INVESTIGATIONS INTO THE ECOLOGICAL DETERMINANTS OF DISTRIBUTION OF *ANOPHELES GAMBIAE* S.S. (DIPTERA: CULICIDAE) LARVAL POPULATIONS IN URBAN ACCRA, GHANA

BY

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A thesis presented in partial fulfilment of the requirements for the degree of M. Phil. Entomology of the University of Ghana

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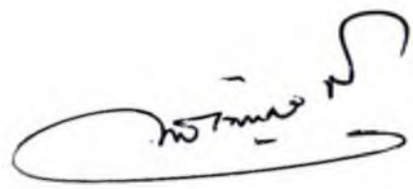
DECLARATION

I do hereby declare that the experimental work described in this thesis was carried out by me with the exception of references to other people's works that have been duly acknowledged. This thesis, either in whole or in part has not been presented elsewhere for any other degree.



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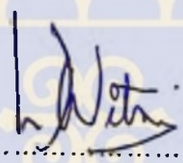

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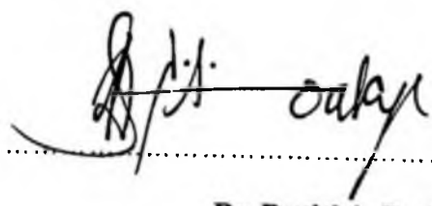

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Dr. Daniel A. Boakye

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DEDICATION

To my wife, Sylvie INSHUTI; my son, Brice Ulrich ISHIMWE

my brother in-law, Aristide BAKUNZI and to the families Francois Xavier

Karamage and Madeleine Hakizimana

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LIST OF ABREVIATIONS

$AgNO_3$	silver nitrate
APS	ammonium persulphate
bp	base pair
BOD	biochemical oxygen demand
$C_6H_5CH_3$	toluene
$CaCO_3$	calcium carbonate
$CHCl_3$	chloroform
CO_3	carbonate
COD	chemical oxygen demand
conc H_2SO_4	concentrated sulphuric acid
$CuSO_4.5H_2O$	copper sulphate
dATP	deoxyradenosine triphosphate
ddw	distilled de-ionised water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DO	dissolved oxygen
DTTP	deoxythymidine triphosphate
EDTA	disodium ethylene diamine tetraacetate. $2H_2O$
EtBr	ethidium bromide
EtOH	ethanol
FAS	ferrous ammonium sulphate

$Fe(NH_4)_2(SO_4)_2.6H_2O$: ferrous ammonium sulphate

$FeSO_4.7HO_2O$: ferrous sulphate septahydrate

GPS global positioning system

$H_2C_2O_4.H_2O$ oxalic acid

H_2O water

H_2SO_4 sulphuric acid

HCl hydrochloric acid

HCO_3 carbonate

HgCl mercuric chloride

HNO_3 nitric acid

K_2CrO_4 potassium chromate

$K_2Cr_2O_7$ potassium dichromate

KAc potassium acetate

Kb kilobase

KI potassium iodide

KOH potassium hydroxide

$KnaC_4H_4O_6.4H_2O$: potassium sodium tetrate tetrahydrate

LDF linear discriminant function

M molar

$MnSO_4$ manganese sulphate

$MnSO_4.4H_2O$ manganous sulphate tetrahydrate

Mw molecular weight

Na_2CO_3 sodium carbonate

$Na_2SO_3.5H_2O$ sodium thiosulphate

NaCl	sodium chloride
NaI	sodium iodide
NaN_3	sodium azide
NaOH	sodium hydroxide
NaOH-NaI	sodium hydroxide-sodium iodide
NH_4	ammonium
NH_4OH	ammonium hydroxide
NO_2^-	nitrite
NO_3	nitrate
PCR	polymerase chain reaction
PH	hydrogen-ion exponent
PO_4	phosphate
rDNA	ribosomal DNA
RNA	ribonucleic acid
Rnase	ribonuclease
rpm^{-1}	revolution per minute
$sddH_2O$	sterile double distilled water
SDS	sodium dodecyl sulphate
s.e.	standard error
SiO_2	silica
s.l.	sensu lato
s.s.	sensu stricto
SO_4	sulphate
TAE	Tris-Acetate EDTA

TBE	Tris-Borate EDTA
TDS	total dissolved solids
TE	Tris-EDTA
TEMED	N,N,N, 'N'-tetramethyl ethylene diamine
Tm	melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3 propanediol
µl	microlitre
µM	micromolar
UPGMA	Unweighted Pairgroup Method with Arithmetic Averaging
$ZrOCl_2 8H_2O$	zirconylchlorideoctahydrate

ABSTRACT

Anopheles gambiae s.s. larval habitats are important determinants of adult distribution and abundance, which also determine the geographical pattern of malaria disease. Information on their habitat characteristics can contribute greatly to a better planning of its control strategies through environmental management. To identify the environmental parameters, which influence the distribution of *An. gambiae* s.s. larval populations in urban Accra, Ghana, 30 habitats with *An. gambiae* s.l. and 24 without but with other mosquito species were studied. Mosquito larvae and pupae were collected and reared to adults. Water samples were taken at the same time of mosquito collection for physico-chemical analyses. The adult mosquitoes obtained were morphologically separated into *An. gambiae* s.l. and other species and counted. Then PCR-based methods were used on 300 mosquitoes to identify the members of *An. gambiae* s.l. and for microsatellite DNA analyses of *An. gambiae* s.s. AGXH7 locus using published oligonucleotide microsatellite primers. The microsatellite DNA data generated was used to determine the population structure and construct the phylogenetic relationships between *An. gambiae* s.s. populations. Twenty-eight physico-chemical parameters of each water sample were measured and those that on comparison were found to be significant were analysed further using linear discriminant function analysis and multiple regression analysis to reveal the best predictors of presence or absence, and abundance of *An. gambiae* s.s. in habitats. Then hierarchical cluster analysis using these water parameters was performed to reveal similar habitats, which was then compared with molecular phylogeny obtained.

All the 300 *An. gambiae* s.l. were identified as *An. gambiae* s.s. Turbidity, pH and calcium were found to be the most significant discriminatory parameters associated with the presence or absence of the *An. gambiae* s.s. in the habitats and were also selected as the best predictors of larval abundance. It was also observed that the molecular phylogeny of *An. gambiae* s.s. populations that bred alone in habitats fitted exactly with the clustering obtained using their water parameters. Also, closer populations were more likely to have relatively similar values of turbidity, pH and calcium concentrations and were also more likely to have similar allelic and genotypic frequencies.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Malaria is the most important disease transmitted by mosquitoes. However, despite tremendous progress made in the acquisition of knowledge of the biology of the malaria parasites, the anopheline mosquitoes and the human host, and the development of anti-malarial drugs and insecticides, the disease has proved far harder to control. At every turn when it was believed that the disease could be eradicated, either the mosquitoes and/or the parasite have eased their way out of extinction. Both, the malaria parasites and the mosquito vectors have become resistant to anti-malarial drugs and to insecticides respectively. For many reasons, we are still far from solving the malaria problem, which is actually getting worse in some parts of the world and even returning to areas from which it has once been eradicated (Dobson, 1999). An estimated two billion people (more than 40 % of the world population) presently live in areas with malaria risk and the global annual incidence ranges between three to five hundred million clinical cases, with a annual death toll of between two to three million (WHO, 1998). Malaria also accounts for 10 % of Africa's disease burden, causing the greatest suffering and impoverishment among poor people, with pregnant women and children under five years of age, being the most vulnerable (Okeni, 1999).

In Ghana, malaria is the most important parasitic disease, accounting for 7.8 % of all certified deaths. About 40 - 42 % of all outpatient attendance in Ghana hospitals is attributed to malaria (Ahmed, 1989)

The disease also is associated with considerable economic burden, including direct cost to governments and patients for hospital admissions and outpatient consultations, cost to households for treatment sought outside the official system, and cost due to absenteeism from productive work or education (WHO, 1997). The estimated direct and indirect cost of the disease in Africa alone is estimated to be $2000 million per year (WHO, 2000).

Human malaria is normally transmitted from one person to another by the bite of female mosquito species infected with malaria parasites. Of the thousands of described mosquito species, only a fraction of those in the genus *Anopheles* are known vectors. Those Anopheline species that do not transmit malaria parasites either do not feed on humans or are not susceptible to human malaria parasites, and a number have life spans that are too short to allow the parasite to fully mature. The vector species that pose the greatest threat are usually abundant, long-lived, commonly feed on humans and typically dwell in close proximity to humans.

There are some 400 species of *Anopheles* mosquitoes, but only about 70 species are known to transmit malaria. About 30 are of major importance, accounting for a significant amount of all malaria cases (Teklehaimanot & Pushpa, 1991). Their role in malaria transmission depends largely on the presence of a favorable environment for larval development and adult survival, and the ability to feed on humans. Transmission

also depends significantly on human habits that promote the host-vector contact (National Academy Press, 1991), characteristics of vectors, such as their abundance, susceptibility to infection, longevity, degree of contact with humans, and the type of species involved in the transmission (Appawu *et al.*, 2001). Finally, it depends also on behavioral attributes of vectors, such as finding and biting of hosts and choice of resting and oviposition sites, that vary both within and between species (Coluzzi *et al.*, 1979), the daily mosquito survival rate, the time between mosquito infection and sporozoite production in the salivary glands, the vectorial competence (even if an uninfected *Anopheles* feeds on an infectious host, either the mosquito may not acquire the viable infections, or *Plasmodium* parasite may fail to replicate within the vector. Furthermore, the mosquito may not transmit the infections onwards at a subsequent meal), and some factor expressing the human recovery rate from infection (Gullan & Cranston, 1994).

In Africa, the most important vectors are members of the *An. gambiae* Giles complex and *An. funestus* complex (Teklehaimanot & Pushpa, 1991). The *An. gambiae* complex comprises six named species, *An. gambiae* s.s., *An. arabiensis*, *An. melas*, *An. merus*, *An. quadriannulatus* and *An. bwambae* (Gillies and Coetzee, 1987), one unnamed species (Hunt *et al.*, 1998) and several incipient species (Coluzzi *et al.*, 1979). The species are morphologically indistinguishable yet genetically and behaviorally distinct. The distinct behavioral characteristics determine their distribution and efficacy as vectors (Favia *et al.*, 1997). *Anopheles gambiae* sensu stricto and *An. arabiensis* are the two most effective vectors of *Plasmodium falciparum* within the *An. gambiae* complex (WHO, 2000). These vectors have proven effective in transmitting the parasite to humans across the region, in rural and urban areas alike. However, all the transmission

characteristics of vectors are influenced by environmental conditions, such as climate, rainfall and vegetation.

Anopheles gambiae s.s. is extremely versatile, regarding tolerance to a wide variety of micro and macro environmental conditions, as evidenced by its wide geographical distribution (Lanzaro *et al.*, 1998). In this species, ecological and behavioral plasticity has been observed to be associated with polymorphisms in the form of paracentric chromosomal inversions, microsatellite DNA and isozyme variability within localized populations (Lanzaro *et al.*, 1995). The spatial distribution of certain gene arrangements also shows strong association with specific regional habitats and their frequencies change seasonally in places with seasonal fluctuations in weather, especially rainfall (Toure *et al.*, 1998; Coluzzi *et al.*, 1985; Bryan *et al.*, 1982; Coluzzi *et al.*, 1979). Clinal geographic and microspatial variation in gene frequencies and arrangements in *An. gambiae* s.s. populations also have been correlated with behavioral differences (Besansky *et al.*, 1994). There is also suggestive evidence that West African populations of *An. gambiae* s.s. are highly structured and that the spatial and temporal distributions of chromosomal inversion polymorphisms in West African populations of *An. gambiae* are non-random (Coluzzi *et al.*, 1979). The strong association of certain karyotypes with specific habitats has led to the description of distinct chromosomal "forms" of *An. gambiae* s.s. called ecophenotypes in West Africa (Lanzaro *et al.*, 1995). Three ecophenotypes, "Bamako", "Mopti" and "Savanna", occur in sympatry at numerous sites in Mali, Burkina Faso, Ghana (Toure, 1991; Lanzaro *et al.*, 1995), Gambia and Senegal (Bryan *et al.*, 1982).

Vectorial capacity can vary tremendously even between members of the same species complex (Coluzzi, 1984) Furthermore, Toure *et al.* (1983) reported that Bamako, Mopti and Savanna forms differ in their vectorial capacity, which means that relevant biological differences are detected not only between species but also between incipient taxa within the same species Small-scale spatial variation and temporal heterogeneity in mosquito densities can have important consequences for disease transmission (Smith *et al.*, 1995).

The strategy of malaria control is based on breaking the chain of transmission of the parasites between humans and mosquitoes either by controlling the parasites with drugs or breaking the contact between people and vectors. Several strategies for malaria management have been evaluated and these include habitat management, chemotherapy, vaccines and vector control using spraying, mosquito nets or future use of transgenic mosquitoes (Fontenille & Lochouarn, 1999; WHO, 2000). However, none of the control strategies has been reported to achieve complete control. Moreover, human behavior including small changes in land use, wars and population movements have complicated, disrupted and frustrated many attempts at control (Dobson, 1999). Malaria control programmes are very expensive and countries where malaria is endemic are often very poor and lack the finances and infrastructure to maintain effective campaigns (Dobson, 1999) To complicate issues further, resistance to anti-malarial drugs has appeared in recent years and it is increasingly becoming widespread, anti-malarial vaccines are eagerly awaited but the problems of developing a cheap and effective vaccine are immense and it is not likely to be available for some years to come (Fontenille & Lochouarn, 1999).

Management through drainage, filling, leveling intermittent flushing of vector breeding habitats are cost-effective options (Rafatjah, 1988) for vector control. Because of insecticide resistance and the environmental impact of spraying, considerable resources have been devoted to search for biological control agents. Vector control by larviciding has been implemented in some circumstances, especially when the use of residual adulticides was not effective or was too expensive. For most malaria vectors, reducing mosquito vector population densities by means of larviciding is generally an inefficient way of affecting transmission, because larval mortality among many anopheline populations may be density dependent, and population reduction at the larval stage does not affect the mean longevity of the surviving adult population. However, when a large proportion of larval habitat can be easily identified and target, larval control can be very effective (Collins & Paskewitz, 1995).

Whatever method(s) is to be adopted, it is critical that there should be a detailed understanding of the basic ecology of the vector, including a sound knowledge of the genetic diversities that may exist within vector species populations. Habits of the local anopheline mosquitoes are what determine the geographical pattern of the disease and information on the micro-ecology of vector breeding sites can contribute greatly to a better understanding of the relationship between the vector, man and the environment in disease transmission.

Despite the importance of *An. gambiae* s.s. as a vector, relatively little is known about its larval ecological preferences. There are two likely reasons given for the dearth of larval

studies on the malaria vectors. The main reason being that malaria control in Africa, traditionally, has been directed at the adult stages, therefore studies of larval ecology have been thought to be irrelevant by some workers (Gemnig *et al.*, 2001). This narrow view clearly is obsolete since an understanding of population dynamics – which includes an understanding of fluctuations in adult populations – requires a thorough appreciation of factors affecting larval abundance. Larval habitats are important determinants of adult distribution and abundance. Although the transient habitats of *An. gambiae* may not be a reasonable target for a vector control, an understanding of dynamics and productivity of larval habitats is required if efforts to model and predict adult abundance are to succeed. The second reason is that until recently such studies would have been impossible to carry out because the tools for characterizing different populations of *An. gambiae* s.s. did not exist. However, microsatellite analyses, which are PCR-based (Lehman *et al.*, 1996; Lanzaro *et al.*, 1995) have made it possible to distinguish populations, study population structures and assess gene flow between them, thus making it possible to type *An. gambiae* s.s. populations to specific habitats.

1.2 Objectives

The main objective of this study was to identify the ecological factors that determine the distribution of populations of *An. gambiae* sensu stricto at breeding sites in a coastal savanna area of Ghana. This was to be achieved by measuring physico-chemical parameters of water of breeding sites, using microsatellite DNA analysis to characterize intraspecific *An. gambiae* s.s. populations at each site and using statistical methods to identify parameters that are significantly influencing their distribution.

Specific objectives

The specific objectives are

i) To measure physical and chemical properties of water samples collected at breeding sites

ii) To collect and rear mosquito larvae and pupae to adults for enumeration and identification

iii) To identify the member species of *An. gambiae* s.l. using molecular methods

iv) To characterize populations and subpopulations using molecular biology techniques to analyze microsatellite DNA of *An. gambiae* s.s.

v) To determine the relationship between populations and subpopulations of *An. gambiae* s.s. and physico-chemical parameters of breeding sites using both univariate and multivariate statistical methods.

CHAPTER TWO

LITERATURE REVIEW

2. 1 Malaria: The Disease and Symptoms

Malaria is caused by infections with protozoan parasites belonging to the genus *Plasmodium* and transmitted by *Anopheles* mosquitoes. It is by far the most important tropical parasite disease, causing immense suffering and loss of life. The symptoms of malaria have been known since man's recorded history with the occurrence of fossil mosquitoes in amber also suggesting its prevalence in the pre-historic times (Smith, 1996) Hippocrates, first described the symptoms of the disease and even related them to the time of the year and to where the patients lived (Bradley, 1996). In describing the disease, a variety of names were given such as shakes, intermittent fever, ague and chills. It was realized then that there was an aetiological relationship between the disease and swamps. This led the Romans to begin drainage programmes because of the bad air that was associated with fever-producing areas and hence the term *mala aria* (name derived from Italian, meaning "bad air"), written *mal'aria* (Smith, 1996). With time the apostrophe was removed to get the present day term *malaria*.

The disease can assume many manifestations in individuals, depending on parasite species and pattern of transmission. The disease follows a course with a pre-patent period between infective bite and patently – the first appearance of parasites in the

erythrocytes (red blood cells). The first clinical symptoms define the end of an incubation period which in the case of *P. falciparum* is 9 days and 21 days for *P malariae* after infection (Gullan & Cranston, 1994). The symptoms include headache, muscular ache, vague abdominal discomfort, lethargy, lassitude and dysphoria. These symptoms precede fever up to 2 days. Then there is fever with temperature rising intermittently (> 37.5°C), shivering, mild chills, worsening headache, malaise and loss of appetite. The periodicity of fever also depends on the type of parasite species that the patient harbours. If infection is left untreated, the fever in *P. vivax* and *P. ovale* infections regularises to a 2-day cycle and for *P. malariae* the fever occurs every 3 days. For *P. falciparum* however, the fever remains erratic and may not regularize to tertian pattern (White, 1996). *Plasmodium malariae* and *P ovale* infections cause little morbidity and almost no mortality, *P. vivax* infections are more severe and debilitating but are usually self-limiting in healthy individuals. *P. falciparum* infections are always life-threatening in non-immune individuals (Collins & Paskewitz, 1995).

WHO (1990) has defined severe malaria, which is the acute form of *falciparum* malaria to include, severe anaemia, renal failure, pulmonary oedema, hypoglycaemia, circulatory collapse, bleeding, convulsions, haemoglobinuria, coma, hyperparasitaemia, jaundice and hyperpyrexia. In highly endemic areas for *P. falciparum*, where entomological inoculation rates can exceed several hundred infective bites per year, severe anaemia in infants is the principal manifestation and major contributor to mortality (Beier *et al.*, 1990). Cerebral malaria is the most prominent feature of severe malaria and is defined strictly as unarguable coma. This is caused by the sequestration of infected erythrocytes in the microvasculature of the brain.

2.2 Global Distribution of Malaria

Malaria is endemic in a total of 101 countries and territories. There are 45 countries in WHO's African Region, 21 in WHO's American Region, 4 in WHO's European Region, 14 in Eastern Mediterranean Region, 8 in WHO's South-East Asia Region, and 9 in WHO's Western Pacific Region (WHO, 1998) (Figure 1). Although this number is considerably less than it was in the mid-1950s (140 countries or territories) more than 2,400 million of the word's population are still at risk (WHO, 2000). The incidence of malaria worldwide is estimated to be 300 – 500 million clinical cases each year, with about 90 % of these occurring in Africa south of the Sahara (WHO, 2000). Outside Africa two thirds of the reported cases are concentrated in only 6 countries, namely (in decreasing order) India, Brazil, Sri Lanka, Afghanistan, Vietnam and Colombia (WHO, 1995).

Malaria is thought to kill between 2 and 3 million people worldwide each year, of whom about 1 million are children under the age of 5 years in Sub-Saharan Africa. These childhood deaths, resulting mainly from cerebral malaria and anaemia, constitute nearly 25% of child mortality in Africa. Fatality rates of 10 – 30% have been reported among children referred to the hospital with severe malaria, although these rates are even higher in rural and remote areas where patients have restricted access to adequate treatment (WHO, 2000). In addition to children under age 5, the others at greatest risk of dying from the disease include pregnant women, people moving from malarious zones for reasons of work, migration, refuge, war or tourism, and travellers who visit endemic countries and return home with the disease (WHO, 1996).

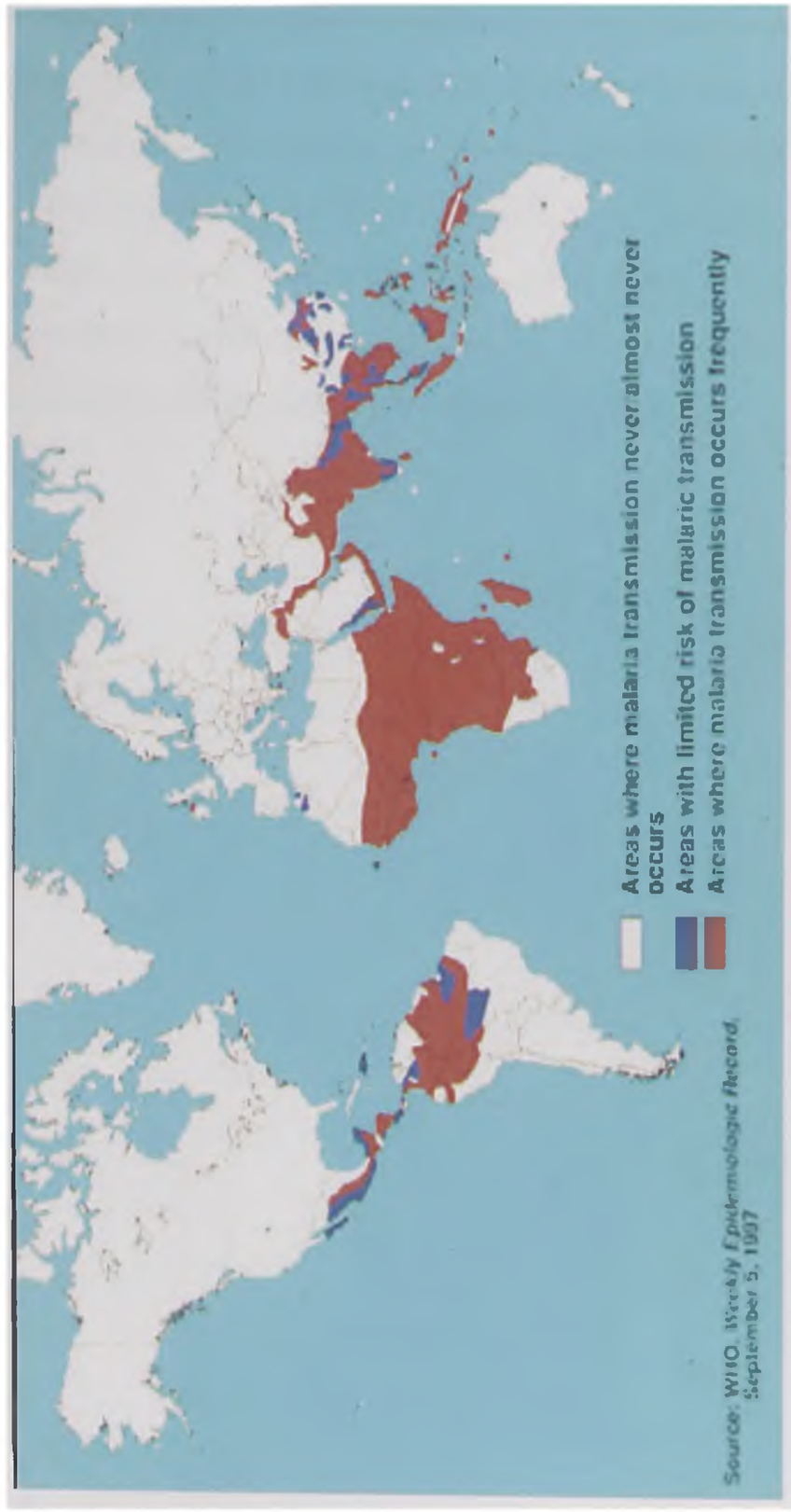

Figure 1: Global distribution of malaria (After WHO, 1997)

The distribution of the disease varies from one country to another and even within countries because of the flight range of the vector, which is thought to be about 2 miles, irrespective of the prevailing wind (WHO, 1998). *Plasmodium vivax* parasite can remain endemic in some Central American populations at very low levels that are difficult to measure in active prevalence surveys, while in some parts of Africa, the prevalence of *P. falciparum* approaches 100% in young children and malaria-specific infant mortality may exceed 20% (Spielman *et al.*, 1993).

2.3 Socio-economic Impact of Malaria

The enormous toll of lives and of days of labour lost, and the costs of treatment, make malaria a major social and economic burden in developing countries (WHO, 1995). Among all infectious diseases, malaria continues to be one of the biggest contributors to disease burdens in terms of deaths and suffering. By undermining the health and capacity to work of hundreds of millions of people, it is closely linked to poverty and contributes significantly to stunting social and economic development (WHO, 1996). Malaria and poverty are intimately connected. Cross-country regressions for the 1965 – 1990 period confirm the relationship between malaria and economic growth (Gallup & Sachs, 2001). Taking into account initial poverty, economic policy, tropical location and life expectancy, among other factors, countries with intensive malaria transmission grew 1.3% less per person per year, and 10% reduction in malaria is associated with 0.3% higher growth (Gallup & Sachs, 2001)

The disease cause 10.6% of lost disability adjusted life years (DALYs), second only to HIV/AIDS. The cost of malaria in economic terms is also high. The cost of a case from society's view point is $ US 9.84 or 12 days equivalent of productivity (WHO, 1999). Treatment ranges in cost between $ US 0.80 and $ US 5.30 depending on local drug resistance (NIAID, 2000) and the total cost in Africa alone is estimated to have reached $ US 2,000 million per year (WHO, 2000).

In many parts of the world, malaria is becoming an even greater problem than before. Epidemics are recurring in areas where transmission had been interrupted and are generally associated with deteriorating social and economic conditions (WHO,

1996). Even when non-fatal, malaria produces considerable impact on the health of the African children mostly during their first five years of life, increasing susceptibility to other infections and hampering their development. It is also a very serious problem, when associated with pregnancy, contributing to maternal and neonatal death and low birth weight (WHO, 1992). The rural communities are the most affected because the rainy season is often a time of intense agricultural activity a time for poor families to earn most of their annual income. The disease can make these families even poorer due to the loss of labour (WHO, 1998). Workers suffering a bout can be incapacitated for 5 – 20 days. It is estimated that malaria stricken family spends an average of one-quarter of its income on malaria treatment, and prevention and such a family can only harvest 40% of crops harvested by a healthy family (WHO, 1990). Malaria also leads to the chronic school absenteeism in children and there can be impairment of learning ability (WHO, 1998).

2.4 The Life Cycle and Transmission of Human *Plasmodium* Parasites

Human malaria is normally transmitted from one person to another by the bite of a female *Anopheles* mosquito infected with malaria parasites. The life cycle of the parasite involve a vertebrate host and an insect vector (Figure 2). There are three phases of development in the life cycle of the parasite namely, exo-erythrocytic stages in the liver, erythrocytic schizogony in erythrocytes and sexual processes in the mosquito (Smith, 1996). The malarial cycle commences with an infected female *Anopheles* mosquito taking a blood meal from a human host. As it feeds, it injects saliva contaminated with sporozoite stage of *Plasmodium* directly into the blood stream. Some sporozoites are killed but the survivors after 30 minutes of their entry into the blood stream migrate to the liver (Smith, 1996). All sporozoites leave the peripheral blood circulation within 45 minutes of injection into the host.

Once in the liver, pre-(or exo-) erythrocytic schizogonous cycle takes place in the parenchyma cells. This leads to the formation of a large schizont, which depending on the *Plasmodium* species may contain from 2,000 to 40,000 merozoites (Gullan & Cranston, 1994). The prepatent period of infection that started with an infective bite ends when the merozoites are released and either infect further liver cells or enter the bloodstream to invade erythrocytes. Invasion occurs when the erythrocytes invaginate and engulf the merozoite, which subsequently feeds as a trophozoite within a vacuole. The first and several subsequent erythrocytes schizogonous cycles produce a trophozoite that becomes a schizont, which releases from 6 to 16 merozoites. The duration of the erythrocyte schizogonous cycle is the duration of the interval between attacks – 48 hours for certain malaria and 12 hours for quartan

malaria (Gullan & Cranston, 1994). Within the erythrocytes, merozoites fuel their activities by consuming haemoglobin and develop into ring-shaped trophozoites. There is another round of asexual production within the erythrocytes, which takes 48 hours and this time when the merozoites are released some invade other erythrocytes whilst others change into sexual forms (micro and macrogametocytes) within 10 – 12 days (Mons, 1985). The synchronous release of merozoites from the erythrocytes liberates parasite products that stimulate the host's cells to release cytokines (a class of immunological mediators), which provoke the fever and other symptoms associated with malaria attack. Normally, a variable number of asexual cycles occur before any gametocytes are produced (Carter & Gwadz, 1980). The gametocytes have no further activity in the human host. These circulate in the blood stream until they are picked up by another mosquito as it takes blood from human. In the gut of the mosquito, there is exflagellation of the microgametocytes and subsequent fertilization of the macrogametocytes. The zygote, which is called ookinete, penetrates the wall of the midgut and develops into an oocyst. Sporogony within the oocyst produces many threadlike sporozoites, which are released as the oocyst ruptures. The sporozoites develop and become up to 1,000 times more infective than when in the oocyst (Smith, 1996). They then migrate to the salivary glands where they reside until injection into another human host.

With the exception of a few cases of transplacental and blood transfusion associated transmission, malaria parasites are transmitted exclusively by mosquitoes of the genus *Anopheles*. Nearly 20% of the almost 400 described species of anopheline mosquitoes have been implicated as vectors, but most of these are probably of minor or incidental importance. With the exception of Southeast Asia, which may have ten

or more important malaria vectors, most malarious regions of the world have only three or four major vectors (Collins & Paskewitz, 1995). In sub-Saharan Africa, where 90% of the world's malaria-infected people are found, most transmission is caused by three anopheline species, *An. gambiae* s.s., *An. arabiensis* and *An. funestus*, with *An. gambiae* s.s. being the most important vector (Collins & Paskewitz, 1995; Gimnig *et al.*, 2001).

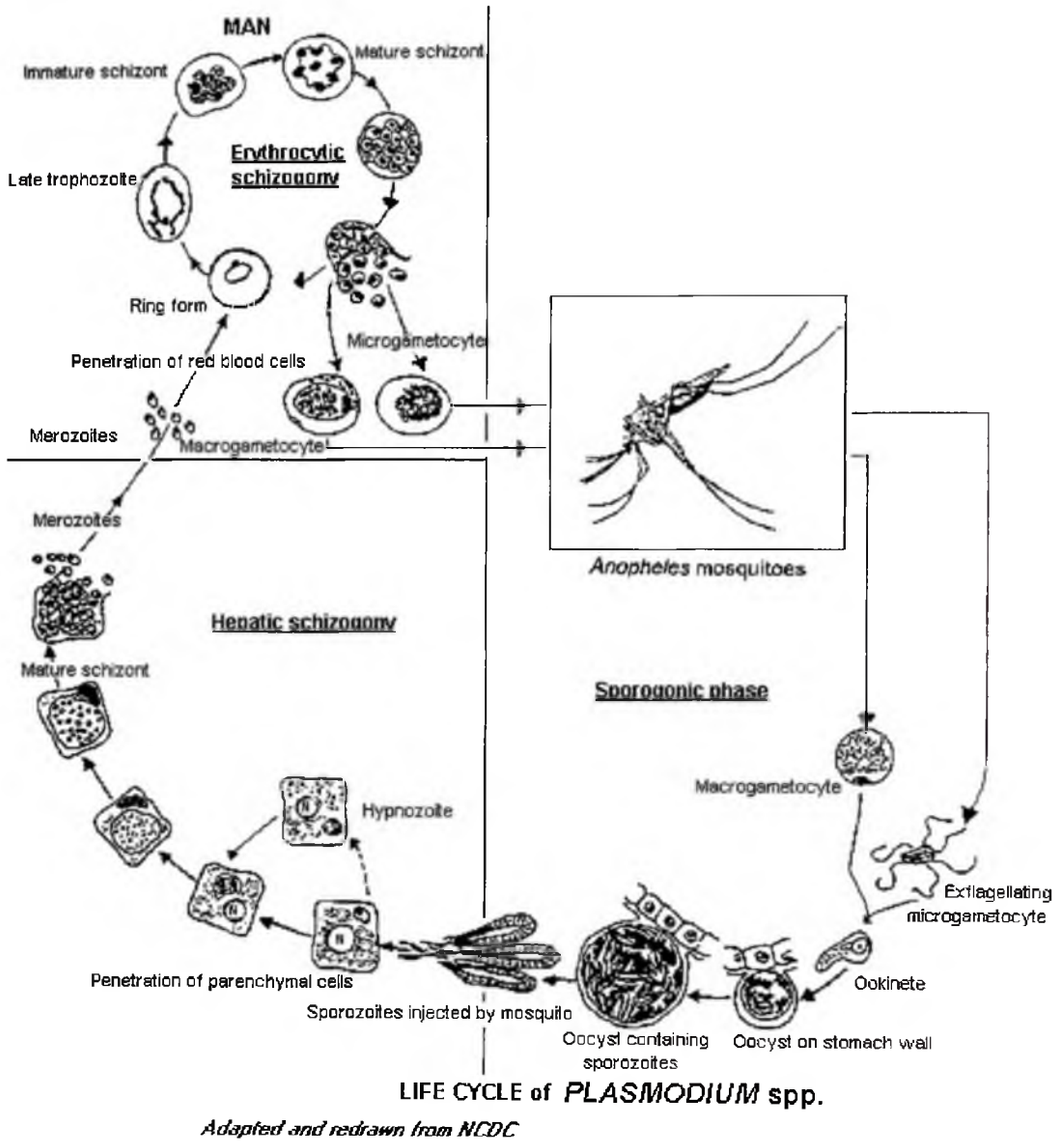

Figure 2: Life cycle of *Plasmodium* species

2.5 The Life Cycle of *Anopheles* Malaria Vectors

Anopheles mosquitoes have four distinct stages in their life history – the egg, the larva, the pupa and the adult (Figure 3). The first three stages occur in water while the adult is an active flying insect. They have a large range of breeding sites but the most common are the shallow open sun lit pools (Service, 1993).

After mating and a blood meal, gravid *Anopheles* lays some 50 – 200 small (1 mm long) brown or blackish boat-shaped eggs on the water surface (Service, 1980). Each blood meal provides enough nutrition for a female mosquito to lay a batch of eggs (White, 1998). In most *Anopheles* there is a pair of conspicuous lateral air-filled chambers called the floats on the eggs, which in few species completely extend round the egg. These floats help maintain eggs floating on the water surface. *Anopheles* eggs cannot withstand dessication and in tropical countries they hatch after 2 to 3 days, but in colder temperate climates hatching may not occur until about 2 to 3 weeks, the duration depending on temperature (Service, 1980). All mosquito larvae are aquatic and metapneustic and pass through four larval instars (Service, 1993). The larvae are filter feeders and unless disturbed remain at the water surface feeding on bacteria, yeasts, protozoa and other microorganisms and also breathing in air through their spiracles (Service, 1980). Rates of larval growth are influenced by environmental factors such as temperature, photoperiodicity, food supply, degrees of overcrowding and the species (Service, 1993). Pupation occurs at the water surface and the whole process takes about 3 to 5 minutes. Pupae are non-feeding and normally spend most of their time at the water surface breathing through the paired trumpets (White, 1998). Pupal duration is mainly determined by temperature. In tropical countries it is usually 2 to 3 days, but can be as short as 26 hours at 30°C

(Service, 1993). The duration also varies according to species. Male larvae generally pupate before females and in most species male pupal life is shorter than female pupal life. When this process is complete, the fully formed adult emerges from the pupal case. Emergence usually takes 12 – 15 minutes and within minutes afterwards the newly emerged adult can make very short flights. The teneral adult usually seeks shelter amongst vegetation until ready for mating, which in the case of the female usually occurs a few hours after emergence or sometimes much sooner (Service, 1993). Males do not mate until their genitalia have rotated through 180°C, a process that take between 20 – 24 hours but in some species as little as 6 – 12 hours (Service, 1963). Mating is often preceded or accompanied by swarming. Most male mosquitoes usually die after mating. The female require a blood meal for ovarian development, followed by the maturation and oviposition of a batch of eggs.

There is a usually heavy mortality, especially among larvae due to predators, disease, drought etc. Larval loss due to predation is one of the factors that reduce the numbers of larvae that develop into adults. It is recognized that predation of larvae in established pools is an important factor in limiting their number. For example, *Culex tigripes* sometimes colonises the same pools as *An. gambiae*, causing a dramatic reduction in *An. gambiae* larval density (Haddow, 1942). It may be noted that the agility displayed by *An. gambiae* larvae in contrast to species such as *An. funestus* tend to increase their vulnerability to attack by predators (Service, 1980). Adult females *Anopheles* can live for several weeks to several months, dependent upon the prevailing environmental conditions.

Adult *Anopheles* usually rests with the body at an angle to the surface with proboscis and abdomen in a straight line. In some species, they rest at almost right angles to the surface, whereas in others such as *An. culicifacies* the angle are much smaller (Kettle, 1992). Most *Anopheles* are crepuscular or nocturnal in their activities, thus emergence from the pupae, mating, blood feeding and ovipisition normally occur in the evenings, at night or nearly in the morning around sunrise. Some species such us *An. albimanus*, a malaria vector in Central and South America, bite man mainly outdoors (exophagic) from about sunset to 21 hours whereas others will rest outside (exophilic) in a variety of natural shelters, such as amongst vegetation, in rodent burrows, cracks and crevices in trees, under bridges, in termite mounds, and other cracks in the ground. Most *Anopheles* species are not exclusively exophagic or endophagic exophilic or endophilic, but exhibit a mixture of these extremes behaviour. Similarly, *Anopheles* do not feed exclusively on either man or animal, most feed on both man and animals but the degree of anthropophily and zoophily varies according to species (Coluzzi *et al.*, 1999).

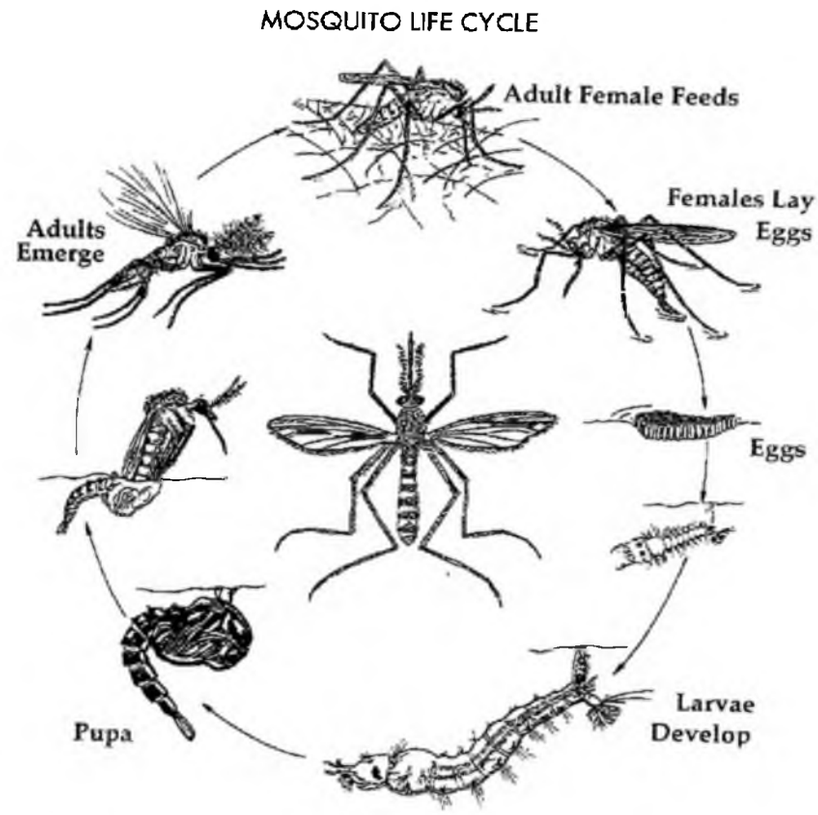

Figure 3: Schematic illustration of the life cycle of *Anopheles* vectors of malaria (After Service, 1980).

2.6 Environmental Factors Influencing the Abundance of Malaria Vectors

The major malaria vectors are characterized by some degree of association i.e. anthropophily with humans, which presumably evolved from primatophilic ancestors and/or as a by-product of adaptation to human environments (Constantini *et al.*, 1997). Humans act as a constant evolutionary challenge, as they provide a source of environmental change and heterogeneity to which anthropophilic *Anopheles* have to respond with a highly vector-host relationship (Coluzzi, 1992). Humans, through activities such as clearing forested areas for agro-related activities either create ideal conditions for the vectors or often exacerbate malaria situation by their moving into areas heavily populated by vectors (Rahman *et al.*, 1993).

Malaria is governed by a large number of environmental factors, which affect its distribution, seasonality and transmission (Snow *et al.*, 1999). Any beneficial impact of development in significantly reducing in transmission potential of *Plasmodium* has only been observed with vector species that have complex larval habitats vulnerable to pollution and/or land exploitation. This is the case for the malaria vectors in the Mediterranean area, namely *An. sacharovi*, *An. labranchiae* and *An. superpictus* (Bruce-Chwartt & Zulueta, 1980). The opposite trend is however observed in various tropical vectors and particularly in the most anthropophilic species of the *An. gambiae* complex, which usually exploit man-made larval habitats such as bare-edged, temporary, freshwater pools etc, exposed to sunlight. The spread of these breeding sites is clearly favoured by agricultural practices associated with deforestation, desalination of coastal areas and irrigation of arid savannas. The result is an increase in recent decades of malaria transmission potential in many areas of

Africa south of Sahara because of a progressive adjustment of the main vectors in the *An. gambiae* complex to man-made ecological changes (Coluzzi, 1984).

Changes in environmental conditions are important determinants of the ecology of living organisms, which adapt to the different ecosystems leading to an evolution of distinct genetic variabilities within species. More obvious modifications of the vectorial system have been reported in relation to man-made ecological changes in the forest zone of Southern Nigeria and in the lagoon area of Cotonou (Coluzzi, 1992). Both instances were the direct consequences of urbanization. Studies in southern Nigeria revealed unexpected concentrations of *An. arabiensis* in urban and periurban situations within the ecological zone formed by the West African forest belt (Coluzzi, 1992). This zone is normally occupied solely by the Forest form of *An. gambiae* s.s. which is characterized by the standard chromosome-2 arrangement. This taxon was however, found breeding in small forest villages around the urban periphery and its absence from the central part of towns was thought to be due to either competitive exclusion by *An. arabiensis* or the possibility that any genetic adaptation to the urban environment was continuously disrupted by gene flow from forest-adapted genotypes. In the lagoon area of Cotonou, *An. gambiae* s.s., polymorphic for the Savanna chromosomal arrangements 2Rb-2La, replaces the less effective vector *An. melas* in lagoon zones where pile-dwelling traditional villages have been converted into unplanned urban settlements (Coluzzi, 1992).

Other observations of adult populations of *An. gambiae* s.s. and *An. arabiensis* indicate a spatial or temporal separation of these species. In Mali, Toure *et al.* (1998) found that the savanna form of *An. gambiae* s.s. predominated in relatively humid

areas with larval production occurring almost exclusively during the rainy periods, whereas *An. arabiensis* prevailed in more arid areas and reproduced throughout the year. In Tanzania, *An. arabiensis* was common during the short rains and just before the long rain, whereas *An. gambiae* s.s. predominated during and just after the long rains (White *et al.*, 1972). Studies carried out in Nigeria, indicated that *An. gambiae* s.s. is common during the long rains, with populations of *An. arabiensis* increasing as the rains receded (White and Rosen, 1973). In Kenya (at Kisumu) Haddow (1942) reported higher populations of *An. gambiae* s.s. with at least five inches of rain per month than with less than that amount.

Anopheles gambiae exists only in frost-free regions (Gilles & de Meillion, 1968), or where the minimum temperature in winter remains above 5°C (Leeson, 1931). The overall relationship between mosquito abundance and rainfall has been demonstrated on several occasions (Molineaux & Gramiccia, 1980; Le Sueur & Sharp, 1988). Rainfall provides breeding sites for mosquitoes and increases the humidity, which enhances their survival. Temperature affects the transmission cycle of *P. falciparum* in many different ways, but the effects on the duration of the sporogonic cycle of the parasite and the vector survival are particularly important (Snow *et al.*, 1999). Temperatures below 22°C are the determining factors of the number of mosquitoes surviving parasite's incubation period, which takes 55 days at 18°C and ceases around 16°C (Detinova, 1962). After 55 days the proportion of a cohort of mosquitoes that survives is only 0.003 (Martens, 1997).

The breeding habitats of *Anopheles* malaria vectors vary from large and usually permanent collections of water, such as fresh water swamps, marshes, rice fields and

borrow pits to smaller collections of temporary water such as small pools, puddles, water filled car tracks, ditches drains, galleys, hoof prints, etc. The most common breeding sites are the shallow open sun lit pools (Service, 1993). Larvae occur in wells and man-made container habitats such as clay pots, motor vehicle tyres, water storage jars and tin cans (Chinery, 1984). Most observations of the larval habitats of *An. gambiae* s.l. have noted a preference for temporary, sunlit pools (Gillies & DeMeillon, 1968; Gillies & Coetzee, 1987). However, not all suitable-appearing, sunlit pools examined in the Freetown, Sierra Leone area, were positive for *An. gambiae*. Generally, many larvae would be found in one pool whereas other similar-appearing pools were completely negative for larvae (Muirhead-Thompson, 1945).

2.7 Control of Malaria

In principle, there are two main approaches to the control of malaria, chemotherapy and vector control. Chemotherapy using antimalarial drugs has been the main method for parasite control and for clinical management of the disease. Vector control aims in eliminating vector or reducing human vector contact using various methods including insecticide, environment management, bednets etc. Various attempts through national programmes have been made to control the disease worldwidely, using these approaches.

2.7.1 Historical background to malaria control programmes

Systematic control of malaria begin after the discovery of the causative parasite by Alphonse Laveran in 1880, the demonstration by Ronald Ross of its complete cycle in 1887 and its specific dependence on *Anopheles* mosquitoes by the Italian malariologistes, Giovanni Battista Grassi, Amico Brignami and Giuseppe Bastianelli (Teklehaimanot & Bosman, 1999). With the discovery of the organochloride insecticide, dichlorodiphenyltrichloroethane (DDT) during World War II, it was thought eradication of malaria globally through vector control was possible (WHO, 1998), therefore the WHO Global Malaria Eradication Programme was created. During this period, Africa hosted several pilot pre-eradication projects, but was never included in the programme, in spite of burden of the disease on the continent (WHO, 1998). The justifications were related to the general under-development of health services, communications and infrastructures (Gramiccia & Beales, 1988). In the end, the major donors withdrew their commitment to malaria eradication and the pilot projects were terminated and countries had to depend entirely on their own untrained technical staff, their limited infrastructure and meagre financial resources.

With the Declaration of Alma Ata in 1978, WHO promoted the idea of malaria control in the context of primary health care, but this vision was not translated into strengthening malaria control efforts in Africa (Teklehaimanot & Bosman, 1999). Because of the resurgence of malaria in various parts of the world and malaria epidemics in Africa, the World Health Assembly in 1990 requested the Director General to reorganize the programme to increase its human resource and develop an appropriate strategy to address the prevailing situation. As a result, the Global Malaria Control Strategy (GMCS) was developed through partnership and consultation involving all major development and bilateral organizations and UN agencies. This started with a meeting on "malaria waiting for the vaccine" at the London School of Hygiene and Tropical Medicine (U.K.), to debate what could be done at that time with the tools available (Targett, 1991). Then the control strategy adopted was further discussed and developed, and was finally endorsed in 1992 by over 92 Ministers of Health from endemic countries and the major international partners during the Ministerial Malaria Conference in Amsterdam, The Netherlands (WHO, 1993). It was subsequently endorsed by the World Health Assembly, the Economic and Social Council of the United Nations, the UN General Assembly and by the Organization of African Unity (Teklehaimanot & Bosman, 1999). The four technical elements of this global strategy were to provide early diagnosis and prompt treatment, to plan and implement selective and suitable preventive measures including vector control, to detect early, contain or prevent epidemics and to strengthen local capacities in basic and applied research to permit and promote the regular assessment of a country's malaria situation, in particular the ecological, social and economic determinants of the disease (WHO, 1998).

Between 1992 and 1996, there was a gap between commitments and real financial support. Initial preparatory activities were completed to support the development of national plans of action and training of programme managers. However, the limited support given did not produce any impact on the malaria situation (Teklehaimanot & Bosman, 1999). Because of resurgence of malaria and the slow progress made to combat the problem, the World Health Assembly requested the Director General to explore the possibility of establishing a special programme on malaria and to mobilize resources to support malaria control activities at country level. Thus, during 1997-8 US$ 20 million were allocated to intensify malaria control in 34 African countries. Then the Accelerated Implementation of Malaria Control was conceived with the aim of establishing sustainable foundations for malaria control in Africa and capacity building of National Malaria Control Programme (NMCP) was given the highest priority with a focus on disease management, involving health professionals from the general health services (WHO, 1998). A significant development was the declaration on malaria by Organization of Africa Unity in 1997, whereby the Heads of State committed themselves to malaria control in Africa, followed by establishment of the African Initiative for Malaria Control which includes the African countries and major partners such as World Bank, UNICEF, USAID, among others. In 1998, the G8 Summit in Birmingham discussed also the need for higher commitment to malaria control, particularly in Africa. During the same year, the Director General of WHO established Roll Back Malaria initiative, as one of the priority projects for the Organization, to be implemented as a global partnership within the context of health sector development (Teklehaimanot & Bosman, 1999).

2.7.2 Chemotherapy

Antimalarial drugs however can be grouped into four categories according to the stage in the life cycle of the malaria parasite that they attack. These are tissue and blood schizonticides, gametocytocides and sporonticidals. Drugs that destroy all exo-erythrocytic forms are referred to as tissue schizonticides. Blood schizonticides act on the asexual erythrocytic parasites and the gametocytocidals act on gametocytes, particularly the immature forms. The antimalarials when taken up in blood meal inhibit the development of oocyst in the mosquito and thereby prevent the production of sporozoites and these are referred to as sporonticidals.

Quinine is a bitter tasting blood schizonticide and in case of *P. vivax* and *P. malariae* also acts as a gametocytocide. It was originally extracted from the bark of *Cinchona ledgeriana* (Cinchona tree) and remains the only drug, which over a long period of time has remained largely effective for treating the disease (Davis, 2001). Quinine was previously widely used but has undesirous side effects including acute massive intravascular haemolysis and haemoglobinuria (black water fever). It is now used only for emergency treatment of *P. falciparum* malaria, when response to Chloroquine and most antimalarials have failed.

In the 1940's, an intensive research programme to find alternatives to quinine lead to the manufacture of Chloroquine and other chemical compounds that are more effective and less toxic (NIAID, 2000). Chloroquine became the most successful antimalarial drug ever synthesised, because of its safety, affordability, ease of use and its great efficacy (WHO, 1967; Ginsburg *et al.*, 1999; Djimde *et al.*, 2001). It was introduced in 1945 and is an excellent blood schizonticide. It is also a

gametocytocide against *P. vivax*, *P ovale* and *P. malariae*. Chloroquine assumed a major role in primary health care because of the rationale of preventing mortality and curbing morbidity and suffering of afflicted persons. However, the efficacy of Chloroquine in malaria chemotherapy has been compromised with the development of the resistance to the drug by the malaria parasites. The first documented cases of *P. falciparum* resistance to Chloroquine were in South America in the 1950s (Peter, 1970), and confirmed in Thailand in 1959 (Harinasuta *et al.*, 1962). Since then, Chloroquine resistance has spread to almost all the malarious areas of the world with heterogenic distribution both in frequency and degree (Bjorkman and Phili-Howard, 1990). Chloroquine now no longer offers protection against South East Asian *P. falciparum* and increasingly in other regions because of resistance (Navy Med. Dept., 2001). In Africa, resistant strains of *P. falciparum* were first recorded in Kenya and Sudan in 1978 (WHO, 1986) and more recently in West Africa (Cheesbough, 1991). In many parts of Africa, the drug is no longer used alone for therapy (Brasseur *et al.*, 1998). While effective in suppressing the *falciparum* in some parts of Africa and most strains of *P vivax*, resistant forms of *P. vivax* are appearing and have been reported in Papua New Guinea, Indonesia, Thailand and India (WHO, 2000). Many studies are under way to discover the cause of resistance in the parasite although how Chloroquine works at the molecular level, the biochemical and molecular mechanisms of drug resistance in *P. falciparum* remain unknown (Wellems, 1992; Wellems *et al.*, 1997; Martiney *et al.*, 1999).

In the 1980s, Halofantrin which belongs to the class of compounds called phenanthrene-methanols and not related to quinine was introduced as a blood schizonticidal against the erythrocytic stages of Chloroquine resistant *P. falciparum*

and also the erythrocytic stages of *P. vivax* but not the hypnozoites. The drug is used to treat acute form of uncomplicated and multi-resistant *P. falciparum* malaria and as a "stand by" drug if when chemoprophylaxis fails and there is no medical aid available. Its short half-life of 1 to 2 days however does not make it suitable as a prophylactic (Davis *et al.*, 2001). Halofantrin has been associated with neuropsychiatric disturbances and also it is contra-indicated during pregnancy and not advised for women who are breastfeeding (WHO, 1998).

Proguanil was first synthesised in 1946 and introduced in 1948 under the trade name Biguanide. It destroys the early tissue stages, especially *P. falciparum* and hence is used as causal prophylactics. It also acts as blood schizonticide working more slowly than chloroquine and as a sporontocide (Navy Medical Dept., 2001) and it is free from unpleasant side effects. Proguanil is combined with chloroquine to prevent transmission by killing gametocytes. It is still used as prophylactics in some countries.

Pyrimethamine was introduced in 1952 as a diaminopyrimidine with similar activities as Proguanil. It is a drug that affects the synthesis and utilisation of folate. Pyrimethamine (2,4, diaminopyrimidine) acts by inhibiting the dihydrofolate reductase necessary for synthesis of tetrahydrofolate, a precursor in the parasite DNA synthesis (Navy Medical Dept., 2001). Some of side effects are skin rashes and higher doses will affect human dihydrofolate releases leading to megaloblastic anaemia. Folate supplement is usually given in pregnancy to reduce the efficacy of the drug (Nwanynwu *et al.*, 1996; Basco & Rignald, 1998). Resistance to Pyrimethamine has been reported in Africa (WHO, 1990).

Fansidar is a blood schizonticide and it is a combination of Pyrimethamine and Sulphadoxine that was pressed into service as the first line of defence against Chloroquine resistant *P. falciparum* parasite in South America and South East Asia (Plowe *et al.*, 1997). It is used for uncomplicated malaria that cannot be cured by chloroquine, or as first line treatment where Chloroquine has been dropped from use (Krogstad, 1996). Pyrimethamine-dapsone (Fansidar) is combined with Chloroquine to prevent transmission by killing gametocytes. In Southeast Asia where parasites are often resistant to both Pyrimethamine and Sulphadoxine, Fansidar is clinically useless (Winstanley, 1996). Resistance to Fansidar is now widespread and serious side effects have also been reported (WHO, 1998).

The infusion of qinghoo *Artemisia annua* has been used in China for at least the last 2000 years (WHO, 1999) and its active ingredient " qinghaosu" also known as Artemisinin has recently been identified. The two most widely used derivates of Artemisinin are Artesunate and Artemether and both are blood schizonticides. While they are widely used in Southeast Asia, they are not licensed in much of the "western world" including Australia. A high rate of treatment failures has been reported and it is now combined with Mefloquine for the treatment of *P. falciparum* malaria (WHO, 1998).

Mefloquine was first introduced in 1971 and is a quinoline methanol derivative structurally related to quinine. It is a blood schizonticide and is also effective in killing hypnozoites if given as a combination treatment with Primaquine. The compound was found to be effective against malaria that was resistant to other drugs

and because of its long half-life it is also a good prophylactic. There have been reports of acute brain syndrome, which is estimated to occur in 1 in 10,000 to 1 in 20,000 of the people taking this drug (WHO, 1998). Multi-drug resistance is an acute problem in some areas, particularly in Southeast Asia, where *P. falciparum* exhibits resistance to virtually all available antimalarials, including mefloquine (Chids *et al.*, 1991; Peters, 1985). Widespread resistance has now developed to Mefloquine and this together with undesirable side effects has resulted in a decline in its use (Fernandez, 2001).

A combination of Proguanil and Atouaquone known as Malarone was first introduced in 1998 in Australia. Atouaquone was introduced in 1992 and it was used with success for the treatment of *Pneumocystis carrinii*. When combined with Proguanil there is a synergistic effect and the combination is at present a very effective antimalarial treatment. Malarone has undergone several large-scale clinical trials and has been found to be 95 % effective in drug resistant *P. falciparum* malaria (WHO, 1998). It has been claimed to be largely free from undesirable side effects even though Proguanil is an Antifolate.

Mepacrine that was introduced in 1935 and used as a prophylactic on a large scale during the World War II (WHO, 1998) is now considered absolute. This drug, which is an effective blood schizonticide has disadvantages such as lading down in the skin and the recipient turns bright yellow. It is now considered to have too many undesirable side effects and is no longer used.

Chemoprophylaxis as a control strategy has been attempted but it still remains a subject of debate. Prophylaxis is generally considered necessary for pregnant women but that for children is debated because of the risk of long-term side effects and of selection for resistant parasite strains (Carnevale & Mouchet, 1987). Countrywide prophylaxis is expensive and also requires strong organization. Maintaining the involvement of the community is also difficult, because at any one time only a proportion of the community participates in drug distribution programs. In addition, drug administration can prevent natural immunity from occurring and may simply delay disease until children are older (Carnevale & Mouchet, 1987).

2.7.3 Vector control

Malaria eradication was achieved in southern European countries in the early 1950s using insecticide spraying (Coluzzi, 1992). Successes with vector control have also been reported in Malaysia, Brazil, Egypt, Cuba and Panama. However, in many other areas attempts to eliminate the vector or reduce transmission have met with limited success and frequent failures (Harrison, 1978; Bynum & Fantini, 1994, 1998). Insecticide formulations with long-term residual activities are sprayed on surfaces, such as walls of houses that the potentially infectious mosquito is likely to encounter. This strategy targets the weak link in the transmission cycle, i.e. the requirement for long-term survival of the infected mosquito for complete parasite development (Collins & Paskewitz, 1995).

Prior to the Second World War, control was based mainly on antilarval measures, including source reduction, while pyrethrum spray as an adulticide was tried on a small scale in certain areas with variable results (Zahar, 1984). During 1940s, with availability of DDT, malaria control by house spraying was initiated on small scale in certain countries and on a larger scale in others such as Madagascar, Mauritius, South Africa (Natal and Transvaal), Swaziland, and Zimbabwe, formerly Southern Rhodesia (Bruce-Chwatt, 1963). DDT became widely used because of its cheapness per unit weight and its durability, which enabled spraying to be carried out twice a year, or only once in areas with a short annual malaria mosquito season (Curtis, 1996). Early studies demonstrated that DDT was also a repellent, irritant and has toxic effects on malaria vectors (WHO, 1998). In 1960s and 1970s there were claims about supposed effects of DDT on human health such as residues in human breast milk, which was usually attributed to contaminated food (Curtis, 1994). Resistance to

DDT developed in the insects, which rendered it ineffective for vector control. DDT resistance in *An. gambiae* s.l. has also been reported in areas in West Africa where it was used in house spraying (Ivorra Cano and Bakri, 1975 unpublished report to WHO). The use of other pesticides in agriculture coupled with the extensive use of DDT in cotton production areas were believed to have contributed to the selection pressure for resistance (Zahar, 1984). DDT was then replaced with other classes of insecticides and later on they were also reported to be ineffective to the resistant mosquito vectors.

Larval control with paris green combined with pyrethrum house spraying was extremely successful in eliminating *An. gambiae* from Brazil (Soper & Wilson, 1943; Soper, 1949; Gratz & Pal, 1988), and paris green was the only agent used in the eradication of *An. gambiae* from a focus in Egypt in 1944-1945 (Soper, 1949; Gratz & Pal, 1988; Russell, 1995). In general, organophosphates (especially temephos) are mostly widely used larvicide, but DDT, dieldrin, larvicidal oils, arsenical compounds, and development inhibitors have all been used with varying degrees of success (Russell, 1995).

Other measures, such as breeding habitat modification and biological control agents, have been effective in limited settings, at eliminating or drastically reducing malaria prevalence in several regions. Synthetic repellents are usually used by residents and visitors to endemic regions. Plants as natural products or synthetic repellents are used in many areas such as the use of Citronella products in India against anopheline mosquitoes (Curtis *et al.*, 1990). In Tanzania, smoke from burning plants are used to protect against mosquito bites. However, the effectiveness of these methods is

probably limited and will depend on both the biology of the local vectors and the intensity of transmission (Collins & Paskewitz, 1995).

Large-scale use of insecticide impregnated bed nets (IIBNs) in endemic countries is being promoted as a means to control malaria. Studies carried out in Senegal (Alonso *et al.*, 1991) and China (Cheng *et al.*, 1995) initially demonstrated the efficiency of IIBNs in reducing infant mortality. These findings were subsequently confirmed by a large-scale multicenter study in six countries across Africa including Ghana (Binka *et al.*, 1996; Lengeler *et al.*, 1996; Nevill *et al.*, 1996). Nevertheless, acquisition of new infections still occurred at a very high rate during the high transmission season. For example, it was estimated that in Kenya 100 % of the children should have been infected with *P. falciparum* with 13.6 weeks in the bed net villages compared with 10.6 weeks for the controls (Beach *et al.*, 1993). Also in Burkina Faso, where transmission levels are also high, the use of bed nets was found not to have impacted on *P. falciparum* malaria incidence (Curtis *et al.*, 1990). The behaviour of the vectors largely affects the success of the control method. During the high transmission season, substantial members of vectors may be feeding outdoors, often during the early evening. Thus, bed nets may be most useful in areas where transmission is less stable, seasonal or of low intensity. Problems include non-compliance in proper use of the bed nets and failure to maintain the insecticide dose, which can be reduced substantially during net washing (Curtis *et al.*, 1990).

Another notable possibility is introducing malaria refractory genes into natural populations of vectors (James, 1992; Crampton *et al.*, 1994). Progress is being made towards identifying refractory mechanisms and their underlying genetics (Vernick *et*

al., 1995) and in developing the technology to introduce genes into the mosquito genome (Miller *et al.*, 1987). However, strategies for integrating selected refractory genes into field populations are lacking. The possibility of population substructuring is an important complication as well as the dynamics of the rate to fixation of a gene introduced into a population that consists of two or more subpopulations maintained by mating barriers, which will be very different than the dynamics in a population of randomly mating individuals (Lanzaro *et al.*, 1995).

Research has also turned toward the vector, with the main aim of using recombinant DNA technology to replace a highly competent vector population with an identical population engineered to be an incompatible host for the malaria parasite (Besansky *et al.*, 1992; Coluzzi, 1993; Crampton *et al.*, 1990; James, 1992; Eggleston, 1991). Several genetic control research projects targeting *Anopheles* mosquitoes have been carried out, none with any particular success (Collins & Paskewitz, 1995). The basic idea is to drive a genetic construct into the existing vector population. This construct carries a parasite-inhibiting gene or genes and will not significantly affect the vector population's fitness. In short, this control strategy targets the parasite within the mosquito rather than the mosquito itself (Graves & Curtis, 1982). This control strategy is unlikely to bear fruit for decades, and the malaria control tools that do emerge from this effort may be entirely unlike what is imagined today (Collins & Paskewitz, 1995).

Considerable resources have been devoted to the search for other alternative methods of controlling malaria vectors. For example, biological control agents have been in use and to date only larvivorous fish have been used successfully in malaria control

projects and theses cases are few. Hackett (1931), Hadjinicolaou & Betzios (1973) and Wickramasinghe & Costa (1986) have all reported that the use of the North American fish *Gambusia affinis* successfully reduced malaria incidence in Italy and Greece, where malaria transmission was unstable. Rishikesh *et al.* (1988) have summarized efforts to identify useful pathogens and parasites, including virus, fungi, nematodes and protozoa. The main pathogens under study include the fungi *Coleomomyces* spp., *Lagenidium* spp., *Culicinomyces clavosporus,* and *Metarhizium anisopliae*, the protozoan *Nosema algerae* and the neamtode *Romanomermis culicivorax*. None of these agents have shown any promise for malaria control, having proven difficult to rear and store, as well as being unstable or inefficient in the field. The bacterial endospore toxins produced by various strains of *Bacillus sphaericus* and *B. thuringiensis israelensis* have been used as larvicidal agents in some situations (de Barjac & Sutherland, 1989). Unfortunately, the *Bacillus* toxins are still relatively expensive and because they have no residual activity, they either require frequent application or are only suitable for environments where a one-time control measure produces a valuable outcome (Collins & Paskewitz, 1995).

Malaria vectors exhibit a wide variety of life history strategies therefore, there is no simple and universal applicable form of vector control. In those parts of the world where malaria has been eradicated the previous important malaria vectors still remains – in many cases, in numbers as great as during the periods when malaria was endemic (Collins & Paskewitz, 1995). Although vector analysis is part of the process of malaria control, this has not always been acknowledged as shown by the recent history of malaria control which has been characterized by the uncritical simplification of success stories that are misleading to the general application of

single control tools (Coluzzi, 1992). The proliferation of "man–made-malaria", which accompanied the push for economic development in most of the endemic countries, spurred the need for the control interventions and while great successes were obtained in many specific projects, the general campaigns proposed by the enthusiasts of vector control faced increasing difficulties in their practical implementation in the field (Najera, 2001). The value and relevance of malaria vector control have not been clearly recognized and its effectiveness has declined for reasons including poor use of available alternative control tools, inappropriate use of insecticides and reduced effectiveness due to vector resistance and lack of an epidemiological basis for vector control interventions.

The problems of malaria control are further aggravated by changing environmental conditions in areas in which exploitation of natural resources and development activities are taking place. Expending agriculture, the cleaning of forest, or the building of dams and irrigation schemes, and unplanned urban development provide mosquitoes with new breeding grounds, while at the same time bringing more people into contact with them. The emergence of resistant vectors has compromised the use of most applicable insecticides and newer generation replacements are consistently more costly and are often less effective or exhibit greater toxicity on non-target fauna.

2.8 Species Identification and Relevance to Vector Control Programmes

Species are considered as biological units, defined by intrinsic mechanisms of reproductive isolation and characterized by some kind of discrete genetic difference that is not necessarily expressed at the morphological level (Mayr, 1996). Although morphological features appear to be in many cases very useful tools for the characterization of species, the genetic analysis of various groups of closely related species or species complexes shows that reproductive isolation may be acquired without or before morphological divergence, resulting in speciation that is undetectable through morphological observation alone (Mayr, 1996). Species complexes containing morphologically cryptic species that vary in their behaviour and vectorial capacity present a very real problem to malaria control programme managers (Coetzee *et al.*, 2000). The identification of sibling species is of major malariological importance (Coluzzi *et al.*, 1979). Morphological similarity generally implies close phylogenetic relationships and recent speciation processes, but it does not imply similarity or identity of bionomics when dealing with sympatric taxa. Such differences are important in the epidemiology of malaria. Failure to recognize sibling species of *Anopheles* may result in the failure to distinguish between a vector and a non vector species of malaria as was the case with the *Anopheles maculipennis* complex (Hackett, 1937; Bates, 1940).

A thorough understanding of the malaria problem and risk, sound knowledge of molecular biology of the vector, human host and environment are prerequisites for effective planning and targeting of vector control interventions (WHO, 2000).

2.8.1 *Anopheles gambiae* complex and its distribution

The *Anopheles gambiae* complex was initially considered as a single species until 1944 and presently, six formally named species, as well as forms within them. There are morphologically indistinguishable yet genetically and behaviourally distinct mosquito species that vary dramatically in their importance as vectors of malaria and in distribution in Africa (Coluzzi *et al.*, 1979; Service, 1985).

Members of *An. gambiae* complex have a wide geographical distribution and have been reported from the most African countries and adjacent islands including Madagascar, as well as Saudi Arabia and Yemen (Coetzee *et al.*, 2000). The two most widespread are *An. gambiae* and *An. arabiensis*. These species breed in temporary water often associated with human disturbances, from the southern limits of the Sahara Desert south to most parts of the continent including Madagascar. They are largely sympatric in the narrowest sense: both are found as larvae in the same pools and as adults in the same huts (Powell *et al.*, 1999). *Anopheles gambiae* predominates in forest and humid savanna zones whereas *An. arabiensis* is more successful in arid savannas and steppes, including those of southwestern part of Arabian Peninsula (Coetzee *et al.*, 2000). *Anopheles arabiensis* is recorded more often than *An. gambiae* in areas where rainfall is less than 1000 mm and the reverse is observed where rainfall is grater than 1000 mm (Gillies & Coetzee, 1987; Hunt *et al.*, 1998).

Anopheles quadriannulatus has a narrower distribution in southeast Africa, Ethiopia and Zanzibar. This geographical discontinuity has apparently produced allopatric speciation, as shown by Hunt *et al.* (1998) through crossing experiments. Both *An.*

quadriannulatus species are generally sympatric with *An. arabiensis* and less frequently with *An. gambiae* s.s.

Anopheles merus and *An. melas* are salt and blackish water breeders confined to the East and west coasts respectively of Africa (Powell *et al.*, 2000), although *An. melas* has been found breeding in fresh water streams in the Gambia (Chinery, 1984). Because of their ecological differentiation into salt water, neither *An. merus* nor *An. melas* are sympatric as larvae with the other members, although adults of these species may encounter both *An. gambiae* and *An. arabiensis* adults in certain situations (Hunt *et al.*, 1998). *Anopheles melas* has a short dispersal range from preferred breeding sites and adults are usually not found at distances more than 3 km from the saline environment (Bryan, 1987). *Anopheles bwambae* is known only from the Semliki forest in the Rift valley near the Zaire border where it breeds in geothermal mineral springs (Coluzzi *et al.*, 1979; White, 1985).

2.8.2 Cryptic taxa within *An. gambiae* s.s. and their epidemiological implications

In the early 1980s studies showed that in West Africa further taxonomic complexity may exist within *An. gambiae* s.s. Subsequent genetic and behavioural variations observed within this species were found to be associated with different cytological forms (Forest, Savanna, Bamako, Mopti and Bissau)which showed restricted or no inter-breeding in the field and whose distribution depended on environmental factors such as climate, breeding sites, etc. (Toure *et al.*, 1994). The evidence for cryptic taxa within *An. gambiae* s.s. is the observation that the various gene arrangements of the 2nd chromosome (differing by inversions) were far from a Hardy-Weinberg equilibrium at certain times of the year in some areas of West Africa (Coluzzi *et al.*,

1999). The strong association of certain karyotypes with specific habitats led to the description of distinct chromosomal forms of *An. gambiae* known as eco-phenotypes. Three ecophenotypes; Bamako. Mopti and Savanna occur in sympatry at numerous sites in Mali, West Africa. Studies of inversion karyotype frequencies at these sites also revealed a deficit of heterokaryotypes relative to Hardy Weinberg expectations (Toure, 1991).

The relative frequencies of these ecophenotypes also vary ecologically and seasonally (Toure *et al.*, 1983, 1994, 1998; Coluzzi *et al.*, 1985). The Savanna form is typically found away from major rivers and flooded or irrigated areas and is fully dependent on rainfall for larval breeding, and therefore, this form usually is absent during the dry season. The Bamako form is closely associated with riverine basins and breeds monthly from mid- to late rainy season. Its distribution coincides with the occurrence of the larval habitats, which are quite unusual for *An. gambiae* s.s. The habitats are mainly slowly moving water and residual pools along the laterite edges of riverbeds. The Mopti form is closely associated with flooded plains and irrigated fields. It is the only form of *An. gambiae* s.s. which is more arid-adapted than *An. arabiensis*, which it could competitively displace (Toure *et al.*, 1998). Two other forms of *gambiae* s.s. namely Forest and Bissau have also been recently proposed. The Forest form refers to forest-breeding An. *gambiae* s.s. which is nearly fixed for the 2R/2L standard chromosomal arrangements. The Bissau form has been recorded in Gambia and Senegal where it is associated with rice fields along the Gambia River (Bryan *et al.*, 1982).

An extremely important implication is that populations of *An. gambiae* s.s. are not homogeneous entities as so often assumed in epidemiological models and in planning of control measures (Coluzzi *et al.*, 1999). The fact that often the vector population is an heterogeneous mix of units, ranging from intraspecific polymorphism to cryptic taxa, affects both the efficiency of disease transmission and the relative value of the control measures (Coluzzi, 1984; 1992). For example, the evolution form of *An. gambiae* that breed through the dry season i.e. the Mopti form can produce year-round transmission when ordinarily it is interrupted in the dry season (Toure *et al.*, 1996). As environments vary spatially and temporally, different genotypes come to predominate, such that the total population fitness is buffered from such perturbances. The fact that different karyotypes have behaviours that differentially place them in contact with humans clearly has important medical implications (Coluzzi *et al.*, 1999). It has also been demonstrated that different karyotypes display differences in frequency of having human or animal blood meals (Petrarca & Beier, 1992). This leads to different karyotypes being differentially infected with *Plasmodium falciparum* (Toure *et al.*, 1986; Petrarca & Beier, 1992). In addition to differences in blood meal choice, these behavioral differences also affect the efficacy of insecticide spraying (Coluzzi *et al.*, 1999).

The adaptive flexibility shown by *An. gambiae* s.s. has important epidemiological implications. The adaptability is clearly exhibited in the species ability to rapidly adapt to man-made environments. While some populations still breed in ancestral habitats, the vast majority of populations of both *An. gambiae* s.s and *An. arabiensis* breed in human disturbed environments. This has meant that as humans clear more forest for agriculture, desalinate more coastal regions, and also irrigate more arid

savanna, they are expanding the geographic range of these highly efficient vectors of malaria (Powell *et al.*, 1999). There has been much discussion recently of genetically manipulating vector populations to make them less efficient vectors of the disease. If species are sharing genes, even if only occasionally, clearly this will have important consequences for any genetic replacement program. The target species could relatively quickly regain the ability to transmit by acquiring the gene or genes from another species. This is especially true if manipulated gene or genes are at a selective disadvantage compared to the wildtype gene they replace.

2.8.3 Characterization of *An. gambiae* s.s. populations by microsatellite DNA analysis

Microsatellites are defined as simple tandemly repeated DNA sequence elements usually as a dinucleotide or a trinucleotide (e.g. $[GT]_n$, $[GAC]_n$, etc.) found abundance in the genomes of just about every known organism and organelle (Zheng, 1997; Chambers & MacAvoy, 2000). Some researchers defined them as 2 – 8 bp repeats (e.g. Armour *et al.*, 1999) and others as 1 – 6 bp repeats (e.g. Goldstein & Pollock, 1997) or even 1 – 5 bp repeats (e.g. Schlotterer, 1998). Microsatellite loci have been described as "ideal" markers to measure population level phenomena (e.g. population structure) due to their high polymorphism, codominance, abundant presence throughout the genome and relative ease in scoring (Bughanan *et al.*, 1994; Scribner *et al.*, 1994; Lanzaro *et al.*, 1995). They have come into prominence over the last decade because scientists have found them to be remarkably versatile molecular tools and applications range from their use as highly accessible genetic markers for chromosome segments identification of individuals to tracking the biological history of populations. They are usually highly polymorphic in length due

to a variation in the number of repeats within a given microsatellite locus as a result of uneven crossover (Jeffrey *et al.*, 1985) or slippage of the DNA polymerase during replication (Tautz, 1989). The high polymorphism of microsatellite results from high mutation rates, estimated to range from 10^{-2} to 10^{-5} locus/gamete/generation (Dallas, 1992; Weber & Wong, 1993) with most estimate being between 10^{-3} and 10^{-4} (Lehmann *et al.*, 1996). This polymorphism also leads to an increased probability of finding heterozygous individuals and the microsatellite markers can be mapped by recombination since they are generally inherited in a co-dominant Mendelian fashion (Weissenbach *et al.*, 1992). The high degree of polymorphism has also made microsatellite DNA useful markers for evolutionary and phylogenetic studies of organisms and in the study of the origins of different human populations (Santos *et al.*, 1997).

Several markers have been used in the characterization of *An. gambiae* s.s. populations from both East and West Africa (Lanzaro *et al.*, 1995; Kamau *et al.*, 1999; Lehmann *et al.*, 1998). The use of microsatellites in *An. gambiae* s.s. studies is now extensive, and these types of markers are increasingly being recognized as valuable for studies on other vectors (Wang *et al.*, 1999). They are becoming the markers of choice for high-density genome mapping for *An. gambiae* (Zheng *et al.*, 1993). Lanzaro *et al.* (1995) have also demonstrated high polymorphism, codominance, abundance throughout the genome of *An. gambiae* and the relative ease in scoring made the authors conclude that microsatellite loci are superior to allozymes for population studies.

Polymorphisms in microsatellite are being used to generate a genetic map of the *An. gambiae* s.s. Microsatellites have all potential as tool for studying the population genetics of this malaria vector. Microsatellite polymorphisms provide a more sensitive measure of divergence and therefore can potentially distinguish more effectively populations that may have recently diverged (Lanzaro *et al.*, 1995). The large number of loci, high degree of polymorphism, and abundance of low and intermediate frequency alleles also suggest that microsatellite will provide a superior tool for estimating gene flow (Wright, 1951) and determining allele distribution (Slatkin, 1985).

Microsatellite DNA analysis may be a PCR-based method that uses primers and scoring the different band sizes of the amplified products for subsequent analysis. Several computer-based algorithms including POPGENE (Population Genetic Analysis) etc, are currently available for analysing microsatellite DNA data. The output from POPGENE includes genotype and allele frequencies, effective allele number, polymorphic loci, genetic distance, expected homozygous, expected heterozygous, differentiation indices (F_{st}), heterozygosity deficiency or excess (F_{is}), Gene flow, etc which aid in characterizing different populations

CHAPTER THREE

MATERIALS AND METHODS

3.1 The Study Sites

Mosquito larvae and pupae together with water from their habitats were collected at fifty-four breeding sites in the Greater Accra Region and in the Apam District in Central Region. The Greater Accra and Central Regions are adjacent to each other and both are located in the southern part of Ghana and all sites are located in the coastal savanna ecological zone, which is characterized by dry climatic conditions receiving the least amounts of rainfall in Ghana (Dickson & Benneh, 1988). It has two rainfall peaks. The first occurring April to June and the second from September to October, with the total amount of rainfall ranging between 740 and 890mm a year. The lowest mean monthly temperature (about 26°C) is recorded during August and the highest (about 30°C) between March and April. The relative humidity throughout the year ranges between 65 and 75% in the afternoons. The vegetation consists mainly of grass with isolated patches of scrub and sparse trees.

3.1.1 Description of larval and pupal habitats

The study area was divided into zones and each zone was surveyed on foot to locate breeding sites. Samples were obtained from a variety of breeding sites, including gutters, marshes, ponds, small pools of stagnant water, muddy water, borrow pits, runoff from bathrooms and irrigated rice fields (Plates 1a-c). Water samples of different qualities were also obtained, but most contained organic debris with filamentous green algae floating on the surface (Plates 2a-b). In addition, most of the breeding sites were also shallow, temporary and exposed directly to sunshine. A global positioning system (GARMIN GPS 40^{TM}, VBA) was used to determine the geographical coordinates of each site (Appendix I).

Plate 1a: Sample collection site at Achimota with a narrow stretched pool of water flowing from a leaking water supply pipe.

Plate 1b: Sample collection site at Madina with a shallow pool of stagnant water flowing from a residential washing area.

Plate 1c: Sample collection site at Adenta with an open gutter containing stagnant water and a mixture of both *Anopheles* and *Culex* larvae.

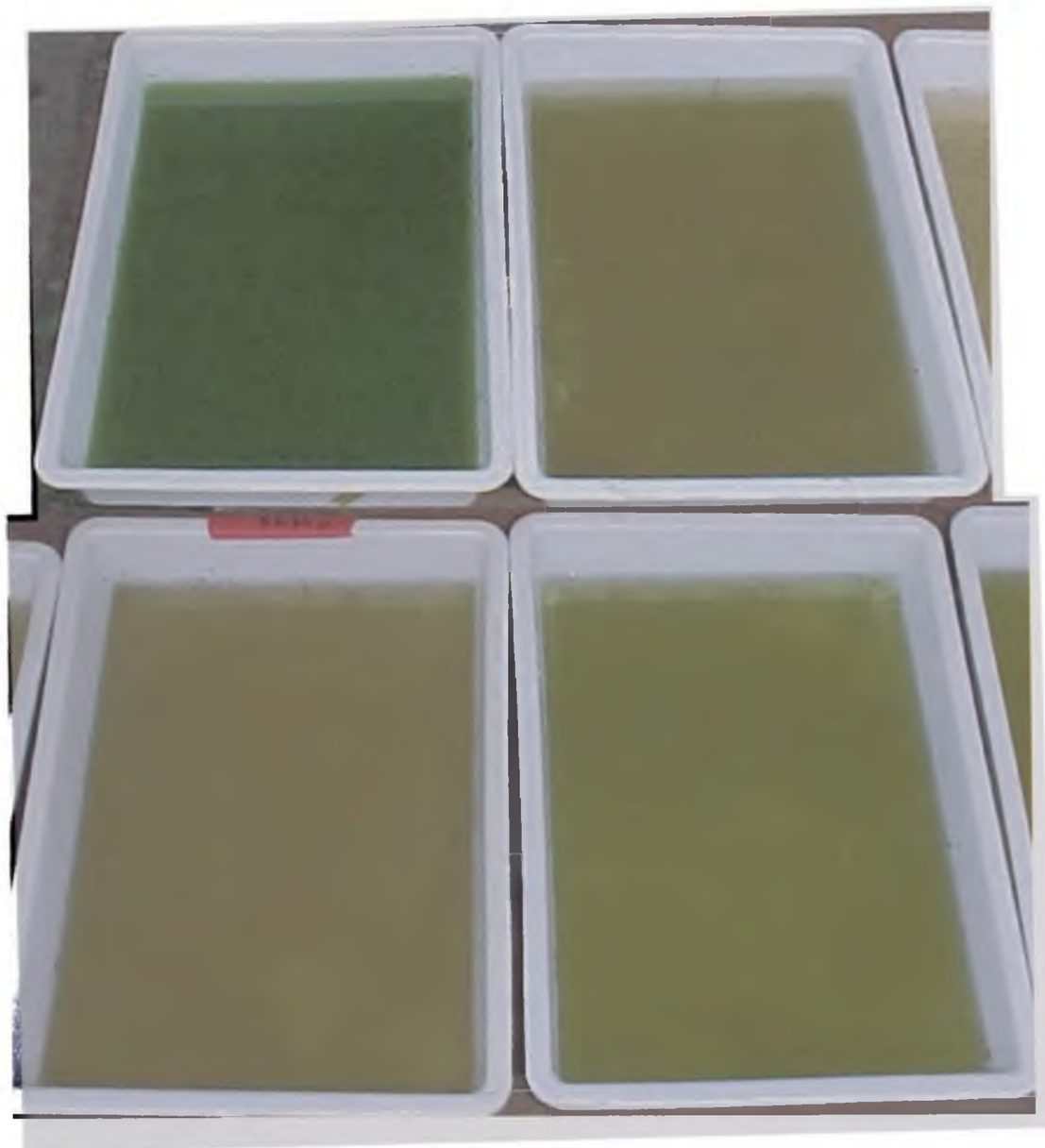

Plate 2a: Examples of type of water from habitats where *An. gambiae* s.s. was found breeding

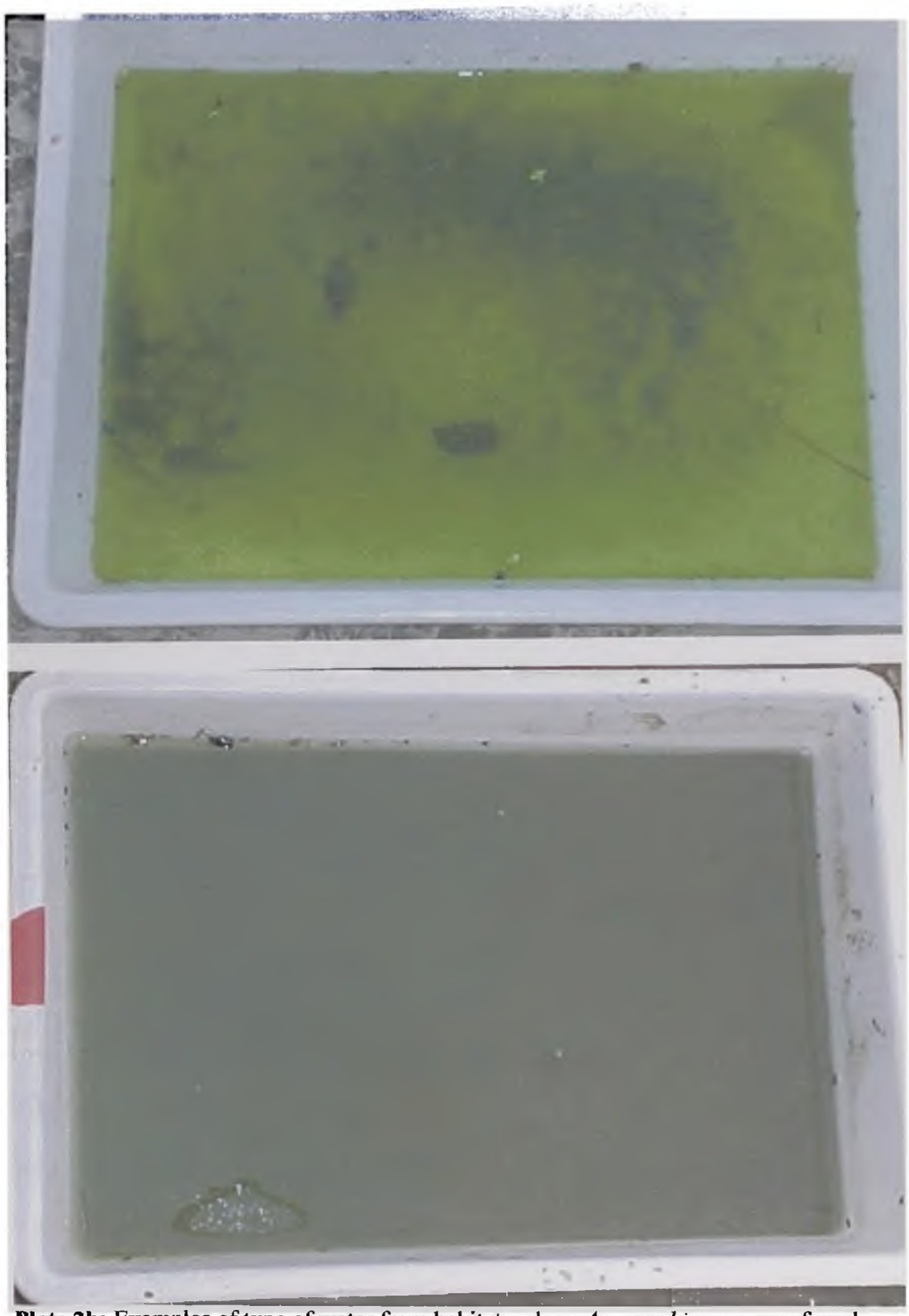

Plate 2b: Examples of type of water from habitats where *An. gambiae* s.s. was found breeding

3.2 Methods

3.2.1 Field sample collections of pre-adult mosquitoes and water

Anopheles larvae identified from their horizontal position on the surface of water and other species identified by their angular position were carefully collected with a 350-ml dipper and transferred into small plastic containers which were loosely capped.

Water samples were also collected thereafter into a 1.5 litre plastic bottles for physico-chemical analyses. For the estimation of dissolved oxygen (DO) a 300-ml glass-stoppered bottle covered with aluminium foil to shield light was filled with the water and 2 ml of manganese sulphate solution, and then 2 ml of alkali-iodide-azide solution added to fix the oxygen. The temperature of each habitat was also recorded. The samples were then transported in iceboxes to the laboratory, making sure that the humidity inside the box was high and that the larvae and pupae did not suffer from excessive heat stress.

3.2.2 Laboratory rearing of mosquitoes

Once in the laboratory, the water with pre-adult mosquitoes were poured into trays to a depth of approximately 2 cm and each tray labelled to indicate the site and date of collection (Plate 3). The trays containing mosquitoes were kept at 27 – 30°C and 76 ± 2% relative humidity with a 12h:12h light and dark cycle. Every two days, about 200 mg of ground Nutrafin goldfish food (Rolf Hagen, USA) were used to feed the larvae in the tray. The development of the larvae was monitored regularly and all those that pupated were collected into shallow plastic cups using rubber pipettes, and

then placed in appropriately labelled cages for adult emergence (Plate 4). All emerged adults were fed on 10 % sugar solution imbibed in cotton until 24 hours after emergence when they were picked from the cages, killed by brief refrigeration at 4°C and then counted. This process was continued until all the larvae and pupae had developed to adults and the total number of adult mosquitoes for each collection was obtained. After sorting them according to species all adult female *An. gambiae* s.l. were preserved dry on silica gel until ready to use for molecular studies. The non-Anopheline adult mosquitoes were however kept at 4°C.

3.2.3 Morphological identification of adult *Anopheles* mosquitoes

Anopheles mosquitoes were separated from other species using morphological key (Service, 1980; Gillett & Smith, 1972). Briefly, the head of *Anopheles* mosquito has prominent compound eyes and a pair of antennae, which are plumose in the male and sparsely feathered in the female. The maxillary palps situated on both sides of proboscis are about as long as the latter in both male and female *Anopheles*, whereas in culicine mosquitoes the female palps are short. The thorax has a rounded single-lobed scutellum, which carries a pair of wings and a pair of halteres whereas in culicine the scutellum is three lobed. *Anopheles* have spotted wings due to the dark and pale scales that are arranged in small blocks or areas on the veins. When resting the abdomen is usually held up with an angle from the surface on which the mosquito is standing, forming a straight line with the proboscis whereas in *Culex* and *Aedes* species the abdomens are usually held parallel with the surface on which they are standing

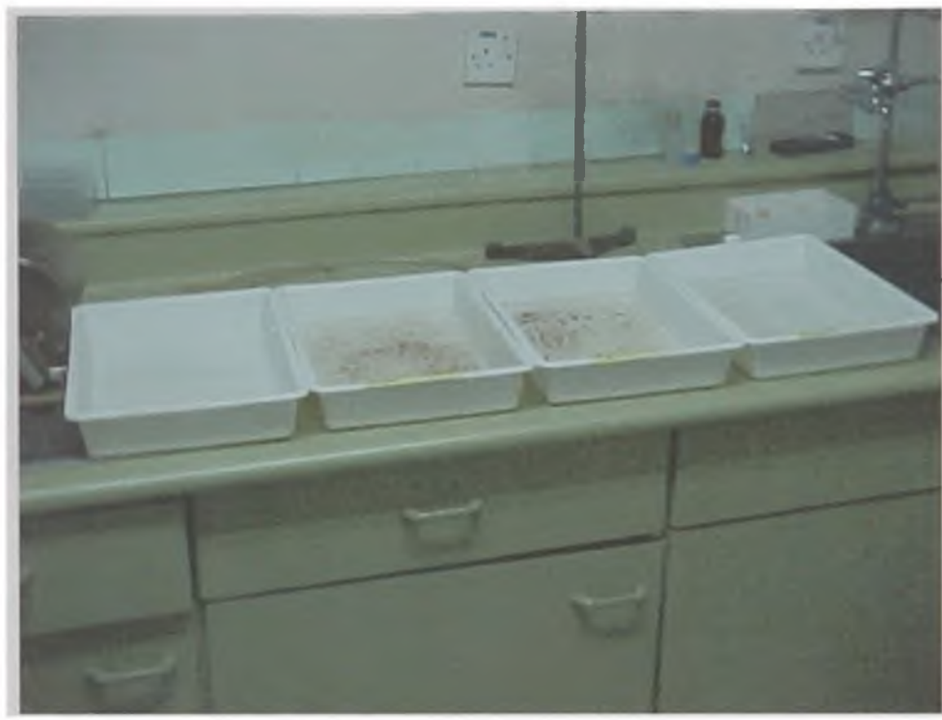

Plate 3: Trays used in the laboratory rearing mosquito larvae and pupae

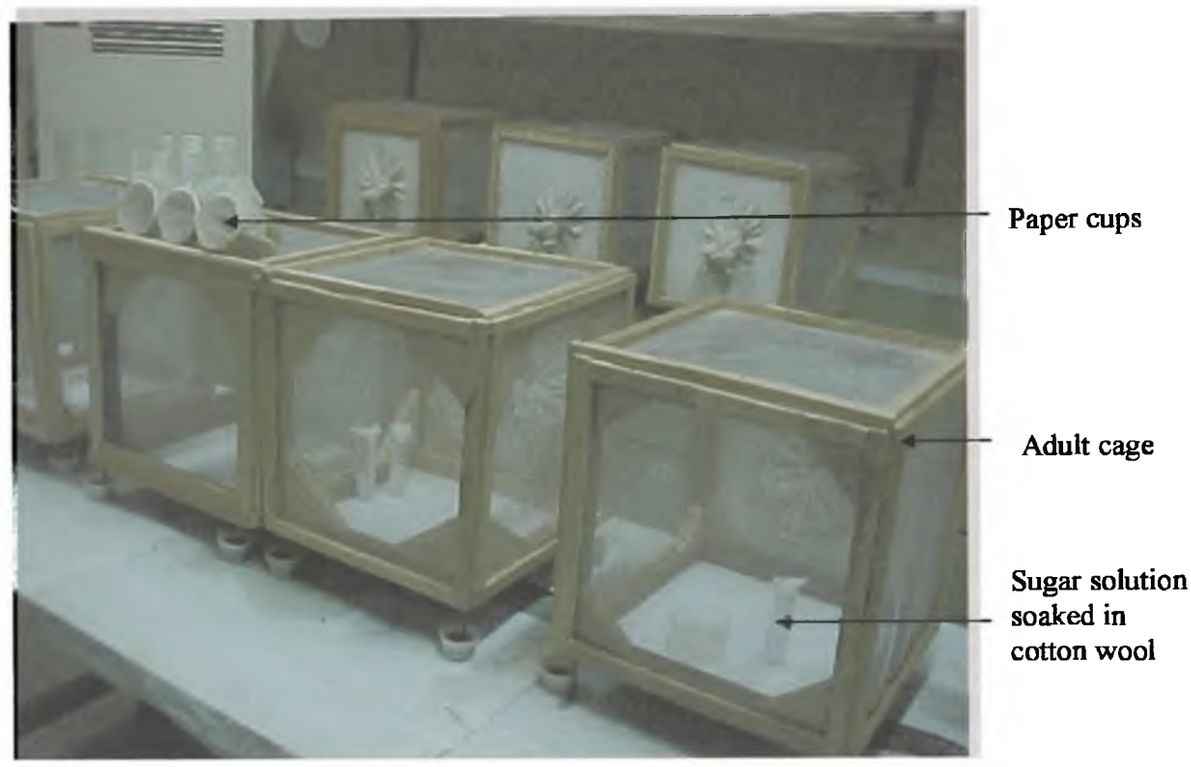

Plate 4: Cages used in the laboratory for holding emerging adult mosquitoes

3.2.4 Molecular biology studies

The various buffers and solutions used in these studies were prepared as outlined in Appendix II

3.2.4.1 The isolation of genomic DNA of *Anopheles gambiae* s.l.

DNA was extracted from adult female mosquitoes using a slightly modified method of Collins *et al.* (1987). Briefly, each specimen was placed in a 1.5 ml eppendorf tube and homogenised with a sterile glass rod in 100µL of Bender buffer (0.1 M Nacl, 0.2M sucrose, 0.1M Tris–HCL, 0.05M EDTA pH 8.0 and 0.5% SDS). The homogenate was then incubated at 65°C for 30 minutes followed by the addition of 15 µl of pre-chilled 8M potassium acetate and mixed well by tapping the tube, then left on ice for 30 minutes. It was then centrifuged for 5 minutes at 14,000 rpm and the supernatant transferred into a fresh tube. To the supernatant was added an equal volume of pre-chilled absolute ethanol, mixed well by tube inversion and then incubated at – 40°C for 2 hours. This was followed by centrifugation at 14,000 rpm for 5 minutes to pellet the DNA. The supernatant was discarded and the pellet was left to dry by evaporation after which it was re-dissolved in 25 µl of sterile double-distilled water and left on ice for 1 hour. The DNA was stored at –20°C until ready for use.

3.2.4.2 PCR identification of species of *Anopheles gambiae* complex

The polymerase chain reaction (PCR) method for the identification of members of *An. gambiae* species complex (Scott *et al.*, 1993) was used for this study. Five

oligonucleotide primers, GA, ME, AR, QD and UN designed from the DNA sequences of the intergenic spacer region of complex ribosomal DNA (rDNA) were used to amplify species-specific DNA sequences. The sequence details of the primers and their expected sizes of the PCR products are given in Table 1. The UN-primer is universal and anneals to the same position on the rDNA sequences of all five species, the GA anneals specifically to *An.* gambiae s.s., the ME anneals to either *An. merus* and *melas*, AR to *An. arabiensis* and the QD to *An. quadriannulatus*.

The PCR reaction mix of 25 µl contained 1 X PCR buffer, 200 µM of each of the deoxyribonucleotide triphosphates (dNTPs), 0.25 µM of oligonucleotide primers, 0.125 units of *Taq* polymerase enzyme (Sigma, USA) and 0.5 µl of the extracted genomic DNA. Sterile double distilled water was added to make up the volume to 25 µl. The reaction mix was spun down briefly at 14,000 rpm and overlaid with mineral oil to avoid evaporation and refluxing during thermo cycling. The amplification reactions were carried out using PTC 100 thermal cycler (MJ Research Inc., USA) and the cycling parameters were as follows: 93°C for 3min (initial denaturation), followed by 35 cycles of 93°C for 30s, 50°C for 30 s, 72°C for 60s. The reaction ended with a single cycle of 93°C for 30s, 50°C for 30s, and 72°C for 10min. For each reaction, a positive control containing 0.5 µl of PCR products of *An. gambiae* s.s. as template DNA and a negative control that contained no DNA template were included.

Table 1: DNA sequence details of the synthetic oligonucleotide primers used for the PCR-based method for the identification of *An. gambiae* s.l. species and their melting temperatures and diagnostic band sizes (Scott *et al.*, 1993).

Name of primer (direction)	Sequences (5' – 3')	Melting temperature T_m (°C)	Expected amplified DNA size (bp)
UN (F)	GTGTGCCCCTTCCTCGATGT	64	468
GA (R)	CTGGTTTGGTCGGCACGTTT	62	390
ME (R)	TGACCAACCCACTCCCTTGA	62	464
AR (R)	AAGTGTCCTTCTCCATCCTA	58	315
QD (R)	CAGACCAAGATGGTTAGTAT	56	153

UN= Universal forward and the reverse primers, GA= *An. gambiae*, ME= *An. melas* and *An. merus*, AR= *An. arabiensis* and QD= *An. quadriannulatus*

3.2.4.3 Microsatellite DNA analysis

Microsatellite analyses of the samples were carried out using oligonucleotide microsatellite primer set AGXH7, which has been found to be the informative in *An. gambiae* s.s. population studies (Lanzaro *et al.*, 1995). The primer set consists of a forward and a reverse primer designed to amplify a microsatellite locus on the X chromosome. The sequence details of the primers and the expected sizes of the PCR products are given in Table 2.

The method of Zheng *et al.* (1993) was used in amplification of the microsatellite sequences. The PCR reaction mix of 20 µl contained: 1 X buffer supplied by the manufacturer (Sigma, USA), 100 µM of each of the 4 deoxyribonucleotides triphosphates (dNTPs) (Pharmacia, Sweden), 0.25 µM of each oligonucleotide primers, 0.125 units of *Taq* polymerase enzyme (Sigma, USA) and 0.5 µl of the genomic DNA. In each reaction, the primer set AGXH7 was used. Sterile double distilled water was used to make up the volume to 20 µl. The reaction mix was spun down briefly at 14,000 rpm and overlaid with mineral oil to avoid evaporation and refluxing during thermo cycling. The amplification was carried out using a PTC 100 thermal cycler and the cycling parameters for the reaction were as follows: 95°C for 5min (initial denaturation), followed by 35 cycles of 95°C 30s (denaturation), 55°C for 30 s (annealing), 72°C for 2 min (extension) and a final cycle of 95°C for 30s, 55°C for 30s and 72°C for 5min. For each reaction, a negative control that contained no DNA template was included.

Table 2: DNA sequences of the oligonucleotide primer set AGXH7 used for the PCR-based amplification of microsatellite sequences in *An. gambiae* s.s. The melting temperature and expected band size of the PCR product is given. The (F) and (R) after the sequences denote forward and reverse primers respectively.

Name of primer	Sequences (5' – 3')	Melting temperature (°C)	Expected amplified DNA size (bp)
AGXH7	CACGATGGTTTTCGGTGTGG (F)	62	99
	ATTTGAGCTCTCCCGGGTG (R)	60	

3.2.4.4 Analysis of PCR products

3.2.4.4.1 Agarose gel electrophoresis

Five microlitres of each PCR product (mixed with 1 µl of Orange G [5 X] gel loading dye) was electrophoresed in a 2 % agarose gel stained with 0.5 µg/ml ethidium bromide to detect the presence of amplified DNA fragments. The gels were electrophoresed in 1 X TAE buffer using a midi gel system (BIORAD, USA) at 100 volts for one hour and photographed over a UV transilluminator (UPC, USA) at short wavelength using a Polaroid camera and type 667 film (Polaroid, USA).

3.2.4.4.2 Polyacrylamide gel electropholesis

Polyacrylamide gel electrophoresis was used for analysing the microsatellite DNA. The composition of the 7% polyacrylamide gel preparation used and how it was prepared are given in Appendix I. The vertical electrophoresis gel tank (BIORAD, USA) was used. Prior to the loading of samples, the wells were flushed with sddH_2O to remove any urea. Fifteen microlitres of the PCR products of each reaction was mixed with 10 µl of bromophenol blue dye and loaded into each well and the gel run at 13 mA and 100 volts for 7 hours. Thereafter, the glass plates were separated and the gel carefully transferred into a plastic tray containing 5 % ethidium bromide for 5 – 10 minutes to stain. The gel was photographed over a UV transilluminator (UPC, USA) at short wavelength using a Polaroid camera and type 667 film (Polaroid, USA).

3.2.5 Physico-chemical Analyses of Water Samples

The various buffers and solutions used for these studies were prepared as outlined in Appendix III. A total of 28 parameters were measured. These were; turbidity, pH, electrical conductivity, suspended solids (SS), total dissolved solids (TDS), sodium, potassium, calcium, magnesium, total iron, ammonium, chloride, sulphate, silica, nitrite, nitrate, phosphate, total hardness, total alkalinity, calcium hardness, magnesium hardness, fluoride, bicarbonate, carbonate, biological oxygen demand (BOD), chemical oxygen demand (COD), dissolved oxygen (DO) and temperature.

3.2.5.1 Measurement of physico-chemical parameters of water samples

The water temperature from mosquito breeding sites was directly measured using mercury-in a glass thermometer each time samples were collected.

The turbidity was measured using turbidimeter (Partech model – DRT 100 B) and the hydrogen ion concentration (pH) was measured using a pH meter. The electrical conductivity was measured using a conductivity meter (JENWAY 4020).

The strong acid titration method was used to determine total alkalinity. 100 ml of water sample was tritrated with 0.02 M Sulphuric acid (H_2SO_4) until it reached pH 8.3. The volume of acid (A) used, which is related to phenolphthalein alkalinity (P) was then recorded. Titration was continued until pH 4.5 was reached and the volume of acid (B) which is related to the total alkalinity (T) was also recorded. The phenolphthalein alkalinity due to $CaCO_3$ (P) and total alkalinity as $CaCO_3$ (T) were given by the equations (1) and (2) respectively.

$$P = \frac{50{,}000 \times A \times N}{V} \text{ mg/l} \qquad \text{(Equation 1)}$$

$$T = \frac{50{,}000 \times B \times N}{V} \text{ mg/l} \qquad \text{(Equation 2)}$$

Where, A = volume of acid added to obtain the phenolphthalein end point of pH 8.3;

B = volume of acid added to obtain the methyl orange end point of pH 4.5;

N = normality of the acid;

V = volume of water sample

The carbonate alkalinity (CO_3^{2-}) and bicarbonate alkalinity (HCO_3^-) were derived from the equations (1) and (2) using the following relationships:

Results of titration	**Alkalinity**	
	CO_3^{2-}	**(HCO_3^-)**
P = 0	0	T
P < ½ T	2 P	T – 2 P
P = ½ T	2 P	0
P > ½ T	2 (T – P)	0
P = T	0	0

The total hardness (due to $CaCO_3$) was determined using EDTA titrimetric method. A mixture of 50 ml of the water sample, 1 ml of buffer solution and a few crystals (0.1 – 0.2 g) of Eriochrome Black T indicator were titrated with a standard 0.01 M EDTA until the solution turned from purple color to bright blue. The total hardness was then given by the equation (3).

$$\text{Total hardness } CaCO_3 = \frac{\text{Volume of EDTA} \times B \times 1000}{\text{Volume of sample}} \text{ mg/l} \quad \text{(Equation 3)}$$

Where, B = mg equivalent of $CaCO_3$ to 1.00 ml EDTA titrant, i.e. ml of $CaCO_3$/ml of EDTA

Calcium ion (Ca^{2+}) concentration was also determined using EDTA titrimetric method. A mixture of 50 ml of water sample, 2.0 ml of 1M NaOH solution and a few crystals of Murexide indicator were titrated with 0.01 M of EDTA until the colour changed from salmon to orchid purple Calcium ion concentration was given by the equation (4).

$$Ca^{2+} = \frac{A \times B \times 400.8}{\text{Volume of sample}} \text{ mg/l} \quad \text{(Equation 4)}$$

Where: A = volume of EDTA;

B (mg equivalent of $CaCO_3$ to 1.0 ml of EDTA titrant at the calcium indicator end point), which is related to the volume of standardized EDTA titrant was calculated using the equation (5).

$$B = \frac{C}{\text{Volume of EDTA}} \quad \text{(Equation 5)}$$

Where, C = volume of standard calcium solution used to standardize the EDTA

The calcium hardness (mg of $CaCO_3$ /l) was determined using the equation (6).

$$\text{Calcium hardness} = \frac{\text{Concentration of } Ca^{2+} \text{ (Equation 4)}}{0.4} \text{mg/l} \quad \text{(Equation 6)}$$

Where, 0 4 = atomic weight of Ca / molecular weight of $CaCO_3$

Magnesium hardness was determined using the equation (7).

Mg hardness (mg/l) = Total hardness (Eq. 3) – Ca hardness (Eq. 6) (Equation 7)

Magnesium ion concentration was determined using the equation (8).

Mg^{2+} = [Total hardness (Eq. 3) – Ca hardness (Eq. 6)] x 0.243 mg/l (Equation 8)

Where, 0.243 = atomic weight of Mg /molecular weight of $CaCO_3$

Chloride ion (Cl^-) concentration was determined using the argentometric method. The pH of 100 ml of the water sample was first adjusted between 7 and 10 using conc.H_2SO_4, then 1ml of potassium chromate (K_2CrO_4) indicator solution was added followed by titration with standard $AgNO_3$ until a pinkish yellow colour endpoint was reached. A blank value was also determined by titration method. The chloride ion (Cl^-) concentration was determined using the equation (9).

$$Cl^- = \frac{(A-B) \times M \times 35{,}450}{\text{Volume of sample}} \text{ mg/l} \qquad \text{Equation 9}$$

Where, A = Volume of $AgNO_3$ used for sample titration,

B = Volume of $AgNO_3$ used for blank titration,

M = Molarity of $AgNO_3$

Potassium (K^+) and Sodium (Na^+) concentrations (mg/l) were determined using flame photometric method. Samples were introduced in a Flame Analyzer (Gallenkamp model FGA – 350L, UK). The calculations of potassium or sodium were given directly by the flame photometer at wavelengths of 768 and 589nm respectively. In instances when either potassium or sodium concentration was very high the water samples were diluted and the concentrations were calculated as follows:

K^+ or Na^+) = (K^+ or Na^+ in aliquot) x D

Where, D = dilution factor, which is determined using equation (10).

$$D = \frac{\text{Volume of sample + distilled water used for dilution}}{\text{Volume of sample}} \qquad \text{(Equation 10)}$$

Ammonia-nitrogen (NH_4-N) amounts (mg/l) were determined by direct Nesslerization. For the calibration curve, 10 ml of the stock ammonium chloride solution was diluted to 100 ml with distilled water. From this intermediate solution, each of 1, 2, 3 , 4 and 5 ml was diluted to 100 ml with deionised water. 5 drops of Rochelle salt were added to 50 ml of each concentration into 100 ml conical flask, mixed well and followed by addition of 2 ml Nessler's reagent. They were then allowed to stand for 10 minutes for colour development. The water sample was left to settle and an aliquot of 50 ml of the supernatant was pipetted into a fresh 100 ml conical flask. Then, 1 to 5 ml of sample was pipetted into a fresh flask and ammonia-free water added until it reached 50 ml mark. For very turbid samples, the water was filtered and the filtrate was used for the analyses. Two drops of Rochelle salt were added to the diluted sample or 5 drops in the case of undiluted sample, mixed well and 2 ml of Nessler's reagent added. They were then allowed to stand for 10 minutes for colour development and the absorbance for samples and blank determined using a UV/VIS spectrophotometer (Philips model PU 8625B, The Netherlands) at a wavelength of 410nm using a 1 cm light path cuvette. The concentration of ammonia-nitrogen in the unknown sample was determined by extrapolating from a calibration curve.

Diazotization method was used to determine the concentration (mg/l) of nitrite-nitrogen (NO_2-N). A series of standards (blank of 0, 0.5, 1, 1.5, 2, 3, 4, 5 and 10 ml of working nitrite solution) was diluted to 50 ml with distilled water. Then, 2 ml of buffer-colour reagent were added to 50 ml of sample in a Nessler tube, mixed well and left for at least 15 minutes to allow colour to develop. The absorbance was

measured in the UV/VIS spectrophotometer at 540nm against the blank. The concentration of nitrite-nitrogen (mg/l) was determined by extrapolating from the calibration curve.

To determine the concentration (mg/l) of nitrate (NO_3-N), the hydrazine reduction method was used. NO_3 calibration standards were prepared by diluting to 10 ml with distilled water the following intermediate nitrate solutions: 0, 2 , 4 , 6 , 8 and 10 ml. In a test-tube, 1 ml of 0.3M NaOH was added to 10ml of the water sample and mixed gently, followed by the addition of 1 ml of reducing mixture and mixed gently. The mixture was heated at 60°C for 10 minutes in water bath, cooled to room temperature and 1 ml of colour developing reagent was added, and mixed well by shaking. The absorbance at 520nm was read and the nitrate concentration was directly computed from a calibration curve. The value obtained however is due to both NO_3-N and NO_2-N, therefore the concentration of NO_3-N was derived by subtracting the concentration of NO_2-N above from the value obtained.

Phosphate (PO_4-P) concentration (mg/l) was determined using the stannous chloride method. Standard phosphate solutions of known concentrations ranging from 0.1 mg/l to 1.0 mg/l were prepared and treated as samples. To a 100 ml sample free from colour and turbidity was added 0.05 ml (approximately 1 drop) of phenolphthalein indicator. To the sample turned pink, a strong acid solution was added dropwise to discharge the colour. Where more than 0.25 ml (5 drops) was required, a small volume of sample was diluted to 100 ml with de-ionised water then a drop of phenolphthalein indicator was added, discharged if the sample turned the pink colour with the acid. A volume of 0.4 ml molybdate reagent I was added with thorough

mixing after each addition and was followed by 0.5ml (10 drops) of stannous chloride reagent. The absorbance at 690 nm was measured after 10 minutes (but before 12 minutes) using a UV/VIS spectrophotometer. The phosphate concentrations in the samples were determined from the calibration curve.

Silica (SiO_2) concentration (mg/l) was determined using the molybdolicate method. From the silica intermediate standard solution, 5, 10, 15, 20, 25 and 30 mg/l concentrations were prepared followed by addition of the colour development reagents as the samples were treated. 1 ml of HCl and 2 ml of ammonium molybdate reagent were added in rapid succession to 50 ml of sample, mixed by inverting at least six times and left to stand for 5 to 10 minutes. This was followed by addition of 2 ml of oxalic acid solution and then mixed thoroughly. Colour was read after 2 minutes (but before 15 minutes after the addition of oxalic acid) at a wavelength of 410 nm on a spectrophotometer. The silica concentration was determined directly from the calibration curve of known standards.

Sulphate (SO_4) concentration (mg/l) was determined using a turbidimetric method. From the sulphate solution, each of 5, 10, 15, 20, 25 and 30 ml was diluted to 100 ml with distilled water and used as standard solution in calibration. 5 ml conditioning reagent were added to 100 ml sample in a 250 ml Erlenmeyer flask and mixed by stirring. Thereafter a spoonful of barium chloride crystals was added still stirring at a constant speed for 60 seconds. After stirring and within 5 minutes the absorbance at 420 nm was measured. The concentration of sulphate was extrapolated from the calibration curve of known standard concentration.

To determine amounts of fluoride (mg/l) the SPADNS method was used. Standard concentrations of fluoride (0.2, 0.4, 0.6, 0.8, 1, 1.2 and 1.4 mg/l) were prepared from standard fluoride solution (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 ml of standard fluoride solution diluted to 50 ml with distilled water). 5 ml each of SPADNS solution and of Zirconyl-acid reagent were added to 50 ml of the water sample and mixed well. The absorbance was then read at 570 nm. If the absorbance was beyond the range of the standard curve, then water samples were diluted with deionised water to 50 ml into a conical flask and the procedure repeated. The concentration of Fluoride in the sample was determined using the calibration curve.

Total suspended solids were determined by gravimetric method. Filter was wetted with 10 ml of deionised water followed by a transfer of suitable volume of sample to the funnel (to yield not more than 200 mg of residue) after the bottle had been vigorously shaken. Filter was washed with three successive 10 ml volumes of distilled water and suction was continued for 3 minutes after filtration was complete. Filter was removed from the filter holder, transferred into a dish and dried for at least one hour at 105°C in an oven. Filter was weighted after cooling in a desiccator. The drying cycle was repeated until a constant weight was obtained. Total suspended solids (mg/l) were determined using the equation (11)

$$\text{Total suspended solids} = \frac{(A - B) \times 10^6}{\text{Volume of sample filtered (ml)}} \text{ mg/l} \quad \text{(Equation 11)}$$

Where; A = weight of filter + dish + residue (g)

B = weight of filter + dish (g)

Total dissolved solids were also determined using gravimetric method. The water sample was shaked and any volume, which was enough to yield between 10 and 200

mg dried residue was taken. Sample was filtered through the glass fibre filter and a vacuum was applied for 3 minutes after filtration to ensure that as much water as possible was removed. After washing with three successive 10 ml volume of deionised water and continuous suction for 3 minutes, total filtrate (with washings) was transferred to an evaporating dish and evaporated to dryness on a water bath. Evaporated sample was dried at 105°C in an oven for at least 1 hour. The filter was removed and placed in a desiccator to cool and then weighed. The drying cycle was repeated until a constant weight was obtained. The total dissolved solids (expressed in mg/l) were determined using the equation (12)

$$\text{Total dissolved solids} = \frac{(A - B)}{\text{Volume of sample filtered}} \text{ mg/l} \qquad \text{(Equation 12)}$$

Where, A = weight of dried residue and dish (g)

B = weight of dish (g)

Chemical oxygen demand (COD) was determined by the closed tube reflux method. 3 ml of standard potassium dichromate solution (0.0167 M) and 7 ml of H_2SO_4 reagent (silver sulphate in sulphuric acid) were added to a sample of 5 ml into a culture tube and mixed well by shaking. The tubes were placed in a COD Heating Digester (VELP Scientifica) preheated to 150°C and refluxed for 2 hours. After cooling to room temperature, 1 to 2 drops of ferroin indicator were added to the products and titrated with 0.1M standard ferrous ammonium sulphate solution (FAS) to change colour from blue-green to reddish brown or wine end point. In the same manner, a blank containing the reagents and a volume of deionised water equal to that of sample was refluxed and titrated COD as mg of O2/l was calculated as follows:

$$COD = \frac{(A - B) \times M \times 8000}{\text{Volume of sample}} \text{ mg/l}$$

Where:

A = volume of FAS used for blank

B = volume of FAS used for sample

M = Molarity of FAS

8000 = milliequivalent weight of oxygen X 1000 ml/litre

Determination of dissolved oxygen (DO) was done using azide modification of Winkler's method. 2 ml conc. H_2SO_4 was added to a sample of 250 – 300 ml in a BOD bottle, shaken till dissolution was complete. From that solution, 100 ml were taken and titrated with M/80 sodium thiosulphate solution to a straw yellow colour, followed by addition of 1 – 2 ml starch solution and titration continued until the blue colour turned to colourless. Dissolved oxygen as mg/l O_2 was calculated as follows:

$$O_2 = \frac{\text{Volume of M/80 thiosulphate} \times 101.6}{\text{Volume of water sample}} \text{ mg/l}$$

Biochemical oxygen demand (BOD) was determined by dilution method. 2 ml of $MnSO_4$ were added to the diluted 250 – 300 ml sample in BOD bottle followed by 2 ml of alkaline-iodide-azide and corked carefully to exclude bubbles. After the precipitate has settled, 2 ml of conc.H_2SO_4 were added, corked and the bottle inverted several times to dissolve the precipitate which gave intense yellow colour. The solution was then titrated with M/80 sodium thiosulphate until a pale yellow colour was obtained. Then 1 ml of starch as indicator was added and the titration was continued until the appearance of blue colour. The rest of the water sample was incubated at 20°C for 5 days after which it was treated likewise. The difference in dissolved oxygen (DO) of days 1 and 5 was used to calculate the BOD as follows:

$$BOD = (D1 - D2) \times P$$

Where:

D1 (mg/l) = DO of diluted sample immediately after preparation

D2 (mg/l) = DO of diluted sample after 5 days incubation at 20°C

P = decimal volumetric fraction of sample used

3.2.6 Data analysis

Statistical analyses of water parameters were performed using SPSS 10.0 software (SPSS Inc., USA). Samples were firstly divided into two groups: group with *Anopheles* species and that without but with other mosquito species. A *t* test was used to compare these groups of observations. Water parameters whose means were significantly different were submitted to a linear discriminant function analysis to identify the best discriminatory parameters between the two groups. To determine the relationship between ecological parameters and the observed variation in the proportions of *An. gambiae* larval populations, correlation and multiple regression analyses were performed. The parameters were first analysed using univariate statistical methods. Then, those which were significantly correlated (Pearson correlation coefficient) to the proportion of *An. gambiae* were included in multiple regression analysis that yielded a regression model containing the best combination of parameters significantly contributing to the variation. To assess the similarity in *An. gambiae* larval habitats whole samples from *An. gambiae* were first analysed together and later divided into two groups of 100% *An. gambiae* and mixed populations. The results of the hierarchical cluster analysis of water parameters was visually compared to the phylogeny tree generated by POPGENE software using the data from microsatellite DNA analysis of *An. gambiae* s.s. for similarities.

3.2.6.1 Test of equality of means

This test deals with comparing groups of observations with respect to continuous data, starting with the simplest case where we wish to compare a single group of observations with some pre-specified value, and moving through to the case where

we have several sets of observations on each a group of individuals. The *t* test is appropriate for this type of analyses.

The most common statistical analyses are those for comparing two independent groups of observations. With these groups, we are interested in the mean difference between the groups, but the variability between subjects becomes important. The two sample *t* test is based on the assumption that each set of observations is sampled from a population with a normal distribution, and that the variances of the two populations are the same. The *F test* or *variance ratio test* was used in situations when the differencies in variations of the two groups were not known. Variance ratio is the ratio of the sample variances or the square of the ratio of the sample standard deviations. The variance ratio observed in the sample is calculated by taking the larger standard deviation divided by the smaller and looks up the square of this value in the table of *F distribution*. The distribution of the *F* statistic has two values of degrees of freedom, one corresponding to each variance. Because we take the ratio of the larger variance to the smaller we consider only the upper tail of the *F distribution*.

The *t* test for equality of means was used to compare the means of parameters two independent groups of mosquito habitats; habitats with *An. gambiae* s.s. and those without but with other mosquito species. Confidence limit was set at 95 %.

3.2.6.2 Discriminant function analysis

The linear discriminant function analysis (LDF) is useful where you want to build a predictive model of group membership based on observed characteristics of each

group. The procedure generates a discriminant function (or, for more than two groups, a set of discriminant functions) based on linear combinations of the predictor variables that provide the best discrimination between the groups. There are several purposes for LDF:

i) To investigate differences between groups

ii) To determine the most parsimonious way to distinguish between groups

iii) To discard variables, which are little related to group distinctions

iv) To classify cases into groups

v) To test theory by observing whether cases are classified as predicted

Basically, a discriminant function score is predicted from the sum of the series of the variables, each weighted by a coefficient. Therefore, there is one set of discriminant function coefficients for the first discriminant function, a second set of coefficients for the second discriminant function and so forth. Cases get separate discriminant function scores for each discriminant function when their scores on variables are inserted into the equation:

$$D_i = d_{i1}x_1 + d_{i2}x_2 + d_{i3}x_3 + \ldots\ldots d_{in}x_n$$

Where D is the standardised score on the ith discriminant function, x is the standardised score on each variable and d_i, is the discriminant coefficient. Just as D_i can be calculated for each specimen, a mean value of D can be calculated for each group. Thus, members of each group considered together have a mean score on a

discriminant function that is the distance of the group in standard deviation units from the zero mean of the discriminant function. This is analogous to multiple regression, but the d_i's are discriminant coefficients which maximize the distance between the means of the criterion (dependent) variable. Cases are also assigned into groups using the basic classification equation:

$$Y_j = b_{j0} + b_{j1}Z_1 + b_{j2}Z_2 + \ldots\ldots b_{jn}Z_n$$

Where Y_j, is the score on the classification function for the group j, Z_1 is the raw score of the variable and b, the associated classification function of the variable, b_{j0}, a constant. This method then can be used to predict to which subgroup a new individual is likely to belong. Discriminant function analysis is also used to select the variables most useful in predicting group membership if performed in a stepwise manner.

The discriminant function analysis was used to select the parameters, which were most useful in distinguishing the habitats of *An. gambiae* and of no *An. gambiae*. Parameters were initially screened by univariate analysis. Those that were significant at $\alpha = 0.05$ were included in multivariate analysis. The probability of F to enter the model was 0.05 and that of removal was 0.051.

3.2.6.3 Multiple regression analysis

The general purpose of multiple regression is to learn more about the relationship between several independent or predictor variables and a dependent or criterion variable. Multiple regression yields a regression model in which the dependent (or

outcome) variable is expressed as a combination of the explanatory variables. The statistical significance of each variable in the multiple regression model is obtained simply by calculating the ratio of the regression coefficient to its standard error and relating this value to the *t* distribution with n-k-1 degrees of freedom, where n is the sample size and k is the number of variables in the model. The *t* statistic, which is calculated as *b/std error*, where *b* is the regression of the coefficient, and is equal to the square root of the F statistic for the extra variability explained by the present model in comparison with the model excluding that particular variable. The principle involves testing the significance of particular regression coefficients and then applying logical approach to select the best set of independent variables, where the best is to be interpreted as including all variables, which really affect the dependent variable and only those remain the same. Thus, for three variables, x_1, x_2, and x_3, the significance of the effect x_1, is tested by comparing the residual sum of squares for the regression on all three variables and for the regression on x_2 and x_3 only. The difference between the residual mean square for the full model and the F-test is used.

Three methods can be employed in this analysis. The forward entry method selects the parameter with the highest level of significance first before any other. The stepwise selection method enters the parameters in their order of significance with the parameter having the highest level of significance being entered first before any other. The backward elimination method removes the parameter with the least level of significance completely out of the model and does not consider it at all in any compilations.

For this study, multiple regression analysis was used to identify which combination of the parameters was significantly contributing to the observed variation in the proportion of *An. gambiae* s.s. larval populations in the various habitats. For this analysis parameters that were significantly correlated (Pearson coefficient) to the proportion of *An. gambiae* s.s. ($\alpha = 0.05$) were included in the multivariate analysis.

3.2.6.4 Hierarchical cluster analysis and phylogeny tree

Hierarchical cluster analysis is a statistical method for finding relatively homogeneous clusters of cases based on measured characteristics. It starts with each case in a separate cluster and then combines the clusters sequentially, reducing the number of clusters at each step until only one cluster is left. When there are N cases, this involves N-1 clustering steps, or fusions. This hierarchical clustering process can be represented as a tree, or dendrogram, where each step in the clustering process is illustrated by a join of the tree. Distance or similarity measures are generated by the Proximities procedure. Our choice of clustering method or clustering criterion will determine the way in which the proximity between two clusters is measured. For example, using single linkage, the proximity between two clusters is the highest similarity (or smallest distance) between any two cases, one from each of the clusters. It's their nearest neighbours. By contrast, with average linkage, the similarity between two clusters is the average of the proximities between all pairs of cases, one from each of the two clusters. It is often recommended to optimize the Euclidean Sum of Squares which involves finding the mean of each cluster and the distance from each case contained in each cluster and its mean, then squaring these distances and summing the squared distances for all the cases in all the clusters. It is

a measure of the within-cluster variance, or the diversity of a particular classification or cluster model.

Phylogeny is the study of the evolution of life forms. It helps to understand the life through time—not just at one time in the past or present, but over long periods of past time. Before we can attempt to reconstruct the forms, functions, and lives of once-living organisms, we have to place these organisms in context. The context of evolutionary biology is phylogeny, the connections between all groups of organisms as understood by ancestor/descendant relationships. Evolutionary history is typically represented by a phylogeny tree, a tree of species with the root being the oldest common ancestor and the children of a node being the species that evolved directly from that node. Each species in a set is represented by a set of traits or character values.

For this study, hierarchical cluster analysis was used to assess the similarity between *An. gambiae* larval habitats. Phylogeny tree was used to assess the ancestor/descendant relationship which connects the *An. gambiae* s.s. populations collected from different habitats. Samples were firstly analysed together and after divided into group of 100% *An. gambiae* and that of the mixture with other species. Visual comparison between hierarchical cluster analysis and phylogeny tree was done.

3.2.6.5 Microsatellite data

The banding profile of alleles of each sample as observed on the gel was scored for the absence or presence of alleles (bands) depending on DNA fragment sizes.

Alphabetic capital letters were used to denote different DNA fragment sizes Single bands were scored as homozygote and recorded as AA whereas bands appearing in pairs or in fours were scored as heterozygote and recorded as AB (Appendix IV). Missing bands were denoted by two dots ". " Population genetics analysis software, POPGENE, Version 1.31 for Microsoft Windows™ (Yeh *et al.*, 1999) was used to analyse the alphabetic diploid and co-dominant data. The output statistics of this analysis included the genotypic frequency, differentiation indices (F_{st}), and heterozygosity deficiency or excess (F_{is}), and allele frequency. F_{st} is an estimate of inter-population genetic differentiation and was considered to be low if the differentiation index (F_{st}) was below 0.05 and high when it was greater than 0.05 (Wright, 1978). Positive F_{is} values indicate heterozygote deficiency while negative values indicate heterozygote excess (Lehmann *et al.*, 1997).

CHAPTER FOUR

RESULTS

Out of 54 samples collected *An. gambiae* s.l. larvae were recorded alone at 5 (9.3%) sites whereas mixtures of *An. gambiae* s.l. with other mosquito species were obtained in 25 (46.3%) sites. Habitats of other species without *An. gambiae* s.l. were recorded at 24 (44.4%) sites.

About 95 % of the larvae and pupae of both *Anopheles* and other species that were reared in the insectary completed their development to adults. However, it was observed that the development from the larval to the pupal stage for *Anopheles* species lasted 3 – 5 days (depending on the larval stage when collected) and adult emergence from pupae lasted 1 – 2 days. It was also observed that in the first batch of *Anopheles* mosquitoes that emerged there were predominantly more males than the subsequent batches, which had more females. A total of 3,652 mosquitoes that were studied were morphologically identified as 2,735 (74.9%) *An. gambiae* s.l. and 917 (25.1%) as other species.

4.1 Molecular Identification of *Anopheles gambiae* Species Complex

Ten morphologically identified *An. gambiae* s.l. mosquitoes per site were studied and PCR identification (Scott *et al.*, 1993) of a total 300 specimens was carried out. PCR amplifications were successful in all cases and revealed only *An. gambiae* s.s. indicated by the diagnostic size of the amplified DNA fragment which is 390 bp (figure 4).

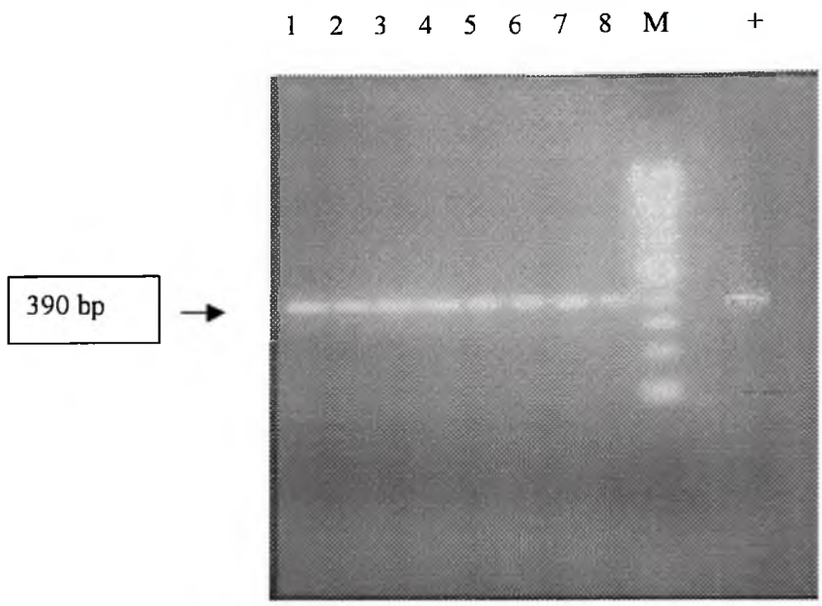

Figure 4: Ethidium bromide stained 2 % agarose gel electrophoresis of PCR products obtained from the amplification of *An. gambiae* DNA for species identification. Lanes 1 to 8 = *An. gambiae* s.s. diagnostic band size; M = 100 bp Molecular weight marker; "-"= negative control; "+"= positive control

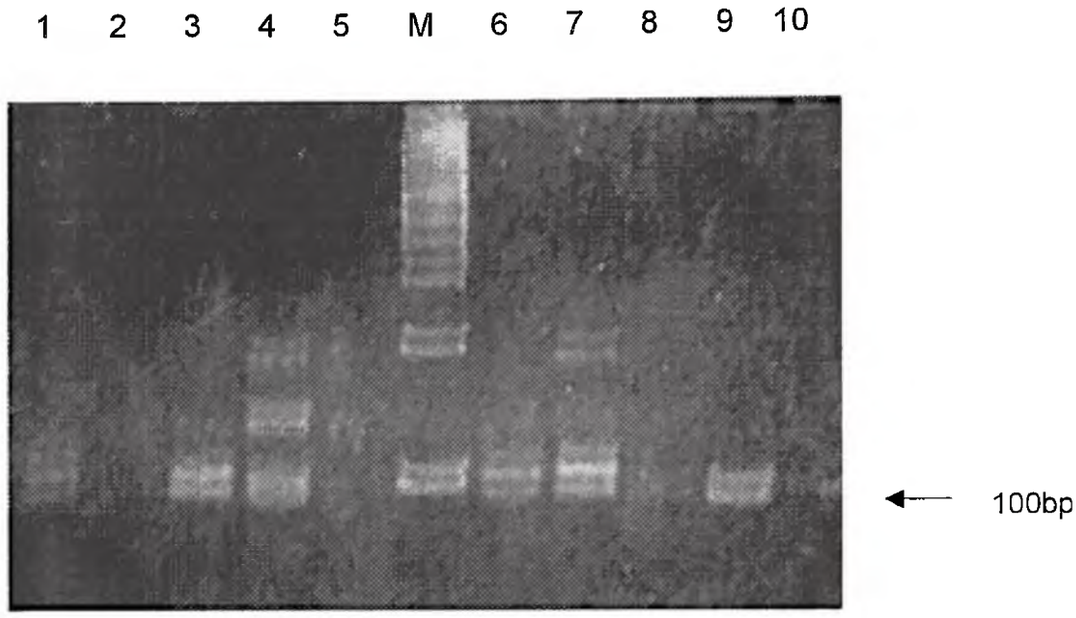

Figure 5: Polyacrylamide gel electrophoresis of PCR products obtained from the amplification of *An. gambiae* s.s. microsatellite DNA with primer AGXH7. Lanes 1, 6 and 7 = Heterozygous set of four bands (AB), Lanes 2, 5 and 8 = No bands (..), Lanes 3, 4 and 9 = Heterozygous double band (AB), Lane 10 = Homozygous single band (AA), Lane M = 100 bp Molecular weight marker

4.2 Genetic Structure of *Anopheles gambiae* s.s. Populations

A total of 270 *An. gambiae* s.s. mosquitoes were studied by means of microsatellite DNA analysis, using the AGXH7 oligonucleotide primer set designed to amplify a locus on the X chromosome (figure 5). Out of the 270 specimens, 260 (96.3%) were successfully amplified on first attempt, 3 (1.1%) were successful on re-amplification and 7 (2.6%) that failed even after re-amplification was taken to be due to their possession of null alleles.

DNA bands that differed in sizes were taken to be distinct alleles. The microsatellite locus AGXH7 studied was found to be polymorphic. Ten loci were recorded for the overall populations and a total of 12 alleles were also recorded and were not uniformly distributed in all the populations. The loci in 100% of *An. gambiae* s.s. populations were found to be 70% polymorphic, whereas those from habitats with 1 to 99% of *An. gambaie* s.s. were 100% polymorphic. A total of 10 alleles were recorded with the 100% of *An. gambiae* s.s population group whereas 12 alleles were recorded with the 1 to 99% of *An. gambiae* s.s. population group. Three (6.0%) out of the overall total of 50 mosquitoes studied from the first group were heterozygous for 99 bp, 150 bp, 340 bp and 430 bp alleles, whilst 49 (22.3%) out of overall total of 220 mosquitoes studied from the second group were heterozygous for different band sizes. In addition 5 mosquitoes were found to be heterozygous for the 99 bp allele.

4.2.1 Genotype frequency distributions

As shown in Table 3, ten alleles were the most common for both groups. However, with the 100% *An. gambiae* s.s. population group, allele 1 was the most dominant

(0.433) and alleles 5, 6, 7, 8, and 9 were found to have the same allele frequency (0.056). With the 1 to 99% of *An. gambiae* s.s. population group, alleles 1 (0.206) and 2 (0.208) were relatively dominant and were present in 17 out of the 22 populations. In the pooled populations, alleles 1 (0.241) and 2 (0.195) were relatively dominant and were present in 21 out of 27 populations.

4.2.2 Population differentiation index (F_{st})

The estimate of inter-population genetic differentiation (F_{st}) indicated a considerable differentiation between populations for both, the group of 100% of *An. gambiae* s.s. populations and that of 1 to 99% of *An. gambiae* s.s. (Table 4). F_{st} was significant, being higher than 0.05 in both cases. Estimates of F_{st} were high for the group of 100% of *An. gambiae* s.s. populations (Fst = 0.9385) than for the other group (F_{st} = 0.9132). A high differentiation between populations also was observed when the analysis was done with the pooled populations (F_{st} = 0.8714).

4.2.3 Estimate of heterozygote deficiency and excess (F_{is})

The heterozygote deficiency or excess which was calculated to determine heterozygote deficiency (positive F_{is} value) or excess (negative F_{is}) for both groups indicated that all these groups had negative F_{is} values (Table 4). The estimated heterozygote excess for the two groups were – 0.2725 and – 0.8081 respectively. The estimated heterozygote excess for the pooled populations was – 0.7574.

Table 3: Frequencies of the most common alleles with the 100 % of *An.gambiae* s.s. population group, 1 to 99 % of *An. gambiae* s.s. population group

Population	Simple size	No of alleles	Frequency of the most common alleles									
			1	2	3	4	5	6	7	8	9	10
100 % *An. gambiae* s.s.	50	10	0.433	0.122	0.067	0.078	0.056	0.056	0.056	0.056	0.056	0.044
1 to 99 % *An. gambiae* s.s.	220	12	0.206	0.208	0.080	0.118	0.102	0.094	0.066	0.036	0.02	0.034
Pooled populations	270	12	0.241	0.195	0.078	0.112	0.095	0.088	0.061	0.039	0.025	0.035

Table 4: Estimates of differentiation (Fst) and heterozygosity (Fis) within populations of *An. gambiae* s.s.

Population	F_{st}	F_{is}
100 % of *An. gambiae* s.s.	0.9385	- 0.2725
1 to 99 % of *An. gambiae* s.s.	0.9132	- 0.8081
All populations	0.8714	- 0.7574

4.3 The Physico-chemical Parameters of Breeding Habitats

The comparison of the mean values of the parameters of habitats with *Anopheles* and non *Anopheles* revealed that 12 parameters; turbidity, pH, conductivity, suspended solids (SS), total dissolved solids (TDS), sodium, calcium, potassium, chloride, sulphate, total hardness and magnesium hardness significantly differed between the two groups. The values of all these parameters were higher for the water of *An. gambiae* s.s. habitats as shown in Table 5.

Table 5: Comparison of water parameters of habitats of *An. gambiae* s.s. larval populations and those with other species only

Water parameters	Habitats				Test of equality of means
	With *An. gambiae* s.s.		With others species only		
	Mean (S.E.)	Range (Min – Max)	Mean (S.E.)	Range (Min – Max)	P value
Turbidity (NTU)	449.8 (70.7)	6.9 – 1000	131.3 (41.7)	5.5 – 1000	0.000
pH	8.0 (0.1)	6.7 – 9.1	7.4 (0.1)	6.7 – 8.5	0.000
Conductivity (µs/cm)	2182.0 (292.2)	274 – 7610	1181.3 (131.3)	150.7 – 2370	0.003
Suspended solids (mg/l)	608.1 (142.3)	15 – 3300	158.8 (41.5)	10 – 970	0.005
Total dissolved solids (mg/l)	1513.4 (231.5)	24 – 5327	561.5 (82.9)	31.4 - 1659	0.000
Sodium (mg/l)	230.1 (35.0)	6.9 - 865	133.7 (21.8)	7 - 490	0.024
Calcium (mg/l)	124.3 (25.2)	12 – 570.7	53.3 (6.4)	8 - 132	0.010
Magnesium (mg/l)	47.5 (8.6)	0.1 - 221	18.5 (2.2)	3 - 51	0.002
Chloride (mg/l)	557.3 (117.2)	12 - 2879	234.1 (51.3)	8.9 - 1072	0.016
Sulphate (mg/l)	45.6 (7.0)	0.3 – 134.4	22.4 (5.0)	0 – 94.2	0.010
Totai hardness (mg/l)	375.2 (47.0)	50 - 1160	208.4 (21.4)	100 - 540	0.002
Magnesium hardness (mg/l)	183.1 (33.8)	0 - 910	71.2 9.2)	11.7 - 210	0.003

4.3.1 Parameters predicting the presence/absence of *An. gambiae* s.s.

The results of the stepwise linear discriminant function analysis revealed that pH, calcium and turbidity were the best discriminatory parameters between presence/absence of *An. gambiae* s.s. larval habitats (Table 6a). pH was first selected followed by calcium and then turbidity in that order. The derived classification function coefficients (Fisher's linear discriminant functions) obtained are shown in Table 6b.

4.3.2 Parameters predicting variation in distribution of *An. gambiae* s.s. populations

The bivariate correlation procedure computed using Pearson's correlation coefficient revealed that the proportions of *An. gambiae* s.s. in larval habitats were significantly and positively correlated to turbidity ($r = 0.515$, $p = 0.000$), pH ($r = 0.429$, $p = 0.001$), conductivity ($r = 0.380$, $p = 0.005$), total suspended solids ($r = 0.384$, $p = 0.004$), total dissolved solids ($r = 0.443$, $p = 0.001$), sodium ($r = 0.310$, $p = 0.023$), calcium (0.256, $p = 0.048$), magnesium ($r = 0.272$, $p = 0.046$), chloride ($r = 0.363$, $p = 0.007$), sulphate ($r = 0.299$, $p = 0.028$), total hardness ($r = 0.269$, $p = 0.050$) and magnesium hardness ($r = 0.287$, $p = 0.036$) (figure 6a-d).

When multiple regression analysis was performed on the dataset of significantly correlated parameters to select the best predictors of proportion of *An. gambiae* s.s. it revealed turbidity, pH and calcium as the best predictors in the model. These together accounted for 42.9% of the total variation observed (Table 7). All the three models that were applied selected turbidity, pH and calcium in that order. However, when

multiple regression analysis was performed using 28 parameters, only turbidity and dissolved oxygen were selected as best predictors associated with proportions of *An. gambiae* s.s. (Table 8). The two parameters however accounted for only 36.7% of the total variation.

To determine which of the non-selected parameters were either counting to or were associated with turbidity, pH and calcium, multiple regression analyses were performed using these as dependent variables. The results obtained are shown in Tables 9a – 9c. The analysis revealed that suspended solids, sulphate, total dissolve solids, magnesium hardness, ammonium and biological oxygen demand were the best variables, together accounting for approximately 79% of the variation existing in turbidity. Furthermore, all were significantly correlated to turbidity ($P < 0.05$ in all cases). However, dissolved oxygen, total dissolved solids, silica and calcium were the best variables associated with pH, together accounting for 47% of the variation existing in pH whereas total hardness and suspended solids were the only variables associated with calcium and accounting for 42% of the observed variation.

Table 6a: Summary of results of stepwise discriminant function analysis performed on dataset set on the significant parameters to select the most discriminatory parameters separating habitats of *An. gambiae* s.s. and those of other mosquito species only

Step	Number of parameters	Exact F statistic	Wilks' Lambda	P value
1	1[a]	14.752	0.779	0.000
2	2[b]	14.558	0.637	0.000
3	3[c]	14.888	0.528	0.000

a. pH
b. pH and calcium
c. pH, calcium and turbidity

Table 6b: Derived classification function coefficients by stepwise discriminant analysis for the separation of presence/absence of *An. gambiae* s.s. habitats (p value to enter = 0.05)

Parameters	Habitats	
	With *An. gambiae* s.s.	Other species only
pH	28.353	25.906
Calcium	6.166^{E-02}	4.913^{E-02}
Turbidity	4.617^{E-03}	1.048^{E-03}
Constant	- 118.962	- 97.966

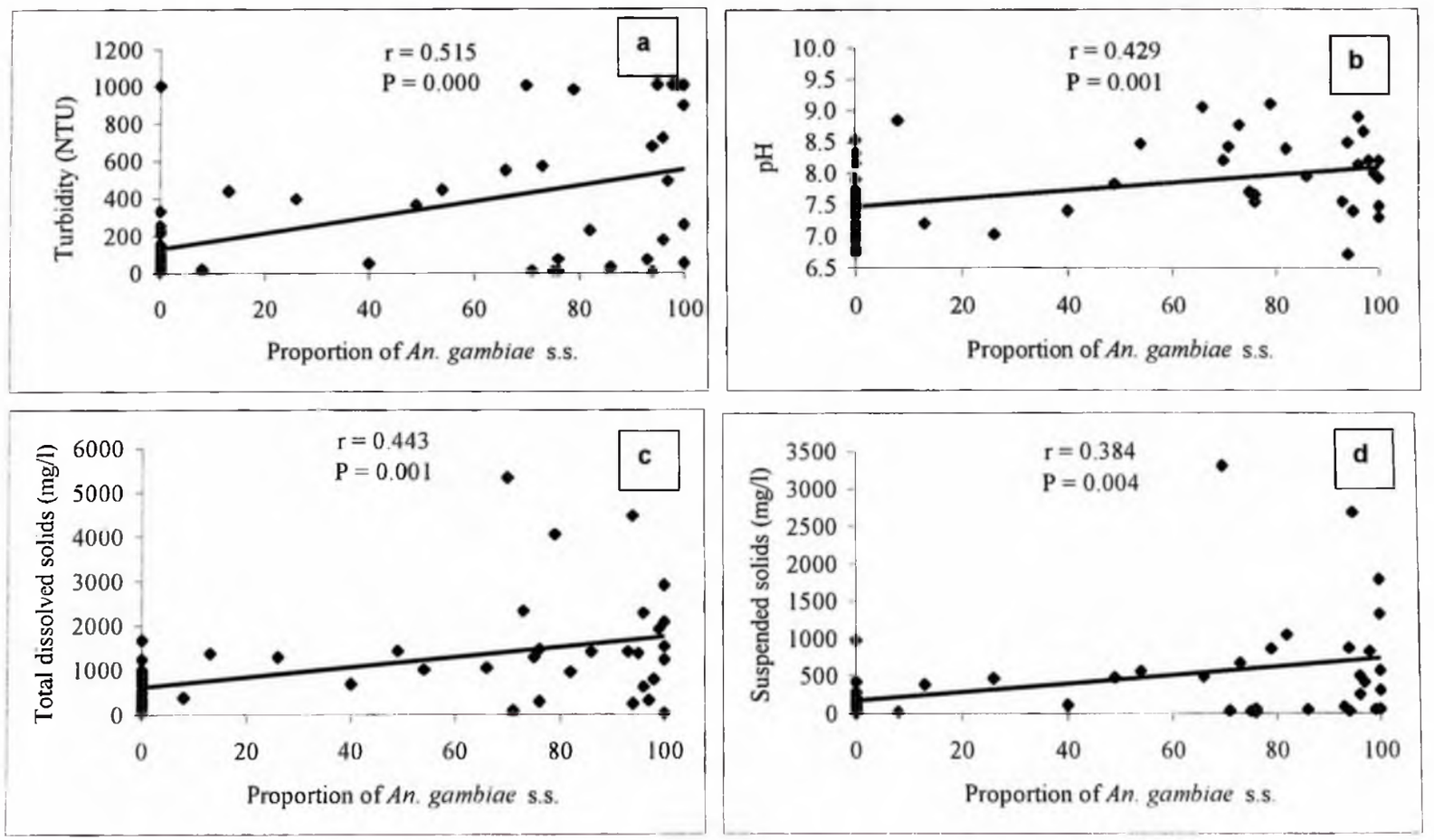

Figure 6a-d: Example of plots showing relationships between the significantly environmental parameters and proportions of *Anopheles gambiae* s.s. in mosquito habitats. The fitted line is drown in bold

Table 7: Summary of results of multiple regression analysis (p value to enter = 0.05) using proportion of *An. gambiae* s.s. as dependent variable and the significant correlated parameters as independents variables

Model	Correlation	R square	Adjusted R square	Standard error	F value	P value
1	0.515[a]	0.265	0.251	37.4293	18.743	0.000
2	0.597[b]	0.357	0.331	35.3619	14.128	0.000
3	0.655[c]	0.429	0.394	33.6536	12.502	0.000

a. Predictors: (constant), turbidity
b. Predictors: (constant), turbidity, pH
c. Predictors: (constant), turbidity, pH, and calcium

Table 8: Summary of results of multiple regression analysis (p value to enter = 0.05) using proportion of *An. gambiae* s.s. as dependent variable and all the measured parameters as the independents variables

Model	Correlation	R square	Adjusted R square	Standard error	F value	P value
1[a]	0.515	0.265	0.251	37.4293	18.743	0.000
2[b]	0.606	0.367	0.342	35.0817	14.764	0.000

a. Predictors: (constant), turbidity
b. Predictors: (constant), turbidity and dissolved oxygen

Table 9a: Summary of results of multiple regression analysis (p value to enter = 0.05) using turbidity as dependent variable and all the measured parameters as the independents variables

Model	Correlation	R square	Adjusted R square	Standard error	F value	P value
1[a]	0.728	0.531	0.521	245.2238	58.757	0.000
2[b]	0.778	0.605	0.590	227.0838	39.079	0.000
3[c]	0.806	0.650	0.629	215.8864	30.968	0.000
4[d]	0.854	0.730	0.708	191.6289	33.093	0.000
5[e]	0.872	0.760	0.735	182.5416	30.376	0.000
6[f]	0.888	0.788	0.761	173.4506	29.064	0.000

a. Predictors: (constant), SS
b. Predictors: (constant), SS and sulphate
c. Predictors: (constant), SS, sulphate and TDS
d. Predictors: (constant), SS, sulphate, TDS and Mg hardness
e. Predictors: (constant), SS, sulphate, TDS, Mg hardness and ammonium
f. Predictors: (constant), SS, sulphate, TDS, Mg hardness, ammonium and BOD

Table 9b: Summary of results of multiple regression analysis (p value to enter = 0.05) using pH as dependent variable and all the measured parameters as the independents variables

Model	Correlation	R square	Adjusted R square	Standard error	F value	P value
1[a]	0.401	0.161	0.145	0.5878	9.964	0.003
2[b]	0.549	0.301	0.273	0.5417	10.973	0.000
3[c]	0.624	0.390	0.353	0.5111	10.647	0.000
4[d]	0.685	0.470	0.426	0.4814	10.842	0.000

a. Predictors: (constant), DO
b. Predictors: (constant), DO and TDS
c. Predictors: (constant), DO, TDS, and silica
d. Predictors: (constant), DO, TDS, Silica, and Calcium

Table 9c: Summary of results of multiple regression analysis (p value to enter = 0.05) using calcium as dependent variable and all the measured parameters as the independents variables

Model	Correlation	R square	Adjusted R square	Standard error	F value	P value
1[a]	0.568	0.322	0.309	91.5114	24.717	0.000
2[b]	0.650	0.423	0.400	85.2630	18.696	0.000

a. Predictors: (constant), total hardness
b. Predictors: (constant), total hardness and suspended solids

4.4 Association between larval habitat types and *An. gambiae* s.s. populations

To determine if the characterised *An. gambiae* s.s populations were specifically associated with certain larval habitats, the phylogeny of the different populations was constructed using the microsatellite DNA data and POPGENE software. Then, a phenogram was constructed to cluster together similar habitat types, using hierarchical cluster procedure in SPSS software version 10.0 (SPSS inc., USA) and environmental parameters that were significantly correlated to proportions of *An. gambiae* s.s. Visual assessment of the phylogenetic tree and the dendrogram did not reveal any association.

Then the two analyses were again performed using microsatellite DNA and environmental parameters of habitats that harboured only *An. gambiae* s.s. In this case there was a perfect match (Figures 7a-b) and in both cases, the *An. gambiae* s.s. proportions and the habitat at one site, Legon village B was more distant (standing alone) from the rest. Table 10 shows the environmental parameters for the five sites where 100% of *An. gambiae* s.s. populations occurred and it shows that the closer values of environmental parameters were clustering together especially, turbidity, pH and calcium as observed at Madina estate A and B sites. The values of the environmental parameters for Legon village B which was standing alone on both trees were lower when compared to those of the rest of this group.

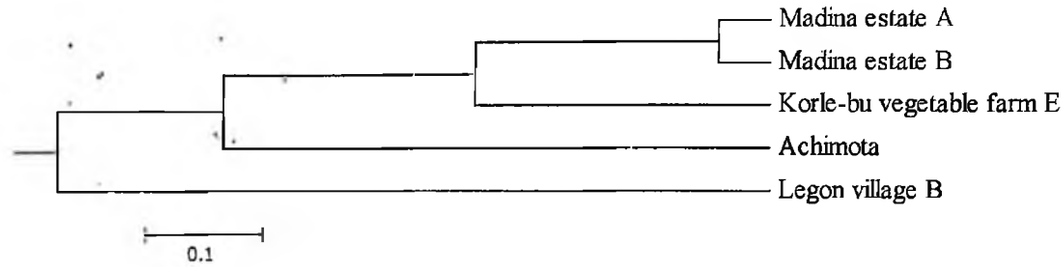

Figure 7a

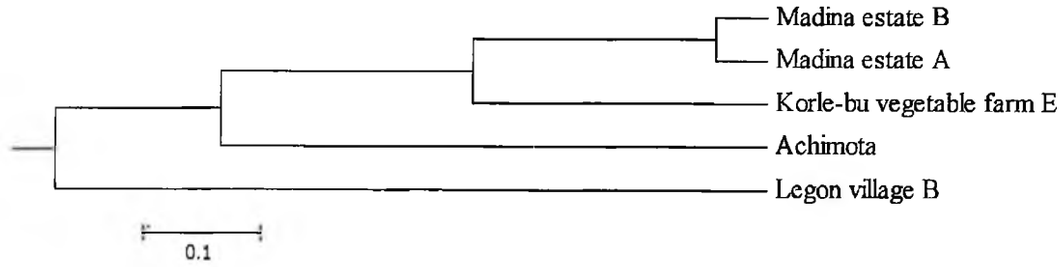

Figure 7b

Figure 7: Molecular phylogenetic trees obtained with microsatellite DNA of *An. gambiae* s.s. of pure populations (a) and hierarchical clustering (b) of their habitats

Table 10: Environmental parameters for the group of 100 % of *An. gambiae* s.s. populations significantly correlated to the proportion of *An. gambiae* s.s.

Sites	Turbidity	pH	Conduct.	SS	TDS	Na	Ca	Mg	Chloride	Sulphate	Total hard.	Mg Hard.
Madina estate B	891	7.48	2430	1175	1515	340	120.0	32.80	581	76.40	435	135
Madina estate A	1000	7.30	2960	1320	2072	258	136.0	55.90	745	120.0	570	230
Korle-bu E	997	7.92	4210	565	2802	485	88.2	0.12	1032	37.27	220	0
Achimota	256.4	8.20	2000	305	1225	192	128.3	31.60	2879	94.0	450	585.6
Legon village B	49.7	7.48	1054	56	24	114	36.1	26.70	228	1.46	200	109.7

CHAPTER FIVE

DISCUSSION AND CONCLUSION

Anopheles gambiae s.s. which is one of the most effective vectors in transmitting the malaria parasite, *Plasmodium falciparum* to humans across the region, in rural and urban areas alike is adapted to a wide variety of micro and macro environmental conditions, as evidenced by its wide geographical distribution. Physical, chemical and biological parameters of water quality may all influence the suitability of certain water bodies for *An. gambiae* s.s. breeding. It is therefore clear that information on breeding sites of this species might contribute greatly to a better planning of effective malaria control strategies.

This study set out to identify the environmental parameters that influence the distribution of larval populations of *An. gambiae* s.s in Accra and found that only *An. gambiae* s.s. occurred there. This finding is in agreement with that of Midega (2001), who also found this species in the study area. However, it contradicts those of Chinery (1984) who found *An. arabiensis* as the dominant species in the same area and further suggested that *An. arabiensis* had replaced *An. gambiae* s.s. as a result of urbanization. In fact, except for this study, all other studies have identified *An. gambiae* s.s. as the dominant vector species at sites in the Greater Region (Appawu *et al.*, 2001; M. Wilson, unpublished data; T. Adeniran, unpublished data and R. Abanja, unpublished data). Studies elsewhere have shown that *An. gambiae* s.s. and *An. arabiensis* can occur in the same habitat (Service, 1970; White & Rosen, 1973; Service *et al.*, 1978; Charlwood & Edoh, 1996; Minakawa *et al.*, 1999; Gimnig *et al.*,

2001) whilst others have demonstrated that the two species exhibit spatial and/or temporal separation of habitats (Charlwood & Edoh, 1996). Moreover, Toure *et al.* (1998) reported that the savanna form of *An. gambiae* s.s. predominated in relatively humid areas with larval production occurring exclusively during the rainy periods, whereas *An. arabiensis* prevailed in more arid areas. Although Greater Accra Region receives the least annual rainfall in Ghana, humidity is high (between 65 and 75%) because of marine influence. Charlwood & Edoh (1996) and Minakawa *et al.* (1999) suggested that the distribution of each species in a larval habitat might be related to the proximity of the preferred hosts of each species rather than to inherent differences in the larval environments with *An. gambiae* s.s. predominating in the habitats near human dwellings and *An. arabiensis* predominating in habitats near cattle. Although cattle are kept very close to houses in some parts of urban Accra no *An. arabiensis* was encountered at these places. Furthermore *An. gambiae* s.s. is believed to be a stronger competitor than *An. arabiensis* (Schneider *et al.*, 2000) therefore supporting our finding of only *An. gambiae* s.s.

The breeding sites of *Anopheles gambiae* s.s. varied from clear to the very dirty water in a variety of habitats but the typical habitats were small and undisturbed temporary pools containing debris, covered by filamentous algae and exposed to sunshine. In addition, *An. gambiae* s.s. were also found breeding in organically polluted waters some with high levels of ammonium, suggestive of runoffs from vegetable farms which apply fertilizers.

The characterization of *An. gambiae* s.s. populations by microsatellite DNA analysis revealed that the distribution of the alleles of the AGHX7 was not uniform. These

observations are supported by the high differentiation index (F_{st} = 0.8714) recorded suggesting that the populations were not related. However, Lehmann *et al.* (1997) reported that F_{st} value (which is an estimate of inter-population genetic differentiation) lower than 0.008 provides no evidence for a division of the gene pool. This is expected since the mosquitoes were sampled from different localities and not at the same time period. The highest number of null alleles was however observed in the *An. gambiae* s.s. only populations. The significance of this finding is not clear. However, null alleles are supposed to be due to mutations in the region complementary with one or two of the oligonucleotide primers (Lehmann *et al.*, 1997), and whether finding them more in these populations are characteristic cannot be concluded from the present study. Moreover, the occurrence of null alleles present a common complication in the interpretation of microsatellite data because there is an apparent reduced level of heterozygosity since they can be detected only in the homozygous state (Pemberton *et al.*, 1995; Lehmann *et al.*, 1996; Donnelly *et al.*, 1999).

The habitat characteristics of *An. gambiae* s.s. larvae were found to be remarkably different from those of *Culex* and *Aedes* species. The *Anopheles gambiae* s.s. breeding habitats were all significantly high in 12 parameters but turbidity, pH and calcium were the most significant discriminatory parameters of presence and absence *An. gambiae* s.s. larval habitats. They were also selected as the best predictors of abundance of *An. gambiae* s.s. larvae in their habitats.

As a rule of thumb, *Anopheles* mosquitoes should breed in fairly clear water and oxygen-rich water (Service, 1980). Turbidity due to organic pollution is believed to

result in a diminished light penetration, which at certain depth anaerobic processes take over. However, numerous exceptions to this including *An. arabiensis* and *An. gambiae* s.s. have been observed breeding in turbid water (McCrae, 1984; Lacey & Lacey, 1990) and Gimning *et al.* (2001) have also found *An. gambiae* s.s. breeding in turbid habitats in Kenya. .

Several biological factors including organic matter, bacterial/fungal/algal contamination and aquatic weeds all contribute to turbidity (Bos, 1991; American Public Health Association *et al.*, 1998). The present study also found that turbidity was associated with suspended solids, sulphate, total dissolved solids, magnesium hardness, biological oxygen demand and ammonium. Except for the ammonium all the other five parameters were positively correlated with turbidity. It is clear how suspended solids and total dissolved solids could affect turbidity, but not for biological oxygen demand (BOD), magnesium hardness, sulphate and ammonium. BOD was found to be comparatively, albeit, marginally significantly high in non-*An. gambiae* s.s. habitats ($p = 0.08$). BOD reflects high densities of algae and other living organisms and high ammonium concentrations also promote bacterial/algal growth, which should therefore increase turbidity yet, the latter parameter was negatively correlated. Drainage waste might contribute to the presence of sulphates in water bodies (American Public Health Association *et al.*, 1998), therefore it is likely that its association with turbidity is indirect and that it is the polluted waste-water that is responsible. As mentioned above, several factors contribute to turbidity and which of them, if any, are attractive to *An. gambiae* s.s. cannot be determined from this study. It is however, unlikely that habitats are selected on the basis of these parameters,

rather, these parameters may be correlated with other characteristics that altogether favour *An. gambiae* s.s. larval development and survival.

In addition to being more turbid, the habitats of *An. gambiae* s.s. were also more likely to have higher pH values than those with other species. The pH of water is dependent on the concentrations of anions, cations, salts and synthetic compounds which indicate its acidic or basic character (Bos, 1991). Therefore, it may be directly or indirectly determinant to the life of any aquatic organism, in occurrence, *An. gambiae* s.s. Apart from direct effect which is not clearly known, anions and cations may also indirectly affect mosquito breeding by favouring certain aquatic vegetation or organisms on which mosquito larvae feed or affect potential biological control agents of mosquito larvae (Bos, 1991). In this study, the determinants of pH were found to be dissolved oxygen, total dissolved solids, silica and calcium. High dissolved oxygen levels are important in maintaining aquatic life (Chapman, 1992) but how it can result in increasing the pH is not clear.

Calcium was found to be last important parameter, and this finding is not unusual because as reported by Pitcairn *et al.* (1987) calcium-rich water favour the growth of the macrophytic alga, *Chara,* whose presence positively correlated with the abundance of *Anopheles freeborni* larvae. The present study also found that calcium concentration was associated with total hardness and suspended solids. The source of calcium in water bodies are the rocks from which it leaches into water and it is also a known cause of water hardness and because of its origin it is not hard to understand its association with suspended solids. Other studies elsewhere used similar parameters but did not get similar results. For example, Mwangangi *et al.* (2002)

measured nitrate, ammonia, turbidity, phosphate, temperature, pH etc but only temperature was found to be significantly associated with *An. gambiae* s.l. habitats along the Kenyan coast. We measured temperature and it was not selected. We do not expect temperature of water to vary significantly – all habitats were exposed to direct sunshine and the topography relief of Greater Accra is flat and low, so all areas will have similar temperature. Moreover, for that study only 10 habitats were studied, and in Kenya temperature can vary significantly depending on where sampling is carried out in valleys or at high elevations etc. The presence of algae, aquatic vegetation and surface film are some of other parameters used in similar studies elsewhere and were found to be useful in describing *An. gambiae* s.s. habitats (Gimnig *et al.*, 2001). Gimnig *et al.* (2001) found that habitats with *An. gambiae* s.s. were more likely to have algal growth than those of *An. arabiensis*. The two types of habitats also differed in temperature (warmer in habitats with both species), the presence of aquatic vegetation (less common in habitats with both species), and the presence of a surface film (less common in habitats with both species). However, although these parameters were known, for logistic and technical reasons they could not be incorporated in the present study.

Interestingly, it was observed that among the five *An. gambiae* s.s. populations that bred alone in habitats the molecular phylogeny constituted fitted exactly with the cluster obtained using their water parameters. On closer inspection it was observed that closer populations were more likely to have relatively similar values of turbidity, pH and calcium concentrations. In addition they were also more likely to have similar allelic and genotypic frequencies. Whether these similarities are real or artefacts cannot be established from this study and will require further studies. There

is however suggestive evidence that West African populations of *An. gambiae* s.s. are highly structured and that ecological and behavioural plasticity are associated with polymorphisms in the form of paracentric chromosomal inversions, microsatellite DNA and isozyme variability with localised anopheline populations (Lanzaro *et al.*, 1995). Moreover, it is also reported that spatial distribution of certain gene arrangements show strong association with specific regional habitats and their frequencies change seasonally in places with seasonal fluctuations in weather, especially rainfall (Coluzzi *et al.*, 1985; Toure *et al.*, 1998). The fact that the fitness of the populations and water parameter clustering could not be produced with the data that included all *An. gambiae* s.s. habitats suggests that the real effect could only be revealed using *An. gambiae* s.s.–specific habitats, but as suggested above, this needs further investigations of a larger sample size.

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APPENDIX I

SAMPLING SITES, DATES OF SAMPLING AND NUMBER OF MOSQUITOES COLLECTED

No.	Study sites	Coordinates		Date of sampling	No. of mosquitoes	
		N	W		*Anopheles*	Other sp.
1	Madina Ritz Hotel A	05°41.120	000°09.917	13/11/01	195	11
2	Madina estate A	05°40.150	000°09.631	13/11/01	163	0
3	Madina Hannah S. A	05°40.463	000°10.263	13/11/01	218	3
4	Madina Hannah S. B	05°40.466	000°10.242	13/11/01	0	*
5	Madina Point Five	05°40.356	000°10.202	13/11/01	173	71
6	Legon Village A	05°35.753	000°10.888	13/11/01	330	7
7	New Achimota	05°37.144	000°14.066	10/01/02	49	8
8	White Cross A	05°38.659	000°14.265	10/01/02	35	37
9	Amanful village	05°18.382	000°50.240	14/01/02	0	*
10	Hwida village	05°15.457	000°48.013	14/01/02	37	1
11	Okyereko village	05°24.854	000°36.167	14/01/02	48	2
12	Muus	05°39.047	000°15.285	17/01/02	1	7
13	Dome	05°39.805	000°14.267	17/01/02	111	36
14	West Legon	05°39.287	000°12.499	17/01/02	48	3
15	Madina Hannah S. C	05°40.367	000°10.254	22/01/02	117	9
16	Madina Hannah S. D	05°40.466	000°10.251	22/01/02	330	109
17	Legon campus	05°38.759	000°11.226	14/02/02	33	361
18	Madina Hannah S. E	05°40.439	000°10.264	14/02/02	81	18
19	Masalaky	05°40.496	000°10.464	14/02/02	0	*
20	Dzorwulu	05°37.049	000°11.704	14/02/02	122	62
21	Korle-bu veg. Farm A	05°32.397	000°14.140	19/02/02	0	*
22	Korle-bu veg. Farm B	05°32.332	000°14.176	19/02/02	0	*
23	Korle-bu veg. Farm C	05°32.284	000°14.147	19/02/02	0	*
24	Industrial area	05°34.633	000°13.275	19/02/02	0	*
25	Korle-bu veg. Farm D	05°32.403	000°14.208	21/02/02	15	1
26	Korle-bu veg. Farm E	05°32.403	000°14.210	21/02/02	76	0

No.	Study sites	Coordinates		Date of sampling	No. of mosquitoes	
		N	W		*Anopheles*	Other sp.
27	Korle-bu veg. Farm F	05°32.389	000°14.202	21/02/02	49	13
28	Korle-bu veg. Farm G	05°32.387	000°14.203	21/02/02	16	6
29	Kokomelmel	05°34.567	000°12.398	07/03/02	0	*
30	North industrial area	05°35.245	000°13.371	07/03/02	0	*
31	Tesano	05°35.602	000°13.799	07/03/02	0	*
32	Alajo	05°35.001	000°13.167	07/03/02	0	*
33	Dzorwulu (GSL)	05°37.024	000°11.921	07/03/02	0	*
34	Legon village B	05°35.734	000°10.906	07/03/02	10	0
35	Adenta Housing D. A	05°42.428	000°08.826	15/03/02	14	21
36	Adenta Housing D. B	05°42.435	000°08.800	15/03/02	37	12
37	Legon campus Valco A	05°38.694	000°11.190	21/03/02	0	*
38	Legon Sabbah An. B	05°38.707	000°11.094	21/03/02	0	*
39	Adenta Lami Dwaahi	05°42.374	000°09.234	21/03/02	0	*
40	Adenta SDA junction	05°41.797	000°10.297	21/03/02	0	*
41	Achimota	05°36.971	000°03.594	21/03/02	84	0
42	Legon –bookshop	05°38.859	000°11.236	12/04/02	0	*
43	Legon campus Valco B	05°38.691	000°11.189	12/04/02	0	*
44	Dome -- Goil station	05°39.810	000°14.298	12/04/02	0	*
45	Dome- Wsion spot	05°39.800	000°14.273	12/04/02	43	2
46	White Cross B	05°38.661	000°14.268	12/04/02	0	*
47	Legon village C	05°35.756	000°10.890	12/04/02	7	70
48	Madina Ritz Hotel B	05°41.190	000°09.817	23/04/02	0	*
49	Madina estate B	05°40.153	000°09.639	23/04/02	159	0
50	GBC – A	05°34.735	000°10.840	23/04/02	99	83
51	GBC – B	05°34.733	000°10.841	23/04/02	0	*
52	GBC – C	05°34.737	000°10.843	23/04/02	0	*
53	Airport residential area	05°35.720	000°10.899	23/04/02	0	*
54	Legon village D	05°35.750	000°10.875	23/04/02	34	14

* Mosquito species not counted

APPENDIX II

STANDARD SOLUTIONS USED IN MOLECULAR BIOLOGY STUDIES

The following standard solutions which were used in molecular biology studies were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lb/sq in. for 15 minutes in an Eyela Autoclave (Rikikakki Tokyo).

DNA extraction

Bender buffer (pH 8.0)

0.01 NaCl, 0.2 M sucrose. 0.1 M Tris-HCl pH 7.5, 0.05 M EDTA pH 9.1, 0.5% SDS stored at 4 °C

0.5 M EDTA (pH 8.0)

186.1 g/l of EDTA in water, pH adjusted with NaOH pellets autoclaved and stored at room temperature.

EtBr (10 mg/ml):

1g of EtBr was completely dissolved in 100ml sddw and stored in the dark at room temperature

KAc (5M K 8M Acetate)

60ml of 5M KAc and 11.5ml glacial acetic acid in 28.5 ml distilled water autoclaved and stored at 4°C.

TE (pH 8.0)

10 mM Tris-HCl and 1 mM EDTA (pH 8.0) autoclaved and stored at room temperature

Solutions for Electrophoresis

Agarose Gels

10 X TAE buffer

242g Tris Base, 57.1ml glacial cetic acid, 100 ml 0.5M EDTA, pH adjusted to 7.7 (with glacial cetic acid) and the volume made to 1litre with sddw.

Urea Polyacrylamide Gels

10 X TBE electrophoresis buffer:

108g/l Tris base, 55g/l Boric acid, 9.3g/l Na_2 EDTA and distilled water added to make up volume to 1 litre. Stored at room temperature on the bench. Diluted to 1X working solution for electrophoresis.

40 % Acrylamide:

38g acrylamide, 2g bis-acrylamide in 100ml sddH_2O. Filtered and stored in the dark at 4 ^{o}C. The required amount of ingredients necessary to prepare the solution for the 7% polyacrylamide running (separation) gel and the plug is given in the following table.

INGREDIENTS	5 % POLYACRYLAMIDE GEL SOLUTION
Water (ml)	30.00
Urea (g)	8.25
40 % acrylamide (ml)	11.66
10 X TBE buffer (ml)	5.50

10 µl ammonium persulphate (APS) and 5µl of TEMED were added to 2.4 ml of the gel to plug the plates to avoid leakage. 60µl of APS and 30µl of TEMED were added to the rest of the solution (48ml) to form the separating gel before pouring.

The table below gives the required amount of ingredients necessary to prepare the stacking gel for a solution of 5% polyacrylamide running (separation) gel and the plug.

INGREDIENTS	4.5 % POLYACRYLAMIDE GEL SOLUTION
Water (ml)	31.00
Urea (g)	8.25
30 % acrylamide/0.8 Bis-acrylamide (ml)	7.50
10 X TBE buffer (ml)	5.50

3.5ml of this solution were taken per gel, 30µl of APS and 10µl of TEMED were added and used as stacking gel. The gel was stained in EtBr

Gel loading buffers

6 X Bromophenol blue

0.25% bromophenol blue was added to 40% sucrose in water and stored at 4°C.

Bromophenol blue xylene cyanol: 1 volume of bromophenol blue xylene cyanol and 4 volumes of cyanide

5X orange G

20% w/v Ficoll, 25 Mm EDTA, 2.5 mM EDTA . 2.5% (w/v) orange G. Stored at room temperature.

DNA molecular weight size markers

The 100 bp DNA molecular weight size marker obtained from Sigma were diluted according to the manufacturers recommendations and used. For the 100 bp ladder, the first band size is 100 bp, the subsequent ones are 200, 300 ..... 1000 bp.

APPENDIX III

STANDARD BUFFERS AND SOLUTIONS USED IN WATER ANALYSIS

Alkali-iodide-azide solution

500g NaOH and 135g sodium iodide (NaI) were dissolved in distilled water and diluted to 1 litre. Then, 10g sodium azide (NaN_3) were dissolved in 40ml distilled water and the solution added to the sodium hydroxide-sodium iodide (NaOH-NaI) solution.

Ammonia-free water

This was usually deionised water which was freshly prepared.

Ammonia molybdate reagent I

25g $(NH_4)_6MO_7O_{24}.4H_2O$ were dissolved in 175ml distilled water. Then 280ml concentrated H_2SO_4 were carefully added to 400ml distilled water. After the solution had cooled molydbate solution was added and the volume made up to 1 litre.

Ammonia molybdate reagent

10g $(NH_4)_6MO_7O_{24}.4H_2O$ were dissolved distilled water with stirring and gentle warming and diluted to 100ml. The pH of the solution was adjusted to 7 – 8 with silica-free NaOH and stored in a polyethylene bottle to stabilise.

Buffer-colour reagent

250ml of distilled water were added to 105ml HCl (density 1.18), 5g sulphanilamide and 0.5g N-(1-naphthyl)-ethylenediamine dihydrochloride, stirred until it dissolved.

136g of Sodium acetate were added and again stirred until it dissolved. The solution was diluted to 500 ml with distilled water and stored in the dark.

Colour developing reagent

25ml of concentrated phosphoric acid and 3.75 g sulphanilamide were added to approximately 150ml of water. After completely dissolving the sulphanilamide 0.19g N-(1-naphthyl)-ethylenediamine dihydrochloride. The solution was diluted to 250ml with water, and stored in a dark bottle in a refrigerator.

Conditioning reagent

50ml glycerol were mixed with a solution containing 30ml concentrated HCl, 300ml distilled water, 100ml 95% isopropyl alcohol and 75 g sodium chloride.

Copper sulphate stock solution

0.625g of $CuSO_4.5H2O$ was dissolved in distilled water and diluted to 250ml in a volumetric flask.

Copper sulphate working solution

5ml copper sulphate stock solution were diluted to 500ml distilled water.

Ferroin indicator

1.485g 1,10 phenanthroline monohydrate and 0.695g ferrous sulphate septahydrate ($FeSO_4.7H_2O$) were dissolved in distilled water and diluted to 100 ml.

Indicator: Eriochrome Black T

0.5g dye and 100g NaCl were mixed together and grinded to prepare a dry powder.

Intermediate nitrate solution (10 mg/l)

5ml of stock nitrate solution was diluted to 500ml in a volumetric flask.

Intermediate silica standard solution (50 mg/l)

5ml of the stock silica solution was poured into a 100ml volumetric flask and diluted to the mark with distilled water.

Manganese sulphate solution

480g manganous sulphate tetrahydrate ($MnSO_4.4H_2O$) were dissolved in freshly boiled water and diluted to 1 ltire.

Methyl orange

0.05g of methyl orange was dissolved in 100ml of deionised water. The solution was stored in a well-covered glass bottle.

Murexide indicator

A mixture of 200mg of murexide and 100g of solid NaCl was grinded to 40 to 50 mesh (0.3 – 0.4 mm).

Nessler's Reagent

35g of potassium iodide (KI) and 12.5g of mercuric chloride (HgCl) were dissolved in about 700ml deionised water and about 50 ml of saturated aqueous solution of HgCl)

with stirring until a light permanent red precipitate was formed. Then mixed with a solution of 120g of sodium hydroxide (NaOH cooled to room temperature) in 150 ml of deionised water. When the mixture has cooled, the solution was transferred to a 1 litre volumetric flask and a further 1 ml saturated mercuric chloride solution was added and shaked. Finally dilution was done to 1 litre mark and shaked again. Before use, the reagent was allowed to settle and only the clear supernatant was used. The solution was stored in a rubber-stoppered bottle in the dark and periodically a portion of the clear supernatant liquid was transferred to a small bottle as required for use. After the solution is stable indefinitely, it was stored at room temperature.

Oxalic acid solution

7.5g oxalic acid ($H_2C_2O_4.H_2O$) were dissolved in distilled water and diluted to 100ml.

Reducing mixture (freshly prepared before use)

20ml copper sulphate working solution and 16ml hydrazine sulphate were added to 20ml 0.3 M NaOH and mixed.

Rochelle salt solution

50g Rochelle salt ($KnaC_4H_4O_6.4H_2O$ – potassium sodium tetrate tetrahydrate) were dissolved in 100ml deionised water, boiled down to 70ml to remove traces of ammonia, cooled and diluted to 100ml with deionised water. This solution was freshly prepared.

Phenolphthalein indicator

50ml of deionised water were added to 0.5g of phenolphthalein dissolved in 50ml of 95% ethanol. Then a dilute (0.05 N) carbon dioxide free solution of sodium hydroxide

was added just a drop at a time, until the indicator turns faintly pink. This solution was stored in a well-covered glass bottle.

Potassium chromate indicator solution

50g of Potassium chromate (K_2CrO_4) were dissolved in little distilled water and followed by addition of silver nitrate ($AgNO_3$) solution until a definite red precipitation is formed. The precipitate was let to stand for 12 hours then filtered and diluted to 1 litre with distilled water.

Saturated aqueous solution of mercuric chloride

Mercuric chloride powder was dissolved in 60ml of distilled water until it dissolved no more.

Sodium carbonate (0.1N Na_2CO_3)

5.30g of anhydrous sodium carbonate previously dried at 250 – 300°C for 1 hour deionised water and make up to 1 litre (1000ml). The solution was stored in a well-covered bottle not more than one week.

Sodium hydroxide (0.05 N NaOH)

0.2g of Sodium hydroxide was dissolved in 100ml of deionised water.

Sodium hydroxide solution (0.3M)

1.2g of NaOH was dissolved in 100ml water in a volumetric flask.

Sodium hydroxide (1M/l)

40g of NaOH were diluted to 1000ml of deionised water in a volumetric flask.

SPANDS solution

958mg of 2-(parasulfophenylazo)-1,8-dihydroxy-3-6-naphthalene disulfonate were dissolved in distilled water and diluted to 500 ml.

Standard calcium solution

1.0g anhydrous calcium carbonate ($CaCO_3$) was weighed into a 500ml Erlenmeyer flask and a funnel was placed in a neck of the flask. A little of 1+1 HCl was added at a time until all the $CaCO_3$ had dissolved. Then 200 ml of deionised water was added, boiled for a few minutes to expel the CO_2, cooled and a few drops of methyl red indicator were added followed by an adjustment to the immediate orange colour by adding 3M NH_4OH. The solution was quantitatively transferred to 1 litre volumetric flask and later on filled to the mark with deionised water.

Standard EDTA titrant (0.01 M)

3.723g of dry powder of ethylene diamine tetra acetate (EDTA) were dissolved in deionised water and diluted to 1000ml in a volumetric flask. The solution was standardised against calcium solution (1.0ml = 0.4008 mg Ca^{2+} equivalent to 400.8mg/l Ca^{2+}).

Standard ferrous ammonium sulphate-FAS (0.10 M)

39.2g of ferrous ammonium sulphate – FAS ($Fe(NH_4)_2(SO_4)_2.6H_2O$) were dissolved in distilled water followed by an addition of 20 ml conc.H_2SO_4. After cooling, the solution

was transferred into 1 litre volumetric flask and diluted to the mark. The solution was standardised daily against standard $K_2Cr_2O_7$ digestion solution as follows:

To 5ml distilled was added 3 ml digestion solution ($K_2Cr_2O_7$), 7 ml sulphuric acid reagent, cooled to the room temperature and followed by 2 and 3 drops ferroin indicator and titrated with FAS solution to a wine endpoint.

Molarity of FAS solution = (A/B) x 0.100

Where; A = Volume of 0.0167 $K_2Cr_2O_7$ solution titrated (ml)

B = Volume of FAS used in titration (ml)

Standard fluoride solution (100 mg/l)

0.221g of anhydrous (NAF) was dissolved in distilled water and diluted to 1000ml in a volumetric flask. 1 ml = 0.1mg F^-

Standard phosphate solution

219.5mg anhydrous KH_2PO_4 was dissolved in distilled water and diluted to 1 litre.

1ml = 50mg PO_4.

Standard potassium dichromate solution (0.0167 M)

4.913 g of $K_2Cr_2O_7$ (previously dried at 102°C for 2 hours) were dissolved in about 500ml of distilled water. 167ml conc.H_2SO_4 were added to the solution followed by 33.3g mercuric sulphate ($HgSO_4$). After cooling, the solution was transferred into 1 litre volumetric flask and diluted to the mark.

Standard silver nitrate titrant (0.0141 M)

2.3952g of silver nitrate ($AgNO_3$) were dissolved in distilled water and diluted to 1litre. The standardized $AgNO_3$ was stored in brown glass bottle.

Standard Sodium chroride (0.0141 M)

0.824g NaCl (dried at 140°C for 1 hour) was dissolved in distilled water and diluted to 1 litre (1000ml = 500µg Cl).

Standard Sodium thiosulphate solution (M/40 i.e. 0.0250 M)

6.205g Sodium thiosulphate ($Na_2SO_3.5H_2O$) were dissolved in distilled water, preserved by adding 5ml of chloroform ($CHCl_3$) and diluted to 1 litre. The solution was stored in a brown bottle.

Standard Sodium thiosulphate solution – working solution (M/80 i.e. 0.0125 M)

500ml of the standard sodium thiosulphate solution into a volumetric flask and diluted to 1 litre.

Standardization of 0.01 M EDTA

1ml of buffer solution was added to 10ml of standard calcium solution into a conical flask and titrated against EDTA (1:1 ratio, 10 ± 1 ml of EDTA were used). Titration was completed within 5 minutes from time of buffer addition.

Standardization of acid against Sodium Carbonate

Two or three drops of methyl orange indicator were added to 50ml of 0.1N Na_2CO_3 into a conical flask (yellow colour obtained).

The solution was titrated with the acid to an orange end-point. The normality of acid was obtained from (NxV)/A where:

N = Normality of Na_2CO_3

V = Volume (ml) of Na_2CO_3 used

A = Volume (ml) of acid used

Standardization of 0.0141 M $AgNO_3$ with NaCl

2 drops of potassium chromate indicator were added to 10ml of standard sodium chloride (0.0141 M) into a flask titrated with the AgNO3 solution to a pinkish yellow end point.

M = (0.014 x 10)/V

Where, M = Molarity of $AgNO_3$

V = ml of AgNO3 used in tritration

Stannous chloride reagent

2.5g fresh $SnCl_2.H_2O$ were dissolved in 100ml glycerol, heated in water bath and stirred with a glass rod to hasten dissolution.

Starch solution

6g of soluble starch were added to a small quantity of distilled water and mixed. This mixture was added to 1000ml boiling water and allowed to boil for 5 minutes. The solution was left to stand overnight. The solution was preserved with 2 drops of toluene $C_6H_5CH_3$).

Strong acid solution

300ml concentrated H_2SO_4 were slowly added to about 600ml of distilled water. The solution was cooled and 4ml Nitric acid (HNO_3) added and the volume made up to 1 litre.

Sulphate solution (1000 mg/l SO_4)

1.47929g anhydrous Na_2SO_4 was dissolved in distilled water and diluted 1 litre mark into a volumetric flask.

Sulphuric acid (H_2SO_4)

2.8 ml concentrated sulphuric acid, density 1.84 was diluted to 1 litre with deionised water and standardized against 0.1N sodium carbonate using methyl orange indicator. This solution was further diluted to 0.02N solution by taking 200ml aliquot of the stock solution and topping to 1 litre. The solution was stored in a covered glass bottle.

Sulphuric acid reagent (silver sulphate in sulphuric acid)

11g silver sulphate were dissolved to 1 litre conc.H_2SO_4

Zirconyl-acid reagent

133mg of Zirconylchlorideoctahydrate ($ZrOCl_2.8H_2O$), were dissolved in about 25ml distilled water. Then 350ml conc.HCl was added to the solution before making up the volume to 500ml with distilled water.

APPENDIX IV

Input data format for the population genetics analysis software POPGENE Version 1.31

/*Diploid data of 27 populations each with 10 loci */

Number of populations = 27

Number of loci = 10

Locus name:

L1 L2 L3 L4 L5 L6 L7 L8 L9 L10

NAME = MADINA HANNAH STREET

BC DE FF GG .. ..

BC DE FF .. .. JJ

BC DE FF .. .. ..

AB DE FF .. .. .. KK ..

BC DE FF .. ..

.. ..

BB DD .. ..

AB DE HH II LL

.. .. DD .. ..

.. BC DD .. JJ .. ..

APPENDIX V

PHYSICO-CHEMICAL WATER PARAMETERS MEASURED

Parameter	Formula	Units	Value	Notes WHO guideline
Temperature		°C		
Turbidity		NTU		5
pH		pH units		6.5 – 8.5
Conductivity		µS/cm		
Suspended solids (SS)		mg/l		
Total dissolved solids (TDS)		mg/l		1000
Sodium	Na	mg/l		200
Potassium	K	mg/l		30
Calcium	Ca	mg/l		200
Magnesium	Mg	mg/l		150
Total iron	Fe	mg/l		0.3
Ammonium	NH_4-N	mg/l		
Chloride	Cl	mg/l		250
Sulphate	SO_4	mg/l		400
Phosphate	PO_4-P	mg/l		
Silica	SiO_2	mg/l		
Nitrite	NO_2-N	mg/l		
Nitrate	NO_3-N	mg/l		10
Total hardness	as $CaCO_3$	mg/l		500
Total alkalinity	as $CaCO_3$	mg/l		
Calcium hardness	as $CaCO_3$	mg/l		
Magnesium hardness	as $CaCO_3$	mg/l		
Fluoride	F	mg/l		1.5
Bicarbonate	HCO_3	mg/l		
Carbonate	CO_3	mg/l		
Dissolved oxygen (DO)		mg/l		
BOD		mg/l		
COD		mg/l		