to this, Krafsur (1977) noted about four times more *An. arabiensis* biting indoors than outdoors and in the Sudan biting was about 4.5 times higher indoors than outdoors (Zahar, 1985). Recently, Ameneshewa & Service (1997) recorded in Ethiopia an overall exophagy of about 55.7%. These differences in man biting behaviour could be attributed, to some extent, to genetic polymorphism with certain karyotypes correlated with biting outdoors, similar to those found in Nigerian populations of *An. arabiensis* by Coluzzi *et al.* (1977, 1979).

3.3.2.4: Host selection

A high percentage (75%) of the indoor resting *An. arabiensis* collected by PSC were fed on humans. One specimen caught resting inside a house had a mixed blood meal (human and bovine), indicating that it went inside the house to feed on human after feeding on bovine outside, probably to complete an interrupted blood meal. The result indicated that 16.7% of the blood fed *An. arabiensis* caught

indoors had entered the houses after feeding on bovines outdoors. In the study area cattle were kept outside. A similar observation was made by Petrarca *et al.* (1991) and Githeko *et al.* (1994) in Kenya, where a substantial number of *An. arabiensis,* that fed on cattle outside, were caught resting inside houses.

Just as bovine-fed females were rare in collections from human dwellings, so the percentage of human blood fed *An. arabiensis* females from pit shelters and animal dwellings was very low, 15.1% and 8.4%, respectively. Similar studies that have been carried out in different parts of Africa indicated that the degree of anthropophily usually decreases when cattle are present (Krafsur, 1971), particularly in *An. arabiensis* which is more zoophagic than *An. gambiae*. In the Kismu area of Kenya, for example, Githeko *et al.* (1993) recorded a HBI for indoor resting *An. arabiensis* as low as 22%, but of all the females collected from granaries, 99.0% had fed on bovine.

The small number of blood samples which were negative to both human and bovine blood meals (12.4%) was probably due to the presence of an alternative host. Within the village there were a considerable numbers of goats, sheep, chicken, dogs and donkeys. On Reunion Island *An. arabiensis* has been shown to prefer dogs above all other possible hosts (Girod *et al.*, 2001)

In general, the preference of *An. arabiensis* for human blood (16.9%) seems to be very low. The present study indicates little difference from previous studies that have been done in East Africa. In Kenya, Ijumba *et al.* (1990) reported 9.5% HBI

for *An. arabiensis* compared to 76.7% for bovine. Boreham (1975) recorded 5% human fed individuals in Ethiopia while Ameneshewa & Service (1997) reported a HBI of 33.3%. Generally the low HBI (16.9%) for *An. arabiensis* in the current study (Table 3.5) might be attributed to their opportunistic feeding behaviour (Costantini, *et al.*, 1999). Moreover, according to these authors, East African populations of *An. arabiensis* are less anthropophilic than West African ones and in Madagascar mainly zoophilic populations of *An. arabiensis* occur independent of the human-bovine ratio (Costantini *et al.*, 1999).

3.3.3: Sporozoite and inoculation rates

There are no baseline data with which to compare the mean sporozoite rate of *An. arabiensis* (0.68%) obtained by the ELISA method in the current study. However, related studies that have been carried out in East Africa have a wide range of sporozoite rates. For example, in the Kisumu area, Kenya, Joshi *et al.* (1975) reported a sporozoite rate of 2.8% to 7.8% while Service *et al.* (1978) and Petrarca *et al.* (1991) found a sporozoite rate of 0.5% and 2.8 to 3.4%, respectively.

The number of infective bites that a person may receive from *An. arabiensis* in this area is very low (0.08 infective bites per person per night). This might be due to the low mean man-biting rate (12.16 bites per person per night). In Kenya, Githeko *et al.* (1993) recorded 0.60 infective bites per person per night with a mean man-biting rate of 52.22 bites per person per night.

In the current study area insecticide treated nets were distributed to the villagers in a credit form and on average there are two bed nets per household per average household of five people (personal observation). This high use of bed nets by the villagers, which reduces the direct contact with gametocyte carriers and sporozoite-positive mosquitoes could be one of the main contributor to the low malaria transmission in this area. Another factor that could be considered as a main contributor for the low transmission might be the location of the area. As compared to the western and southwestern lowlands, it is located in a relatively low transmission risk

Since the ELISA based sporozoite rate was obtained from the head/thoraces of *An. arabiensis* females and not directly from the salivary glands, the sporozoite rate obtained could be slightly elevated. The inoculation rate obtained in the current study area is therefore not an absolute measure of *P. falciparum* malaria transmission risk. However, it can provide an index of potential malaria risk in time and from place to place, and consequently can be used to assess variation in transmission levels in the malaria control programme.

CHAPTER FOUR

THE SUSCEPTIBILITY OF *ANOPHELES ARABIENSIS* TO INSECTICIDES

4.1: Introduction

Insecticides have played a central role in controlling the mosquito vectors of diseases since the early 20th century. Although important advances continue to be made in the development of alternative control measures, insecticides will remain a vital part of the integrated vector control programme in the foreseeable future (Ferrari, 1996; Collins *et al.*, 2000). Insecticides have been used in malaria control programmes in Africa since the 1930's. Initially, pyrethrum extracted from C*hrysanthemum* flowers was used as a short-term knockdown insecticide until replaced with long-lasting DDT after the Second World War (Coetzee *et al.*, 1999). The cyclodiene insecticides, dieldrin and gamma BHC, were also introduced for malaria control shortly after DDT. Due to mammalian toxicity, they are no longer in use. The extensive and widespread use of these insecticides in Africa resulted in the emergence of insecticide resistant strains of malaria vector mosquitoes (Coetzee *et al.*, 1999). The increasing incidence of this disease is largely due to the development of these resistant strains (Roberts *et al.*, 2000).

Insecticide resistance is rapidly eroding the number of suitable insecticides for malaria control. Close surveillance of the susceptibility of a vector population to insecticides is therefore necessary. Determining the susceptibility status of vectors to insecticides has three objectives: 1) Provide baseline data for programme planning and insecticide selection before the start of control operations; 2) Detect

resistance at an early stage so that timely management can be implemented; 3) Continuously monitor the effect of control strategies on resistance (Brogdon & McAllister, 1998).

4.1.1: Common insecticides in malaria control

Dichloro-phenyl trichloroethane (DDT)

DDT is one of the organochlorine insecticides which has been used extensively as an indoor residual spray and continues to be used in some parts of Africa. It is cheap and effective against indoor resting and feeding malaria vectors and has a long residual life. Resistance to DDT was relatively slow to develop and its current distribution among malaria vectors covers limited regions of Africa, mainly in West Africa (Coetzee *et al.*, 1999; Roberts *et al.*, 2000). The first case of DDT resistance involving *An. gambiae* was observed in 1967 in Burkina Faso. Soon after, it was also observed in *An. arabiensis* from Senegal (Chandre *et al.*, 1999a). In West Africa, DDT resistance was observed to show cross-resistance to pyrethroids (Martinez-Torres *et al.*, 1998). In Zanzibar, Tanzania, no cross-resistance to pyrethroids in the DDT resistant strains was observed (Prapanthadara *et al.*, 1995).

Dieldrin

Dieldrin was another organochlorine insecticide used in the control of malaria. Resistance to this insecticide was first reported in the 1950s from a population of *An. gambiae* in Nigeria (Davidson 1957). Since then resistance to this insecticide

has been reported from other parts of Africa (Coetzee *et al.*, 1999). However, considering its toxicity, it is unlikely that it would ever be considered for use in malaria control programmes again.

Hexachlorocyclohexane (HCH)

Hexachlorocyclohexane, also known as benzenehexachloride (BHC), is another member of the organochlorine groups used in the control of malaria. In its technical grade, there are five isomers: *alpha, beta, gamma, delta* and *epsilon* (Ware, 1999). However, only the gamma isomer has insecticidal properties. It is also highly toxic to humans and not used in malaria control these days.

Malathion

Malathion is the principal organophosphate insecticide in use for malaria control at present. In Africa, resistance to malathion has been reported only in *An. arabiensis* from a cotton growing area in Gizera, Sudan (Hemingway, 1983; Coetzee *et al.*, 1999).

Pyrethroids

Pyrethroids are synthetic structural analogues related to the six biologically active compounds known as natural pyrethrins (Zerba, 1988). They are extracted from *Chrysanthemum* flower heads, particularly *Chrysanthemum cinerariaefolium*, grown commercially in most parts of Africa and Asia (Miller, 1988). The use of mosquito nets impregnated with pyrethroid insecticides such as permethrin, deltamethrin and lambda-cyhalothrin, is an important advance in malaria control.

They are preferred for bed-net impregnation because they are highly effective and fast acting with a strong excito-repellent effect on mosquitoes and low mammalian toxicity (Lengeler & Snow, 1996; Magbity *et al.*, 1997). This intervention has contributed to the reduction of malaria morbidity and mortality in tropical Africa (Choi *et al.*, 1995). Pyrethroid treated bed nets were observed to have a major impact by reducing the indoor-resting density, man vector contact and the entomological inoculation rate (Curtis *et al.*, 1990; Lindsay *et al.*, 1991; Mbogo *et al.*, 1996). Furthermore, reduction in the sporozoite rate and the parity/mosquito survival has been recorded in Tanzania which is attributed to the mass killing effect of the nets (Curtis *et al.*, 1990). However, no such effect was observed in Kenya and The Gambia (Thomson *et al.*, 1995; Mbogo *et al.*, 1996). According to these authors (Thomson *et al.*, 1995; Mbogo *et al.*, 1996), the reduction in indoor density, man-vector contact and entomological inoculation rate is mainly attributed to the excito-repellency of the insecticides and not to the reduction of the number of vectors coming to feed.

Resistance to pyrethroids in the malaria vector *An. gambiae* has been recorded from several West African countries and is mainly attributed to the use of pyrethroids for domestic and agricultural use (Chandre *et al.*, 1999b). In East Africa, the reduced susceptibility of *An. gambiae* to pyrethroids was attributed to the use of these insecticides for bed net impregnation (Vulule *et al.*, 1994). However, in The Gambia, despite the extensive use of these insecticides for bed net impregnation, no change was observed in the susceptibility status of *An.*

gambiae (Hemingway *et al.,* 1995). Recently, resistance of *An. funestus* to pyrethroids has been reported from South Africa (Hargreaves *et al.*, 2000).

4.1.2: Insecticide resistance

Insecticide resistance is defined by the World Health Organization (1992) as an inherited characteristic that imparts an increased tolerance to a pesticide, or group of pesticides, such that the resistant individuals survive a concentration of the compound(s) that would normally be lethal to the species. Behavioural changes (change of behaviour that results in reduced contact with the insecticide), vigour tolerance (cuticular change that reduces the rate of penetration of insecticides), target site insensitivity and increase in the rate of insecticide metabolism are the four classes of insecticide resistance. The last two are the primary causes of insecticide resistance and will be discussed briefly below.

4.1.2.1: Target site insensitivity

Most of the conventional insecticides for which resistance has developed are neurotoxins. Hence, the mechanisms of resistance involving target site insensitivity are related to modifications of the nervous system. Such modifications on the neural target site involve:

Insensitive acetylcholinesterase (AChE)

Acetylcholinesterase is the target site of the organophosphate and carbamate insecticides. These insecticides exert their effect by inhibiting the enzyme AChE

which hydrolyses the neurotransmitter acetylcholine (Hemingway *et al.*, 1986). Inhibition of AChE by these insecticides results in paralysis or death of the insect. Insects have developed resistance to these insecticides by structural modification of their AChE that results in a decrease in the sensitivity to inhibition (Hemingway & Ranson, 2000).

Reduced neural sensitivity to organochlorine and pyrethroids

The voltage-sensitive sodium channel is considered to be the principal target site of action for DDT and pyrethroids. Structural changes in the insecticide receptors in the sodium channel are responsible for reduced neural sensitivity (Zlotkin, 1999). This reduction in the sensitivity of the insect's voltage-gated sodium channels to the binding of insecticides causes the resistance phenotype known as knockdown resistance (*kdr*) (Hemingway & Ranson, 2000).

Reduced neural sensitivity to cyclodienes

The cyclodiene insecticides target the neuronal gamma aminobutyric acid (GABA) receptors. GABA is an inhibitory neurotransmitter receptor at the synaptic junction of the central nervous system and at the neuromuscular synapses of insects (Hemingway & Ranson, 2000). Resistance to this class of insecticides is associated with reduced sensitivity of a GABA receptor to the toxic effects of the insecticide (Soderlund & Bloomquist, 1989)

4.1.2.2: Increased rate of metabolism

Increased rate of metabolism can result either from modification of existing enzyme forms, making them more suitable for degradation of insecticides, or by the action of regulatory mechanisms increasing the production of detoxification enzymes which were already available in susceptible insects but in very small amounts (Brogdon & McAllister, 1998). Resistance involving increased rate of metabolism includes:

Esterases

Esterase is a collective term for the enzymes which hydrolyse carboxylic esters (Hemingway & Karunaratne, 1998). Elevated expression of the esterase in question serves to sequester and degrade the insecticide absorbed preventing it from binding to its target site (Hemingway & Ranson, 2000). The organophosphate and carbamate insecticides have a high affinity for the esterase enzymes. Therefore, the esterases rapidly bind to these insecticides and slowly hydrolyse the new ester bond (acylated enzyme) to produce two insecticide metabolites, alcohol and acid (Hemingway & Karunaratne, 1998).

Glutathione *S* transferases (GSTs)

Glutathione *S* transferases are a large family of enzymes involved in intracellular detoxification of mutagens, carcinogens and other xenobiotic compounds (Prapanthadara *et al.*, 1995). The GSTs of *An. gambiae* are primarily of interest

because of their role in DDT resistance (Ranson *et al.*, 1997). Elevated levels of GST activity in insecticide resistant insects results in detoxification of DDT to a non-toxic product that can be readily excreted (Hemingway & Ranson, 2000).

Mixed function oxidases (MFO's)

The mixed function oxidases are a large and complex group of enzymes that hydrolyse, oxidize, and conjugate injurious compounds into less toxic, more water soluble products that can be readily excreted (Brogdon, *et al.*, 1997). Increased MFO activity is associated with metabolism of virtually all insecticides leading to detoxification (Hemingway & Ranson, 2000).

4.2: Materials and methods

4.2.1: Mosquito rearing

Anopheles arabiensis (identified by the PCR method of Scott *et al.*, 1993) collected from pit shelters by hand catch were used to establish a colony from Eritrea. Mosquitoes were reared in a climate-controlled room at 25±2 °C and 71-85% relative humidity, with LD 12: 12 hour photoperiod at the South African Institute for Medical Research, Johannesburg. Adults were kept in 15cm by 20cm high cages and were provided with a 10% sucrose solution. In addition to this, females were offered a blood feeding at least twice a week. Eggs were collected three or four days following the blood meal and transferred to small plastic bowls (5cm by 10cm) containing distilled water for hatching. After the eggs hatched and

larvae reached at least second instar, they were transferred into big plastic trays 30cm x 25cm x 13cm. Larvae were fed on a mixture of finely ground dog biscuit and brewers yeast. Emerging *An. arabiensis* adults were collected using an aspirator and transferred into the cages.

4.2.2: Test procedures

4.2.2.1: Mosquito selection

As recommended in the World Health Organization (1981) guidelines, two to three day old unfed female *An. arabiensis* were used for insecticide susceptibility tests.

4.2.2.2: Test conditions

All tests, except the exposure period which was conducted at room temperature, were carried out in an insectary at a temperature of 25±2 °C and 71-85% relative humidity. A one hour pre-testing holding period was used so that any dead or damaged individual could be removed before conducting the test. In each test, laboratory colonies of *An. gambiae* (from Nigeria and Cote D'Ivoire) known to be susceptible to all insecticides were used for comparison purposes. These colonies were tested for the same insecticides during the period these tests were carried out. This is why they were used for comparison purposes against *An. arabiensis*. Exposure of samples of mosquitoes to papers treated only with the solvent of insecticide in question were also included as controls.

4.2.2.3: Details of the procedure

Each test was conducted according to the World Health Organization (1981) protocol for determining the susceptibility or resistance of adult mosquitoes to different classes of insecticides. In each test, ten test tubes: four (with red dot) for exposing the mosquitoes to the insecticide, one (with green dot) for the control, five (with green dot) as holding tubes for the pre-test and post-exposure observation, were used. In each of the holding tubes, a piece of clean white paper (12 x 15 cm) rolled in to a cylinder was inserted to line the wall. The papers were fastened in position with a spring-wire clip.

Mosquitoes were transferred gently into the holding tube lined with a piece of clean white paper (Fig. 4.1, C) and left for one hour to ensure that damaged specimens were not included in the test. In each test 20-27 mosquitoes were used per tube. After one hour, the exposure tube lined with impregnated insecticide paper was inserted in to the holding tube in order to introduce the mosquitoes into the exposure tube (D), the slide lever was fully pulled out to align the opening of the slide with the opening of the exposure and holding tubes and the mosquitoes were gently blown from the holding tube into the exposure tube. The slide lever was pushed in to close the exposure tube (D). The exposure was run for one hour with the tubes standing upright at room temperature (E). Mosquito knockdown was recorded every 10 minutes during the one hour exposure time. Final mortality was recorded after a 24 hour holding period during which time a 10% sucrose solution was provided for the survivors (F) (Fig. 4.1).

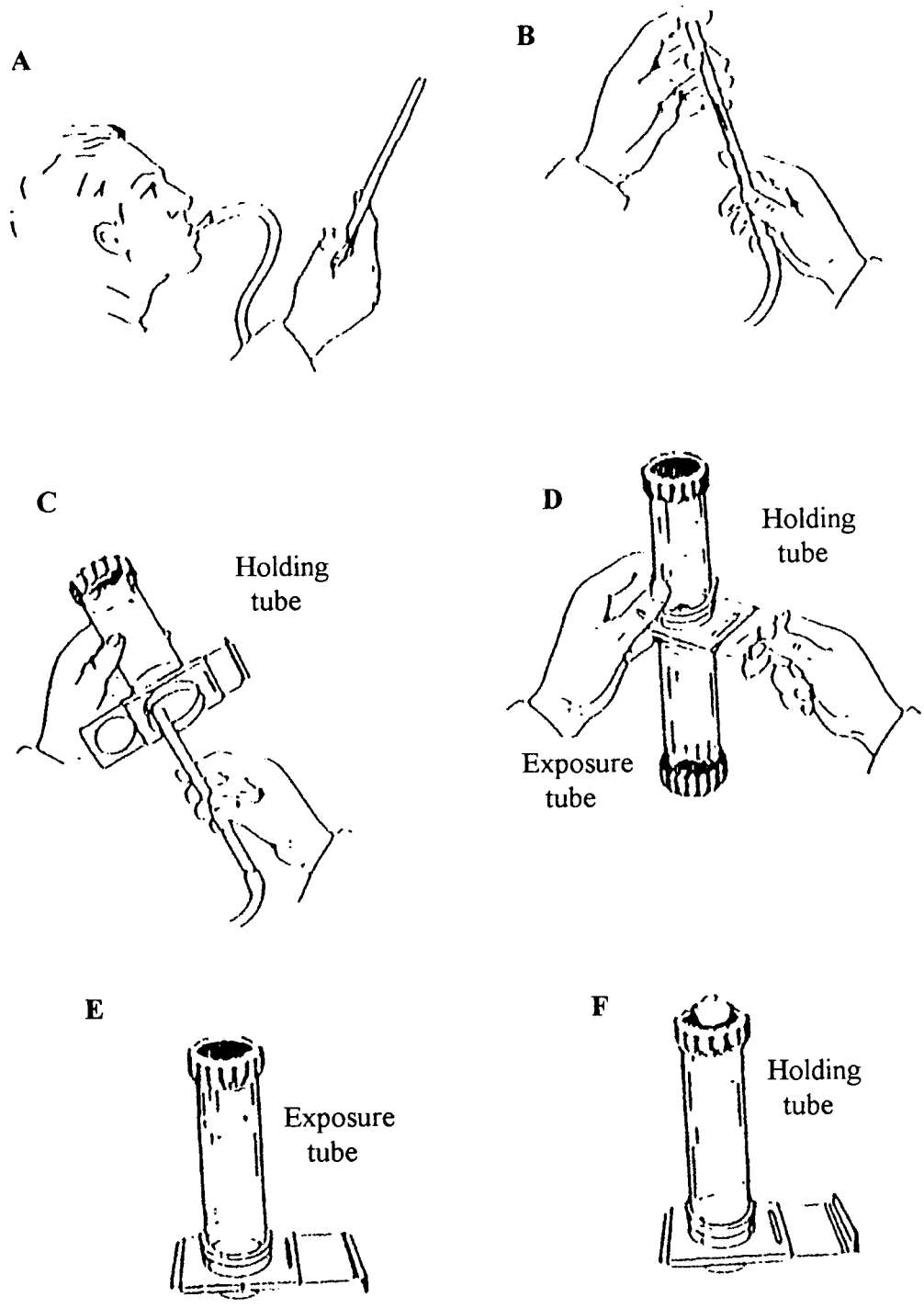


Fig.4.1: Method for determining the susceptibility or resistance to insecticides (World Health Organization, 1981).

4.3: Results

The percentage mortality 24 hours post-exposure of *An. arabiensis* to permethrin, DDT, propoxur, deltamethrin and lambda-cyhalothrin insecticides along with the susceptible reference strains used, is given in table 4.1. The percentage knockdown mortality was 100% for all insecticides tested, except for 4% DDT which was 95.9% and 90.0% for the tested colony and susceptible reference strain, respectively. Post-exposure mortality with permethrin at the diagnostic concentration of 0.75%, proposed by World Health Organization (1981) was relatively low (Table 4.1). By contrast, 100% mortality was obtained when the samples were exposed to 1% permethrin papers. There were two survivors (2.7%) from a sample size of 73 after exposure to the World Health Organization (1981) discriminating dosage of 4% DDT for one hour. Following the exposure of *An. arabiensis* to deltamethrin, lambda-cyhalothrin and propoxur using WHO discriminating dosages, 100% mortality was recorded (Table 4.1).

Table 4.1: Susceptibility tests on the *Anopheles arabiensis* (ARER) colony reared from wild-caught females.

Insecticides	Conce-ntration	Total Tested	% knockdown at 60 min			% Mortality 24h post-exposure		
			ARER	ARER Control	Reference strain	ARER	ARER Control	Reference strain
Organochlorine								
DDT*	4.0%	73	95.9	0	90	97.3	0	100
*Carbamate**								
Propoxur	0.10%	68	100	0	100	100	0	100
*Pyrethroids***								
Deltamethrin	0.05%	139	100	0	100	100	0	100
Lambda-cyhalothrin	0.05%	68	100	0	100	100	0	100
Permethrin	0.75%	138	100	0	100	72.5	0	90.2
	1.0%	72	100	0	100	100	0	100

The susceptible reference strains used were insectary colonies from Cote D'Ivoire* and Nigeria**

All ARER mosquitoes that survived the 0.75% permethrin diagnostic concentration were offered a blood meal and kept for oviposition. The F_1 progeny were tested along with the parental colony. In both colonies, 100% mortality was recorded (Table 4.2)

Table 4.2: Susceptibility tests on *An. arabiensis* progeny reared from 0.75% permethrin survivors.

Colony	Number Tested	% Knockdown at 60mi	% Mortality 24h post-exposure
ARER*	26	100%	100%
$ARER_1$**	25	100%	100%
NAG***	30	100%	100%
$ARER_1$ control#	24	0	0

ARER*: The Parental colony (See table 4.1)
$ARER_1$**: F_1 progeny of females that survived the original exposure (see table 4.1)
NAG***: Perimethrin susceptible colonies from Nigeria

4.4: Discussion

By applying the Davidson & Zahar (1973) criteria (98-100% mortality indicates susceptibility; 80-97% mortality means verification is required; <80% suggests that resistant individuals are present), it was found that *An. arabiensis* from Elabered sub-zone were susceptible to deltamethrin, lambda-cyhalothrin, propoxur and DDT, although verification was required for DDT due to two survivors (2.7%) after the 24 hour post-exposure period. Although the percentage mortality using 0.75% permethrin papers was low (75,5%), the 100% mortality observed after exposure of the progeny reared from the survivors indicates that the species is susceptible to permethrin. A possible explanation for the survivors

observed during the first tests is that the test papers were not functional, which is supported by the reduced mortality of the susceptible reference strains (Table 4.1). As widespread use of permethrin impregnated nets is promoted for long term use by the National Malaria Control Programme in Eritrea, there will be a need to do further studies at field level in order to evaluate the current test results and monitor routinely the susceptibility status of the vector population in order to detect any change in this situation.

Agricultural land use is often closely associated with an increase in insecticide resistance (Chandre *et al.*, 1999b) resulting in an increase in the prevalence of vector-borne diseases (Brooke *et al.*, 2001). The current insecticide resistance observed in Africa may have resulted from intensive use of these insecticides in crop protection (Hemingway 1983, Chandre *et al.*, 1999b). In Elabered sub-zone, insecticides have been used for agricultural purposes and are still in use both by the farming estate and individual farmers. Dimethoate, cypermethrin, ultracide, malathion, diazinon and actellic are some of the pesticides that are used by the farming estate. No study has been carried out to determine the role of these pesticides in selecting resistance in mosquitoes in this area. However, no spraying against any vectors of any disease has been done and permethrin impregnated bed nets were only introduced recently. As the extensive use of pesticides in this area continues, it is likely that such resistance will evolve regardless of the organised use of pyrethroids in a properly managed malaria control programme. Therefore, there will be a need to do routine monitoring of the resistance status of the vector

population and determine the protective effectiveness of the insecticide treated nets.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

1. This study, which was carried out in Elabered sub-zone, Eritrea, allowed us to define some aspects of malaria transmission, particularly the vector responsible for the transmission of malaria, its behaviour, efficiency in transmission of malaria and susceptibility status to insecticides. The results will serve as a base line for further detailed and broader studies.

2. In this sub-zone, the anopheline fauna was composed of at least five species. *Anopheles arabiensis,* identified by PCR, was the only vector species found during the study period. Unlike in most parts of Africa where malaria transmission is complicated by the presence of two or more vector species, some of them being cryptic species, transmission in this area takes place by *An. arabiensis* only.

3. As in most parts of Africa, *An. arabiensis* in this area rather prefers to rest outdoors than indoors. Therefore, vector control operations might benefit from including enviromental management and chemical control in order to reduce, to some extent, the total population rather than just the indoor resting density.

4. It appears that in the study area, although *An. arabiensis* readily bites outdoors, most man-vector contact takes place indoors because the villagers do not sleep outdoors and most of them spend their evenings indoors. Nevertheless, outdoor

man-vector contact is probably not negligible due to the early peak biting habits of the species both indoors and outdoors.

5. Biting by *An. arabiensis* is extensive during the first half of the night before most of the villagers go to beds. Therefore, the villagers could benefit from the use of additional protective measures (beside bed nets) such as repellents. Furthermore, children should be encouraged to go to bed very early so that they can benefit from the use of insecticide treated bed nets.

6. The infection and entomological inoculation rates recorded in this study area were very low. However, it is hard to postulate the exact cause for these low rates. One of the primary contributors could be the high percentage use of bed nets by the villagers. Therefore, there will be a need to evaluate the protective effect of the impregnated nets on the villagers.

7. *Anopheles arabiensis* is susceptible to the insecticides that are in use for the malaria control programme in this area. However, as the extensive use of pesticides in this area continues, it is likely that resistance could evolve regardless of the organised use of insecticides in properly managed malaria control programmes. Therefore, as the use of insecticide treated nets is increasingly promoted by the national malaria control programme, routine monitoring of the susceptibility status of the vector population should be undertaken to detect any changes in this situation.

8. There will be a need to extend the study by including more villages, collection days and collection methods so that information on the dynamics of malaria transmission between villages could be gathered.

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