STUDIES ON INSECTICIDE USAGE AND PYRETHROID RESISTANCE IN POPULATIONS OF *ANOPHELES GAMBIAE* SENSU STRICTO (DIPTERA: CULICIDAE) IN THE GREATER ACCRA REGION OF GHANA

BY

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DECLARATION

I do hereby declare that the experimental work described in this thesis was carried out by me and that all cited references have been duly acknowledged. This thesis, either in whole or in part has not been submitted for any other degree in any institution or organisation elsewhere.

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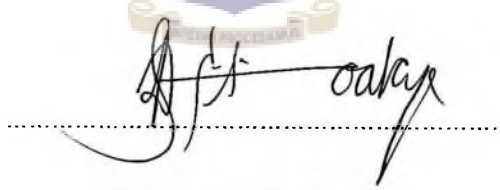

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DEDICATION

To my family

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bp	base pair
dATP	deoxyadenosine triphosphate
ddw	double distilled water
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTPP	deoxythymidine triphosphate
EDTA	disodium ethylene diamine tetraacetate
EtBr	ethidium bromide
EtOH	ethanol
H_2O	water
KAc	potassium acetate
kb	kilobase
kdr	knockdown resistance
KOH	potassium hydroxide
M	Molar
Mw	molecular weight
NaOH	sodium hydroxide
PCR	polymerase chain reaction
pH	$-\log_{10}[H^+]$
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute

ssdw	sterile double distilled water
s.l.	sensu lato
s.s.	sensu stricto
TAE	Tris-acetate EDTA
T_m	melting temperature
Tris	2-amino-2-(hydroxymethyl)-1,3 propanediol
µl	microlitre
µM	micromolar

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ABSTRACT

Insecticide resistance due to knockdown resistance *(kdr)* mutations in *Anopheles gambiae* populations has been reported in neighbouring West African countries, such as Côte d'Ivoire, Burkina Faso and Benin despite the absence of large-scale mosquito control in these countries. The hypothesis is that agricultural or household use of insecticides is responsible for the evolution of resistance. However, studies relating such uses of insecticides and the development of resistance in *An. gambiae* have not been carried out in Ghana. Meanwhile, Ghana is one of the countries where insecticide treated bednets is a major component of the Roll Back Malaria programme. Pyrethroid insecticide resistance could hamper this mass control effort; hence there is the need to establish baseline information on insecticide use pattern and vector susceptibility levels for this country. To achieve these objectives a questionnaire survey was conducted to obtain information on the household insecticide usage and habits in the Accra Metropolis. Then adult *An. gambiae* reared from larvae collected at geopositioned sites were tested at diagnostic doses of permethrin (0.75%) and deltamethrin (0.05%) using WHO adult mosquito test kit to assess the impact of insecticide usage on levels of resistance. The presence of gene mutations associated with knockdown resistance (*kdr)* in the test mosquitoes was detected using polymerase chain reaction (PCR) with already published oligonucleotide primers Agd1, Agd2, Agd3 and Agd4. Polymerase chain reaction was also used to identify the species of the *An. gambiae* complex. A total of 171 households were surveyed. Results of this survey revealed that the frequency of pyrethroid insecticide application was high. Most

respondents said the available aerosols were not as effective as they expected. However, respondents still preferred aerosols to coils and that the aerosol of choice was RAID* which contains tetramethrin (0.15%), allerthrin (0.25%), and deltamethrin (0.015%). There was no significant difference between the reported incidences of malaria in all the socio-economic classes of the respondents. All bioassays showed mortalities ranging from 68-88% (mean: 78%) indicating the existence of resistance in *An. gambiae* populations in Accra. The specimens identified by PCR were all *An. gambiae* s.s. and *kdr* mutations were found in 52/195 (26.7%) of the sample population and 39/48 (81.3%), thus colloborating the results of the bioassay. The importance of this study, being the first of its kind in Ghana, was to obtain baseline data essential for monitoring resistance in *An. gambiae* s.l for future malaria vector control and the implications of findings in the context of malaria control in Ghana are discussed in Chapter five.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 Introduction

Malaria is by far the world's most important tropical parasitic disease. In many developing countries and in Africa especially, malaria exacts an enormous toll in lives, in medical costs and in days of labour cost. There are four different species of the malaria parasite. *Plasmodium falciparum*, (malignant tertian), which is found globally but is commonest in Africa, is the most important species responsible for severe and fatal malaria. *Plasmodium vivax*, (benign tertian) which ranges widely throughout Asia, Africa, the Middle East, Oceania and the Americas (and is resurgent in Eastern Europe), can cause recurring and debilitating infection, but rarely kills. The other two are *P. malariae* (quartan malaria) and *P. ovale* (ovale benign malaria). Mosquitoes belonging to the genus *Anopheles* transmit all these parasites.

1.1 Malaria – The Scope of the Problem

Malaria is a public health problem in more than 90 countries inhabited by some 2,400 million people -- 40 percent of the world's population (WHO, 1998). It is estimated to cause up to 500 million clinical cases and over one million deaths each year. Snow *et al.* (1999) estimated that, among populations exposed to stable endemic malaria in

sub-Saharan Africa, approximately 987,466 people might have died in 1995 from direct consequences of *P. falciparum* infection. This included 765,442 children below the age of 5 years. It is estimated that, a child somewhere dies of malaria every 30 seconds and that in any given year; nearly ten percent of the global population will suffer a case of malaria (WHO, 1998). Most survive after an illness of 10-20 days. In many parts of the developing world, malaria and acute lower respiratory tract infections (ALRTI) are the two commonest causes of child morbidity and mortality (TDR News, 1991). In comparison to other infectious diseases, malaria kills about as many persons per year as AIDS has done in the last 15 years. About 30 times as many persons die of malaria every day, as died in the Ebola virus outbreak of 1995 in Zaire (Annual Report of the Institute of Medicine of the National Academy of Sciences of the United States, 1996).

Over a quarter of a very poor family's income can be absorbed in the cost of malaria treatment, quite apart from the cost of prevention, or the opportunity cost of labour lost through illness. Being the most prevalent disease in poor rural regions, malaria is debilitating causing its victim to forego, on average, 12 days of productive output. The economic consequences of malaria-related diseases are high. The direct and indirect costs of malaria in sub-Saharan Africa was more than US$800 million in 1987 and more than US$2,000 million at the end of 1997. Cost to countries is estimated to be 1-5% of gross domestic product (GDP) in Africa (WHO, 1988).

In afflicting entire races and nations, malaria hinders development, discourages inward capital investment, stultifies global trade, and generally depresses the standard and quality of life for the world's most disadvantaged persons. Yet the

marginal cost of reversing malaria's toll is, comparatively speaking, small. (Desowitz, 1991).

Although malaria had been eliminated or effectively suppressed in many parts of the world, it is now returning to areas, from which it had been eradicated, and spreading geographically into new areas, such as Central Asia, and Eastern Europe, which previously had low or no transmission (WHO, 1998). Thus, more people are now dying of malaria than thirty years ago. There are several factors that contribute to this but the most important ones are insecticide resistance in vectors, drug resistance in parasites and human activity. Mosquitoes have either developed or are developing resistance to the major classes of insecticide that have been used so far in vector control. In most areas, malaria parasites resist at least one drug whilst in others they resist all known drugs. For example, chloroquine is an extremely safe and cheap drug, but in Asia and an increasing area of Africa and South America the resistance levels are high. In some areas of Asia there is resistance to all the major drugs (Kashirsagar *et al.*, 2000). There are no fail-safe treatments, a state of affairs not known since the discovery of quinine in the 17th century. In malaria endemic parts of the world, a change in risk of malaria can be the unintended result of economic activity or agricultural policy that changes the use of land, for example, creation of dams, irrigation schemes, commercial tree cropping and deforestation (WHO, 1998).

1.2 Rationale

Due to the failure of past vector control efforts, there has not been any organised large-scale insecticide treatments to control malaria in recent times. Nevertheless, because of the biting nuisance of mosquitoes coupled with their importance as vectors of diseases, household control of mosquitoes using insecticide sprays, coils and chalks has been on the increase. This indiscriminate use of insecticides varies from area to area and depends on demographic factors including the economic status of the residents and location.

There is renewed interest for the use of insecticides in malaria control because of the parasites' resistance to anti-malarial drugs and the observation that the use of pyrethroid-treated materials can reduce considerably malaria transmission and morbidity (Choi *et al.,* 1995; Binka *et al.,* 1999). Unfortunately, pyrethroid resistance due to *kdr* mutations in mosquitoes has been reported in *An. gambiae* species complex in a number of places including Côte d'Ivoire, Burkina Faso and Kenya (Martinez-Torres *et al.*, 1998; Chandre *et al.*, 1999a, 1999b). Its geographical distribution has been thoroughly investigated in Côte d'Ivoire where it occurs in most wild populations, occasionally at very high frequency (>90%) (Chandre *et al.*, 1999b). The existence of resistance could render ineffective the mass use of the insecticide-treated bednets for malaria control. Reduced susceptibility levels in wild vector populations caused by application of household or agricultural insecticides in different demographic settings or variation among different cytological forms of the same species may not provide the expected control (Chandre *et al.,* 1999a). These notwithstanding, the observation is that insecticide-treated materials (ITMs) can still be

considered an excellent method for personal protection in areas with resistant *An. gambiae* s.s (Darriet *et al.*, 1999).

It is obligatory that tests on mosquito susceptibility to insecticide be performed before the introduction of insecticide-treated bednets. Therefore, routine testing with a standardised protocol for resistance before bednet trials and operations has been advocated (Anonymous, 1997). However, this cannot be carried out for all areas if bednets are to be used over a wider area. As a result, each country may be divided into sections according to similar geographical and demographic parameters and baseline susceptibility data collected at representative sites. Based on such baseline data, susceptibility levels can be extrapolated for areas with similar socio-economic status, demographic and ecological characteristics. Various studies on the pyrethroid resistance gene (*kdr*) have been undertaken in West Africa particularly in the French-speaking countries (Chandre *et al.*, 1999a, 1999c). These studies have mapped the distribution of the *kdr* gene, the frequency of the gene in certain areas as well as relating resistance to agricultural and to a limited extent household use of insecticides.

Identification of vector species of diseases is an essential component of any vector control strategy. It is now known that levels of susceptibility to insecticide can vary between different populations of the same species even under the same level of insecticide pressure. For example within the *Simulium sanctipauli* s.s. in the Onchocerciasis Control Programme, the Comoé form is the population found to maintain a stable resistance to the insecticide temephos (Boakye *et al.*, 1999). Recently, Touré *et al* (1994) have used chromosomal features to identify population variation within the *An. gambiae* s.s. in Mali. These chromosomal variants in

Anopheles may show differences in the susceptibility levels to pyrethroid insecticides but there is only one report of this happening in the *An. gambiae* complex (Chandre *et al.*, 1999a) and more information is needed.

Molecular techniques such as those developed by Martinez-Torres *et al.* (1998) will be useful to determine the initial allele frequency when the mechanism for resistance has been determined and also monitor resistance. Once a resistance allele has been established, its rate of spread or time to fixation will depend on other conditions such as the generation turnover of the species, migration (gene flow), formulation of insecticide, frequency of application and persistence of insecticide (selection). Other important factors are the dominance of the allele coding for resistance, relative fitness of resistance genotypes and initial allele frequency. Understanding these parameters requires a population genetics approach to the problem of insecticide resistance. There is therefore an urgent need to develop a reliable tool to detect and monitor pyrethroid resistance in mosquito populations in Ghana where such studies have not been carried out.

Since the 1970s, pyrethroids have been extensively used in urban areas (as domestic coils and aerosols) as well as for agricultural purposes in rural areas. In both cases, the selection pressure exerted on *An. gambiae* s.l. populations was not negligible: the first case of pyrethroid resistance in *An. gambiae* s.l. was recorded in Bouaké (Côte d'Ivoire) and was attributed to the domestic use of aerosols (Elissa *et al.*, 1993). Later, reduced susceptibility was observed in an area of Kenya where permethrin-impregnated bednets were used (Vulule *et al.*, 1994; Beach, 1997).

Insecticide resistance in vector mosquitoes has serious implications for malaria control. This is because the *Anopheles gambiae* complex contains very efficient vectors of malaria. Thus, reduced susceptibility to the commonly used insecticides for their control will invariably contribute to higher transmission rates. The experience with DDT and other insecticides for which resistance has evolved and also with avoidance behaviours in arthropods requires that each chemical be studied early to define types of action against vector species by geographic area before large-scale use especially for impregnated bednet applications (Roberts and Andre, 1994). Efforts to combat insecticide resistance sometimes fail because loss of susceptibility is detected only when resistance frequencies are so high that corrective action is futile (Brogdon, 1991). This means that constant monitoring is imperative in vector control programmes.

This project is therefore intended to establish baseline information for insecticide use and to carry out resistance studies in vector mosquitoes in the Accra Metropilis.

1.3 Overall Objective

The main objective of the proposed study is to investigate by questionnaire, respondents' habits, attitudes and knowledge of insecticide use and whether pyrethroid resistance due to the *kdr* gene is present in *Anopheles gambiae* s.l. populations in the Accra Metropolis.

1.3.1 Specific objectives

The specific objectives are:

1. To carry out survey to determine the types of insecticides used for household mosquito control.
2. To find out information about knowledge, attitude and behaviour of respondents to insecticide use.
3. To carry out bioassays with WHO test kit on adult female *An. gambiae* s.l. mosquitoes obtained from the study site.
4. To identify each test mosquito to the level of species within the *An. gambiae* complex using PCR.
5. To detect *kdr* mutation in each test mosquito using PCR.
6. To analyse the data to establish the knowledge, attitude and perception of respondents to insecticide usage in the study area.
7. To analyse the bioassay and *kdr* data to determine any relationships that may exist between them.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria - The Disease Symptoms

Symptoms of malaria include fever, shivering, pain in the joints, headache, repeated vomiting, generalised convulsions and coma. Severe anaemia (exacerbated by malaria) is often the attributable cause of death in areas of intense malaria transmission. Malaria infection can be chronic and unremitting in parts of the world where transmission is high, such as coastal Africa (Giha *et al.*, 2000). People living in these areas may receive hundreds of infectious mosquito bites a year, with the result that they are perpetually infected with the *Plasmodium* parasite.

Fever, caused by the release of merozoites and consequent destruction of the infected red blood cells, is the first symptom. At its peak, a person's body temperature can soar to 41°C. Several hours later, the fever drops and chills set in. Two to four days later, the cycle repeats. Progression of symptoms from initial fever to death can take as little as 24 hours (WHO, 1998). When the disease is severe, the brain and kidneys become involved.

Cerebral malaria is the most dreaded form of the disease, and is unique to *P. falciparum* (WHO/TDR, 1990). Infected red blood cells are sticky and can gum up the capillaries of the brain. The victim enters into coma, and if he happens to recover, may suffer brain damage as a result.

Not all persons with the parasite have the disease, but some are carriers without symptoms. A mosquito may pick infected blood from these persons and transmit the parasite to another person, who may be more vulnerable.

2.2 Epidemiology of Malaria

The epidemiology of malaria results from the interactions of three gene pools: parasite, human host and mosquito vector and their environment (Koram *et al.*, 1995). The severity and cause of an attack of malaria depend on the species and strain of infecting parasite and hence on the geographical origin of the infection. It also depends on the age, genetic constitution, state of immunity, general health and nutritional status of the patient and on the chemoprophylaxis or chemotherapy used (Warrell, 1993). The distribution of malaria within human populations is also closely linked to species-specific characteristics such as vectorial capacity of the vector population.

The distribution and frequency of malaria in tropical Africa depend on the climate and, among the various climatic factors involved, two surpass the others in importance. These are rainfall and temperature. Thus, the duration of the transmission period of malaria, as well as its intensity, is closely related to the amount of rainfall and its distribution throughout the year (Mouchet *et al.*, 1998). Rainfall provides breeding sites for mosquitoes and increases the humidity, which enhances their survival. An average rainfall of 80mm per month, for at least 3-5 months, is minimum for stable malaria transmission (Craig *et al.*, 1999).

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 vector survival are particularly important. At temperatures below 22°C the determining factor is the number of mosquitoes surviving the parasite's incubation period, which takes 55

days at 18°C and ceases at around 16°C (Detinova, 1962). After 55 days the proportion of mosquitoes that survives may be as low as 0.003 (Martens, 1997). The mean temperature varying from region to region and decreasing as the altitude increases, also affects the limits of the transmission period, or at least modifies the intensity of transmission (Bruce-Chwatt, 1974). For example, the endemicity is less intense in the southern part of Angola and Mozambique, where the altitude is above 1500m. It is however intense in the greater part of West Africa, particularly in Benin, Ghana, Guinea, the eastern part of Guinea-Bissau, Côte d'Ivoire and Nigeria (Bruce-Chwatt, 1984). This is probably due to the temperature regimen and rainfall distribution pattern.

Anopheles larvae occur in many different types of habitats. This can be small and temporary or large and more permanent, ranging from fresh and salt water marshes, mangrove swamps, grassy ditches, rice fields, edges of streams and rivers, ponds, burrow pits, puddles, hoof prints to wells and discarded tins. A few species sometimes occur in water storage pots. Some *Anopheles* prefer habitats with aquatic vegetation, others habitats without vegetation, some species like exposed sunlit waters while others prefer more shaded breeding places. In general, however, *Anopheles* mosquitoes prefer clean and unpolluted waters and are usually absent from habitats that contain rotting plants or are contaminated with faeces (Service, 1980).

2.2.1 Human host

The epidemiology of malaria also depends on human immunity and behaviour. Innate and acquired immunity are the two types of human immune responses. In general human behaviour affects malaria transmission.

2.2.1.1 Innate and acquired immunity

Innate immunity involves many intrinsic factors that govern the ability of malaria parasites to enter and multiply within the erythrocytes. Individuals who lack the Duffy blood-group antigens on their erythrocytes (i.e. Duffy genotype Fy-fy) are resistant *to P. vivax* infection, because the receptor of the *P. vivax* merozoites on the red blood cell is associated with antigens of the Duffy blood group (Miller *et al.*, 1973, 1975, 1978; Barnwell *et al.*, 1989). A typical example is seen in indigenes of West Africa (Mason *et al.*, 1977). Heterozygotes for haemoglobinopathies and other disorders of the red cells such as thalassemia and glucose-6-phosphate dehydrogenase deficiency promote innate resistance to *P. falciparum* infection (Weatherall, 1987). Such protection is probably triggered by modification of parasite development within the erythrocytes of these individuals. However, it has been proposed that these physiological effects may foster processes that lead to an enhancement in the intensity and/or specificity of the adaptive immune response, enabling individuals carrying these genes to acquire clinical immunity to malaria faster than others (Allison, 1984; Bayoumi, 1987). In support of this hypothesis Bayoumi *et al* (1990), found that lymphocytes of individuals carrying the HbAS genotype showed higher reactivity to

malaria antigens in proliferative assay than lymphocytes from HbAA control individuals living in the same area.

Protection to malaria, acquired by individuals living in endemic areas is largely governed by the transmission pattern. MacDonald (1957) identified two epidemiological extremes within which the disease exists. In one case, instability of transmission is the dominant feature where the disease is characterised by the incidence of substantial seasonal fluctuations, producing cyclical epidemics. In the other case, transmission and morbidity remain stable over the seasons and years. In those parts of the world where malaria occurs in its unstable extreme, the entire population regardless of age is vulnerable and infections tend to be followed by clinical disease. In areas where populations are exposed to frequent heavy inoculations, individuals acquire protection against clinical attacks after several years (Christopher, 1924) and the disease therefore, mainly affects children. McGregor (1960) proposed that individuals living in areas with stable malaria pass through five stages before immunity is acquired. The age at which the individual will pass through each stage depends upon the endemicity of malaria in that area. The age intervals given for each stage below are therefore only indicative and vary considerably from region to region. In stage one (age group: 0-2 months), infants are protected against malaria and parasites are only found in the peripheral blood of approximately 10% of the children. During stage two (2-6 months) parasites are found more frequently in the peripheral blood and the children will often experience the first clinical attacks of malaria (Giles, 1961). These attacks are normally mild (McGregor, 1960). Stage three (7 months - 2 years) is characterised by repeated malaria attacks, associated with severe clinical illness. Parasites are almost always found in the peripheral blood during these years

and often appear in high densities. At this stage malaria disrupts the normal pattern of growth, producing profound anaemia and high mortality rates (McGregor *et al.*, 1956). Stage four (3 - 4 years), represents the acquisition of the first phase of clinical immunity, an anti-toxic immunity, characterised by a rapid decline in the severity and frequency of clinical episodes, despite the persistence of relatively high parasitaemia. In the fifth and final stage, clinical malaria episodes are rare and mild. However, immunity is not a sterile one. Adults living in holoendemic areas will often harbour the parasite without clinical symptoms.

2.2.1.2 Behaviour

Behaviours such as the use of insecticide-treated materials (ITMs), mosquito repellents, insecticidal coil and aerosols as well as prophylactics also influence the incidence of malaria in humans. The severity of the malaria pandemic in the tropics is aggravated by the ongoing spread of parasite resistance to antimalarial drugs. The advent of drugs such as 4-aminoquinolines in the 1940s provided for the first time, reliable and acceptable means for treating acute malaria and for malaria prophylaxis (Laing, 1984). However, widespread use of these drugs has resulted in parasite resistance to the major drugs for prophylaxis or therapy for malaria. Synergistic drug combinations such as pyrimethamine-sulfadoxide (Fansidar) have unpredictable half-life of efficacy before the development of resistance reduces their value (Wensdorfer, 1983). Drugs like halofantrine (Halfan) are effective, but very expensive for the majority of people in developing countries.

2.2.2 The vector

Anopheles mosquitoes belonging to the order Diptera, family Culicidae and subfamily Anophelinae, in general transmit malaria. However, the transmission of the disease depends significantly on the characteristics of the vectors, such as their abundance, susceptibility to infection, longevity and their contact with humans.

Anopheles species have a worldwide distribution, occurring not only in tropical areas but also in temperate regions; they are, however, absent from Polynesia and Micronesia (Service, 1980). In sub-Saharan Africa, the members of the *Anopheles gambiae* complex and *An. funestus* are the most important vectors of *P. falciparum* (Mekuria, 1983). *Anopheles funestus* also remains the main vector in southern Africa (Coetzee *et al.*, 2000). Appawu *et al.* (1994) have implicated *An. pharoensis* in malaria transmission at Dodowa, in southern Ghana where it occurs in sympatry with *An. gambiae* s. l., but its relative importance as a vector has not been established.

Members of the *An. gambiae* species complex are *An. gambiae* sensu stricto, *An. arabiensis, An. merus, An. melas, An. quadriannulatus and An. bwambae.* Three of these sibling species are adapted to fresh water breeding sites. *Anopheles gambiae* s.s. predominates in humid areas and is highly anthropophilic. *Anopheles arabiensis* extends more into savanna areas, and in many areas is more zoophilic and exophilic. *Anopheles quadriannulatus* is zoophilic and consequently does not transmit malaria in humans. The last fresh water species, *An. bwambae* has a very restricted distribution in a small area of the Rift Valley, west of Ruwenzori, where it breeds in geothermal waters (Coetzee *et al.*, 2000)

The two brackish water species, *An. merus* of East Africa and *An. melas* of West Africa are generally more exophagic and zoophilic and thus less efficient as vectors than *An. gambiae* s.s.

In tropical Africa the main *Anopheles* species, which bite humans, are *An. gambiae* s.l. and *An. funestus*. These bite predominantly late at night between the hours of 22.00HR and 05.00HR (Curtis, 1997). Some species such as *An. albimanus*, a malaria vector in South America, bite humans mainly outdoors (exophagic) from about sunset to 21.00HR whereas *An. gambiae* complex bite indoors (endophagic) (Bryan *et al.*, 1987).

Sporozoite rates, that is, the percentages of infective female vectors vary considerably not only from species to species of mosquitoes, but also according to locality and season. Sporozoite rates are often about 1-5% in species such as *An. gambiae* s.s. and *An. arabiensis*, but less than 1% in many other species such as *An. albimanus* and *An. culicifacies* (Mendis *et al.*, 2000).

Both before and after blood-feeding some species will rest in houses (endophilic) whereas others will rest outdoors (exophilic) in a variety of natural shelters, such as amongst vegetation, in rodent burrows, in cracks and crevices in trees, under bridges, in culverts, and cracks in the ground (Julvez *et al.*, 1997). Most *Anopheles* species are not exclusively exophagic or endophagic, exophilic or endophilic but exhibit a mixture of these extremes of behaviour. Similarly, few *Anopheles* species feed exclusively on either humans or non-humans, most feed on both humans and animals but the degree of anthropophilism and zoophilism varies according to

species. For example, *An. culifacies* an important malaria vector in India frequently feeds on cattle in addition to humans, whereas in Africa *An. gambiae* s. s. feeds more rarely on cattle and thus maintains a stronger vector-human contact. This is one of the reasons why *An. gambiae* is considered a more efficient malaria vector than *An. culifacies,* for example. However, there are no differences in nocturnal biting cycles or endophagous rates between *An. gambiae* and *An. arabiensis* (Lemasson *et al.*, 1997). In tropical countries adult mosquitoes probably live on average only about two to three weeks, but in temperate climates the average life may be four to six weeks or longer. It seems that males usually have a shorter life span (Service, 1980).

2.3 Life Cycle of *Plasmodium* and Transmission of Malaria

Female *Anopheles* mosquitoes ingest male and female *Plasmodium* gametocytes during blood-feeding. The gametocytes are passed into the mosquito's gut where they undergo cyclical development that includes a sexual cycle termed sporogony. Only gametocytes (male and female) survive in the mosquito's gut (Service, 1980). All other blood forms of the malaria parasite) are destroyed. Male gametocytes extrude flagella, by a process of exflagellation to become the male gametes (microgametes). The microgametes break free and fertilise the female gametes (the macrogametes), which have formed from the microgametocytes. As a result of fertilisation a zygote is formed which elongates to become an ookinete. The ookinete then penetrates the wall of the mosquito's gut to reach the outer membrane where it becomes spherical and develops into an oocyst (Craig *et al.*, 1999).

Oocysts can be seen on gut walls of vectors about four days following an infective blood-meal (Warrell, 1999). The nucleus of the oocyst divides repeatedly to produce numerous spindle-shaped sporozoites. When the oocyst is fully grown (about 60-80μm), it ruptures and thousands of sporozoites are released into the haemocoel of the mosquito. The sporozoites are carried in the insect's haemolymph to all parts of the body, but most penetrate the salivary glands. The mosquito is now infective and sporozoites are inoculated into man the next time the mosquito bites. It has been estimated that there may be some 70 000 sporozoites in the salivary glands of vectors (WHO/TDR, 1990). Sporozoites are usually found in the salivary gland after 9-12 days, but the time required for this cycle depends on both temperature and the species of *Plasmodium*. For example, at 24^{0}C sporogony in *P. vivax* takes only nine days, in *P.*

falciparum 11 days, in *P. malariae* 21 days, and at 26°C sporogony of *P ovale* is completed in 15 days (Menard, 2000).

After inoculation of the sporozoites into man, many are destroyed by phagocytes, but some enter the parenchymal cells of the liver, develop and asexually multiply in a process called pre-erythrocytic schizogony. When a schizont matures it bursts into the blood stream and most of them invade the red blood cells present in the sinusoid of the liver. On entering the red blood cells they form trophozoites that absorb the haemoglobin of the red blood cells, leaving as the product of digestion a pigment called haemozoin (WHO/TDR, 1990). The trophozoite undergoes another asexual multiplication (erythrocytic schizogony) and then a cytoplasmic division to form a schizont. When the erythrocytic schizogony is completed the red blood cells burst and release merozoites into the blood stream. Many may be destroyed by the host's immune system, but others survive as androcytes in which another cycle of erythrocytic schizogony begins (Fig. 1).

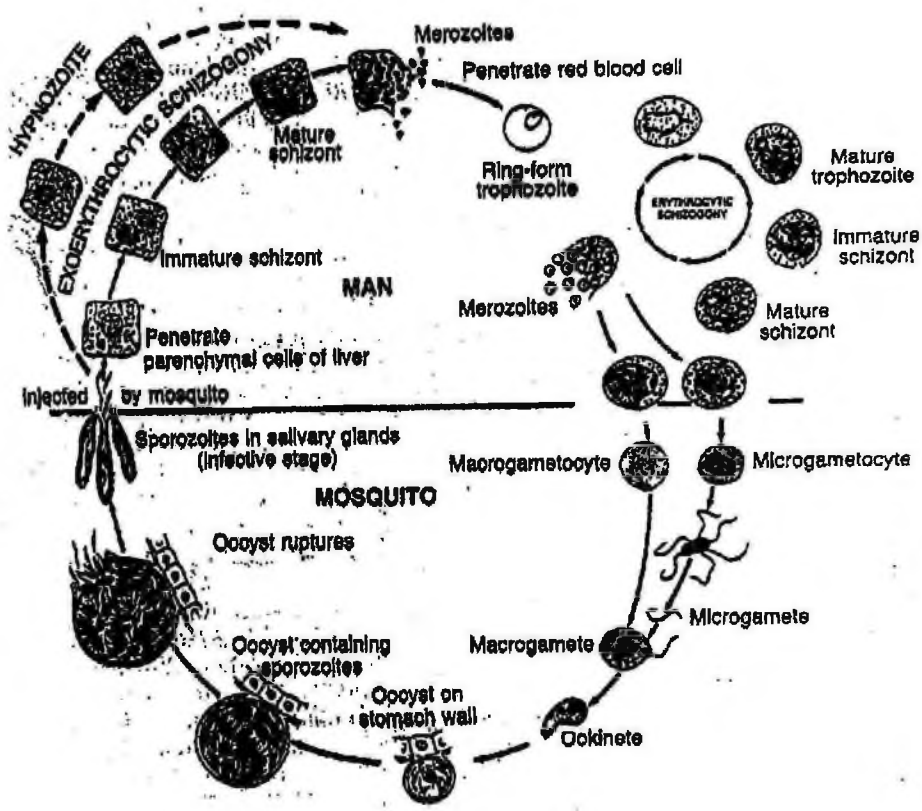

Fig. 1 Life cycle of malaria parasite based on *Plasmodium vivax* (Modified from Melvin, 1979).

2.4 Life Cycle of *Anopheles*

The life cycle of *Anopheles* involves aquatic pre-adult stages and a terrestrial stage. It is holometabolous, that is, the egg hatches into larva that develops into pupa. The imago emerges from the pupa.

2.4.1 Egg stage

Most *Anopheles* species lay about 50- 200 small (1mm long) brown or blackish boat-shaped eggs on the water surface. In a few species the eggs are not boat-shaped, but are oval in outline. In most of them there is a pair of conspicuous lateral air-filled chambers called the floats on the eggs, which in a few species completely extend round the egg (Fig. 2). These floats help maintain the eggs on the water surface. *Anopheles* eggs cannot withstand desiccation. In tropical countries they hatch within two or three days. In colder temperate climates hatching may not occur until about two to three weeks depending on temperature (Service, 1980).

2.4.2 Larval stage

Anopheles larvae have a dark brown or blackish sclerotised head and a roundish thorax with numerous simple and branched hairs and a single pair of thoracic palmate hairs. There are nine visible abdominal segments of which segments one to six or seven usually have dorsally a pair of palmate hairs, which help maintain the larva in a

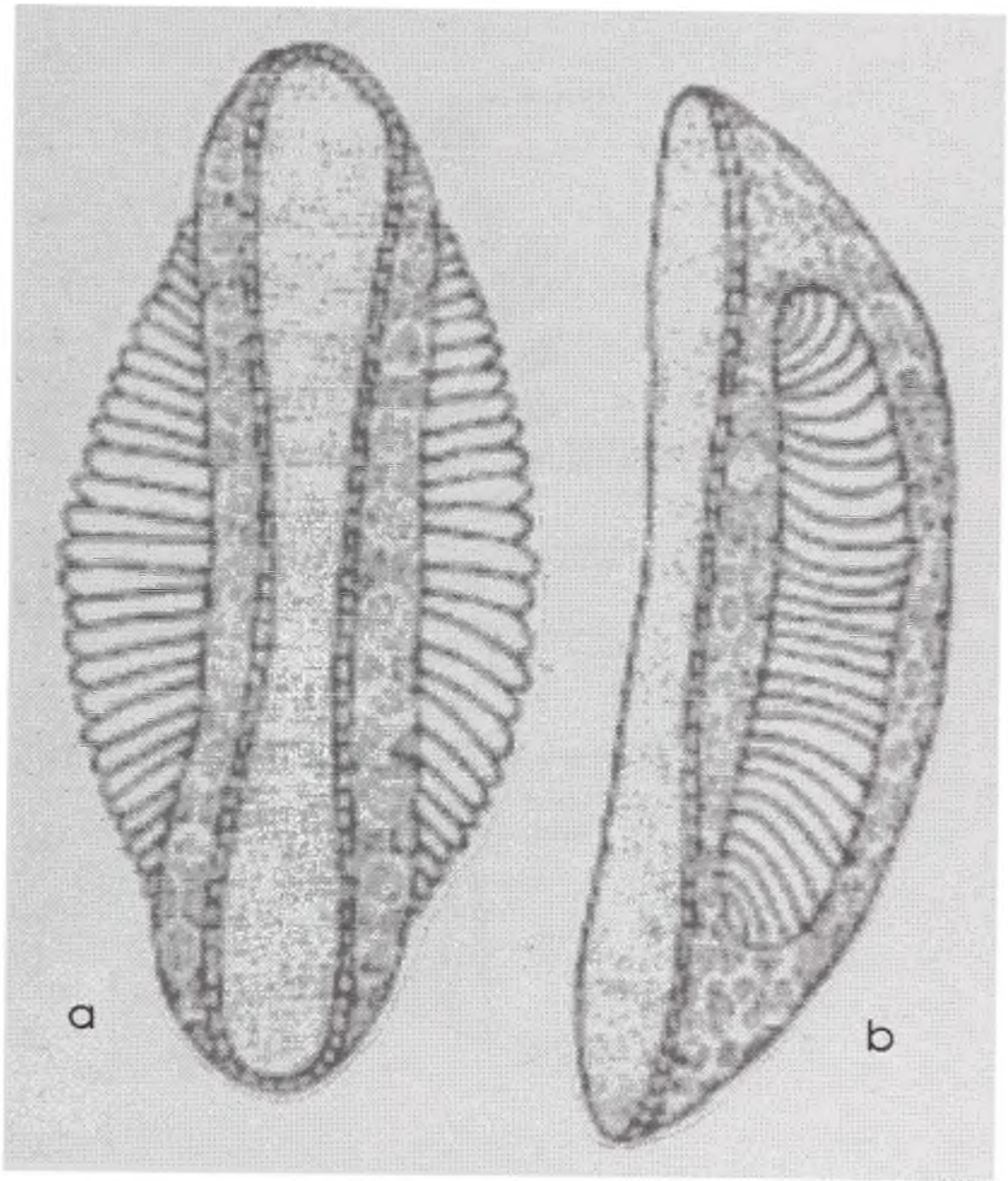

Fig 2 Dorsal (a) and (b) lateral views of *Anopheles* egg (After Service, 1980).

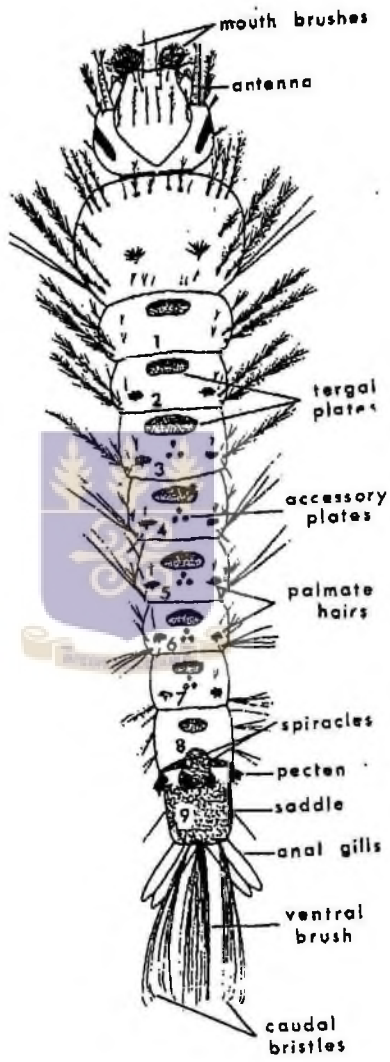

Fig. 3 Dorsal view of an *Anopheles* larva (After Service, 1980)

2.4.3 Pupal stage

In the comma-shaped pupa, the head and thorax are combined to form the cephalothorax, which dorsally has a pair of short trumpet-shaped breathing tubes, with broad openings (Fig. 4). There are eight visible abdominal segments each having numerous short setae. Segments two or three to seven have distinct short peg-like spines. The last segment terminates in a pair of oval paddles. Pupae normally remain floating at the water surface with the aid of the pair of palmate hairs on the cephalothorax, but when disturbed they vigorously swim down to the bottom with characteristic jerky movements. The pupal period lasts two to three days in warm climates (Coluzzi, 1993).

2.4.4 Adult stage

Most adult *Anopheles* (Fig. 5) are crepuscular or nocturnal in their activities, thus emergence from the pupae, mating, blood-feeding and oviposition normally occur in the evenings, at night or in the early morning around sunrise. Both before and after blood-feeding some species will rest in houses (endophilic) whereas others will rest outside (exophilic) in a variety of natural shelters such as amongst vegetation, in rodent burrows, in cracks and crevices of trees, under bridges and in culverts (Kettle, 1992). Most *Anopheles* species are not exclusively exophagic or endophagic, exophilic or endophilic, but exhibit a mixture of these extremes of behaviour. Similarly, few *Anopheles* feed exclusively on man or other vertebrate hosts, most feed on both

humans and animals but the degree of anthropophilism and zoophilism varies according to species.

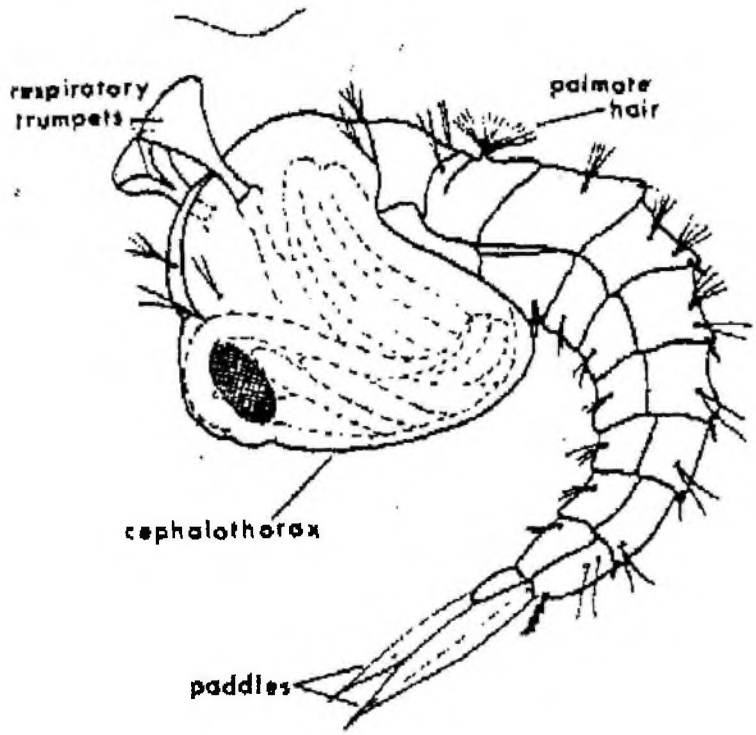

Fig. 4 Pupa of *Anopheles* mosquito (After Service, 1980)

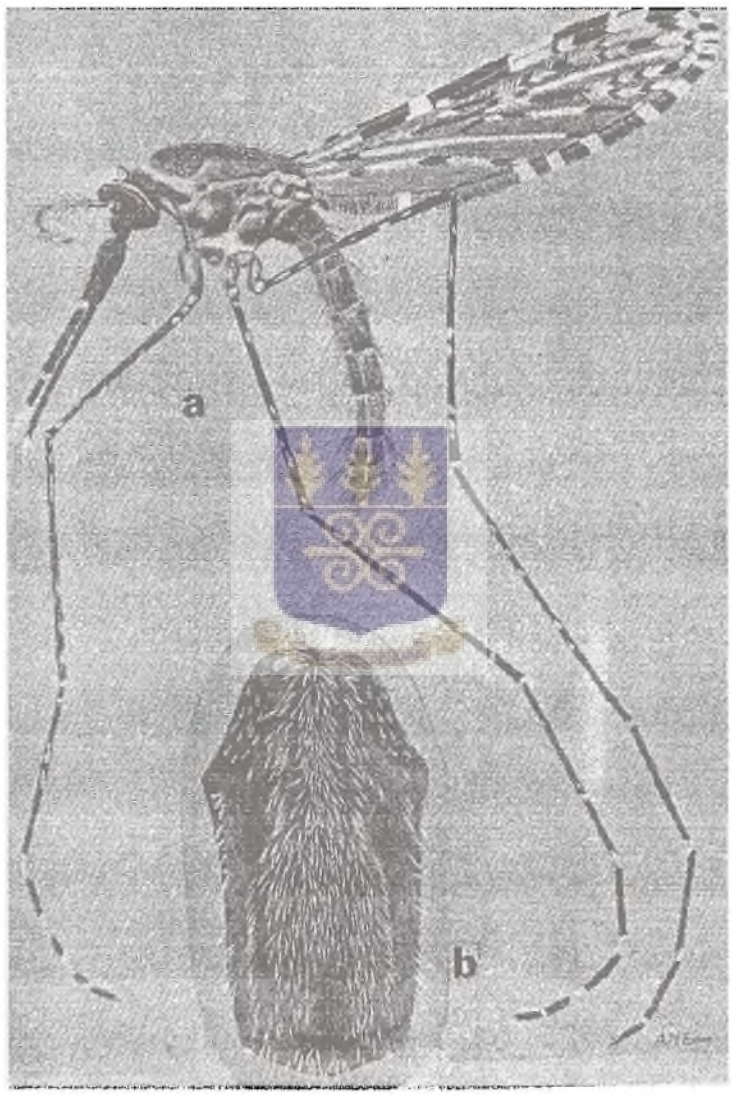

Fig. 5 *Anopheles gambiae* (a) side view of female (b) female dorsum of mesonotum (After Evans, 1927).

2.5 Prevention and Treatment of Malaria

2.5.1 Prevention

Prevention of malaria encompasses a variety of measures that may protect against infection or against the development of the disease in infected individuals. Measures that protect against infection are directed against the mosquito vector. These can be personal (individual or household), for example, protective clothing, repellents, bednets, or community protection measures, such as use of insecticides or environmental management to control transmission. Measures, which protect against disease but not against infection, include chemoprophylaxis.

Disease management through early diagnosis and prompt treatment is fundamental to malaria control. Whereas formerly malaria control depended to a large extent on insecticide spraying, now the selective use of protection methods, including treated bednets, is proving cost-effective and more sustainable.

2.5.2 Chemotherapy

A limited number of drugs for treatment of malaria are available. Due to the worsening problems of drug resistance in many parts of the world, adequate treatment of malaria is becoming increasingly difficult. Some of the new drugs that have appeared in the last twenty years are mefloquine, halofantrine, artemisinin derivatives, malarone, atovaquone + proguanil and co-arthemether (WHO, 1998).

In Africa, with increasing levels of chloroquine resistance and fears of toxicity and decreased efficacy for sulfadoxine/ pyremethamine, amodiaquin is being reconsidered as a first or second line drug in areas where chloroquine resistance has developed. Artemisinin derivatives presently show no cross-resistance with known antimalarial drugs and as such are important for treating severe malaria in areas of multidrug resistance. They however require long treatment courses and when used alone, recrudescence may occur (WHO, 1998).

2.5.3 Vaccines

The three main vaccines being developed are: "anti-sporozoite" vaccines, designed to prevent infection, "anti-asexual blood stage" vaccines, designed to reduce severe and complicated malaria and "transmission-blocking" vaccines, designed to arrest the development of the parasite in the mosquito, thereby reducing or eliminating transmission of the disease (WHO/TDR, 1998).

2.6 Malaria Control initiatives

There are presently two major malaria control initiatives: The WHO's Roll Back Malaria (RBM) and the Multilateral Initiative on Malaria (MIM). On March 13, 1998, the World Health Organisation announced a global campaign, allying the WHO, the World Bank and governments of both the developing and developed countries, to roll back malaria. The programme aims to halve malaria deaths by 2010, and halve them again by 2015, primarily through rebuilding health care and malaria control in developing countries (Shiff, 2000).

The Multilateral Initiative on Malaria began as a joint African-American-European project that brought together representatives from thirty-seven countries, three charities and three intergovernmental agencies in Senegal in 1997. It has already led to increase in the co-ordination of research efforts on the continent (Annual Report of the Institute of Medicine of the National Academy of Sciences of the United States, 1996).

The control of malaria in Africa will require a long-term collaboration between scientists in the North and South and commitments from the industrialised countries to funding, and from African leaders to support scientists and health and research infrastructures in their countries. A comprehensive malaria control strategy requires three interdependent and complementary components: disease management, surveillance, and prevention including environmental management (The World Resources Institute/ United Nations Environment Programme/ United Nations Development Programme/ the World Bank (1998). If people are taught how they can

wage war on mosquitoes: by filling ditches or covering containers where water stagnates and mosquitoes breed; by stocking ponds with fish that eat mosquito larvae; by using insecticides judiciously and in the right places; by insect-screening their homes; and by planting water-hungry trees to dry out muddy soils. These are just a few of the measures to destroy mosquito-breeding sites. Avoiding mosquito bites is a cheap and very effective way to reduce deaths. Mosquitoes bite at night, therefore, by sleeping under a mosquito net impregnated with a natural, biodegradable insecticide derived from chrysanthemums lowers one's risk of disease greatly (Curtis and Lines, 1985).

2.7 Vector Control

2.7.1 Use of insecticides

Decades of intensive use of synthetic organic insecticides to control disease vectors and agricultural pests have led to the selection of insecticide resistance. The deleterious consequences of this include increased level of environmental contamination, higher rates of insecticide application and increases in vector control costs. There is also disruption of ecologically sound vector control strategies and increased incidence of human and animal diseases in which transmission depends on insect vectors (Georghiou, 1986).

In the past, increasing the amounts of insecticide applied or replacing older chemicals with new, more effective compounds controlled resistance populations. Both of these strategies are of limited value today. This is due to the high cost of increasing the amounts and the declined rate of discovery and development of new insecticides, so that alternatives are not available for populations that are resistant to organophosphorus (OP), carbamate, and pyrethroid insecticides (Hammock and Soderlund, 1986).

For the control of larval mosquitoes, a combination of developed resistance and environmental unacceptability has eliminated the chlorinated hydrocarbons (e.g. DDT), the cyclodiene compounds and γ- HCH. The principal larvicides are now the phosphorodithioate, malathion, and the phosphothioates fenthion, chlorpyrifos and temephos (Brown, 1983). Other effective compounds include fenitrothion, parathion-

methyl, bromophos, jodfenphos and pirimiphos-methyl. Carbamates as a group are insufficiently potent as larvicides. The only new materials recommended for larval control are the insect growth regulator methoprene, and the refined oil FLIT MLO. Diflubenzuron is also highly effective. For adulticide mists, the organophosphates (OPs) malathion, fenthion, chlorpyrifos, the phosphate naled and a carbamate (propxur) are recommended (Rathburn, 1979). For residual adulticides against anophelines (for which DDT may still be used where not yet ineffective) the recommended OP compounds are malathion, fenitrothion, chlorphoxim and pirimiphos-methyl and the effective carbamates are propoxur, Landrin and bendiocarb (WHO, 1980). Among the pyrethroids, resmethrin and synergised pyrethrins are recommended for ultra-low-volume (ULV) adulticide sprays (Rathburn, 1979). Permethrin and decamethrin have proved effective as residual adulticides against anophelines. Certain aliphatic amines such as Armeen L_{15} (the primary C_{15} β-amine) – which kill pupae as well as larvae – are used as additives to increase the effectiveness of larvicidal oils (Brown, 1983).

2.7.2 Bednets

2.7.2.1 Historical overview of bednet use

The belief that bednets offer protection from malaria arose independently in different places more than 70 years before Sir Patrick Manson suggested in 1894 that mosquitoes transmitted malaria (Lindsay and Gibson, 1988).

The first bednets were typically made of flax, although hemp or even palm fibres were sometimes used (cf Russel, 1955). However, one deluxe model owned by Holofernes, an Assyrian General, was reputed to be 'purple interwoven with gold, emeralds and precious stones (Apocrypha, 1970). Plutarch in 1579 published that Cleopatra had a natty bednet made of cloth of gold whereas in Japan many were made of silk. Today, bednets are generally less ostentatious and are made of cotton or synthetic material, woven into netting or sheeting.

The earliest recorded use of bednets was in the 6th century BC in the Middle East (Apocrypha, 1970). Different ethnic groups have used them in precolonial times. In West Africa the Fulani and Hausa slept under fine-mesh grass mats (cf MacCormack, 1984).

2.7.2.2 Impregnated bednets

Insecticide-impregnated bednets are currently widely promoted for vector control (Curtis, 1990; Lengeler *et al.*, 1996). Pyrethroids are presently the only practical insecticides used for impregnating bednets. In Ghana they constitute the main components of household insecticides. Synthetic pyrethroids, such as permethrin, deltamethrin and λ-cyhalothrin are photostable analogues of natural pyrethrum, with low mammalian toxicity and are highly insecticidal as well as excito-repellent (Elliot *et al.*, 1973). Permethrin has been used for the impregnation of clothing as a protection against mosquitoes (Lindsay and McAndless, 1978; Schreck *et al.*, 1978) and ticks

(Schreck *et al.*, 1982). The aims of the impregnation are twofold namely individual effect and mass effect.

The individual effect - to improve the personal protection conferred by nets by overcoming the problems of damaged or badly used nets. The mass effect – to use insecticide more efficiently, than is the case with house spraying, to shorten the mean lifespan of the vector by placing the insecticide on a surface which mosquitoes are bound to encounter in their attempts to bite sleeping people. The treated net could be considered as a trap baited with human odour. This effect will benefit nearby non-users of nets or people when outside their nets (Curtis and Lines, 1985; Mouchet, 1987).

Recently there has been great interest in the use of insecticide-treated bednets and other materials, such as curtains to screen doors, windows and the eaves of houses as a strategy for controlling malaria. Clinical studies of the impact of insecticide-treated bednets on malaria have shown a reduction in spleenomegaly and parasite rates in young children in Mali (Ranque *et al.*, 1984) and a reduction in *P. falciparum* incidence in Papua New Guinea (Graves *et al.*, 1987).

A trial of permethrin-treated bednets in Gambia (Snow *et al.*, 1989) reduced the incidence of clinical attacks with heavy parasitaemia by 72% in children sleeping under insecticide-treated bednets compared with those with untreated bednets (Lindsay and Gibson, 1988). In Kenya and in Ghana use of impregnated bednets reduced all child-cause mortality by 33% and 17% respectively (Binka *et al.*, 1994).

The largest trials of treated bednets undertaken so far have been in China, and these resulted in significant reduction in malaria (cf Targett and Greenwood, 1998). In Africa, the results of trials have been particularly encouraging. In a series of trials carried out in areas with different levels of endemicity, the number of clinical attacks of malaria in children protected by treated bednets was reduced by 30-60%. In subsequent trials, the effects on mortality were not quite so dramatic, but there were still reductions of mortalities between 15% and 33%. All these studies were carried out in areas of stable malaria transmission where control was most difficult (Targett and Greenwood, 1998).

Nets cost $5-10 and a year's natural insecticides under $1, making bednet donation programmes cheap and well within the means of governments and large companies. With will and commitment, properly managed integrated programmes with the incorporation of bednets, better use of available drugs, and further needed research particularly in insecticide resistance, could all be a reality.

For the community as a whole, use of bednets by a few individuals will not be an advantage since unprotected people will be made more likely to contract malaria because mosquitoes will be diverted to them. If the whole community is using bednets, the targeted mass killing of mosquitoes has been shown to reduce the density of the population of mosquitoes that have survived long enough to carry infective *Plasmodium* sporozoites (Curtis, 1997). Thus, the organised community action can provide protection to some people even when they are not under nets.

Advocates of use of bednets agree that this is only a temporary measure since persistent use of these insecticides will invariably select for resistance.

Pyrethroid resistance or tolerance in the malaria vector *An. gambiae* s. s. has been reported from both West and East Africa (Elissa *et al.*, 1993; Vulule *et al.*, 1994, 1996). Recent studies in Burkina Faso and Cote d'Ivoire (Martinez-Torres, *et al.*, 1998; Chandre *et al.*, 1999a, b, c) and Kenya (Vulule *et al.*, 1999; Ranson *et al.*, 2000) revealed extensive pyrethroid resistance in wild populations of *An. gambiae*, involving at least three mechanisms: increased esterase and hydrolase metabolism, and knockdown resistance (*kdr*).

2.7.2.3 Effects of pyrethroids on humans

Permethrin and deltamethrin have rat dermal LD_{50} values of more than 4000 and 2000mg/kg of body weight respectively. They are classified by the WHO as moderately hazardous (WHO, 1988). Permethrin was recommended for use on bednets by the WHO Expert Committee on Vector Biology and Control (WHO, 1986).

Some pyrethroids, particularly the most active α-cyano-substituted compounds such as deltamethrin and λ-cyhalothrin provoke a sensation on the skin or mucosae (Lindsay and Gibson, 1988). Safety aspects are of greater concern to persons impregnating large numbers of bednets than for the owner of a bednet who impregnates his own net (WHO, 1988).

2.8 Insecticide Resistance in Anopheline Vectors of Malaria

The use of chemical pesticides to control arthropods leads to the development of resistance, which may be as a result of physiological, biochemical, or behavioural change (Georghiou, 1986).

Resistance is an inheritable character developed in a population of normally susceptible insects. It is not a characteristic acquired during the insects' lifetime, and it cannot be induced in a population by completely sublethal doses of the insecticide (Brown, 1983). It derives from the selective effect of exposures that kill or disable a portion of the population, the subsequent generation originating from survivors. Among the survivors are those that carry preadaptations (genes, or gene alleles) for resistance to that insecticide. With repeated applications and generation after generation, the population comes to consist mainly of resistant strains due to selection. A population is usually termed resistant only when it has reached a level that results in a control failure in the field with the recommended dosage of the insecticide, and when a marked divergence from the normal has been confirmed by a standard test of a sample of the insects (Chadwick *et al.*, 1984).

Resistance to the DDT deposits sprayed on house walls for malaria control was first discovered in 1951 in *An. sacharovi* at Naupolion in Greece, where DDT had been applied to rice fields since 1946 (Georgopoulos, 1951). Then in 1954 a 23-fold adult resistance to DDT was detected in *An. sandaicus* populations in Jakarta, Indonesia. In 1955 DDT resistance in excess of 10-fold was found in *An. stephensi* in the Al Hasa oasis of eastern Saudi Arabia, where DDT had been applied for malaria control since

1948. In all these instances no resistance to dieldrin or HCH were detected. However, dieldrin-resistance extending to γ-HCH later appeared in *An. sacharovi* in 1952 in the Greek population mentioned above, in *An. quadrimaculatus* in 1952 in Mississippi, USA, and in *An. arabiensis* in 1955 in the western Sokoto district of northern Nigeria (Brown and Pal, 1971). Then between 1955 and 1969, the WHO launched a series of campaigns aimed at eradicating malaria, through spraying inside of homes with insecticides. On the inauguration of the WHO global malaria eradication campaign however, DDT-resistance and dieldrin-resistance came to be found in an increasing number of species. By 1961, the tonnage of organochlorine insecticides applied annually reached 64,000 for DDT, 4,000 for dieldrin and 500 for γ-HCH. By 1962, DDT-resistance had appeared in 12 species and dieldrin-resistance in no less than 29 species. In five of these anophelines (*sacharovi, stephensi, aconitus, albimanus, quadrimaculatus*) both types of resistance were present simultaneously. In 1967, dieldrin-resistance was joined by DDT-resistance in populations of *An. gambiae* in West Africa.

In 1955, *An. gambiae* in northern Nigeria was reported to be resistant to dieldrin and partly so to HCH, but it remained susceptible to DDT in most parts of Africa until 1968 when a high level of resistance was reported in Burkina Faso. In 1972, a trial was carried out in Togo and no mortality of mosquitoes was observed with standard exposure to DDT, nor was there any reduction of man-biting or house-resting. Similar results were obtained from Senegal after DDT spraying (Bruce-Chwatt, 1984).

Malathion-resistance in *Anopheles albimanus* was first detected on a cotton-growing estancia in the Department of La Libertad, El Salvador in 1969 (Breeland *et al.*, 1970).

Studies carried out in June 1970 showed larval resistance ratios of 2.9 to malathion and 3.4 to parathion-methyl (Ariaratnam and Georghiou, 1971). Subsequent studies in February 1971 showed increased larval resistance ratios, which were 11.7 to malathion, 26.2 to parathion-methyl, 4.8 to fenitrothion, 1.2 to fenthion and 164.4 to propoxur with no cross-resistance to chlorpyrifos and temephos (Georghiou, *et al.*, 1972).

In 1977, malathion-resistance was found in adult *A. arabiensis* at Bakarat, a swampy area in the Gezira cotton-growing tract in Sudan, where malathion house sprays and temephos larvicide had been introduced in 1975 (Hemingway, 1980). In Kenya, fenitrothion was used successfully, although transmission could not be fully interrupted (Bruce-Chwatt, 1984).

2.8.1 Resistance mechanisms

The intoxication of an insect by an insecticide encompasses three levels of pharmacokinetic interactions: penetration of barrier tissue; distribution, storage and metabolism in internal tissues; and molecular interaction with the ultimate target site (Welling and Paterson, 1985). The measured toxicity of an insecticide therefore depends not only on its intrinsic potency at its site of action but also on the pharmacokinetic parameters that describe its uptake following exposure, its distribution and partitioning among insect tissues, and the extent of its metabolic activation or detoxification in these tissues. Mutations that affect the rate constants of any of these processes may reduce either the delivery of the ultimate toxicant to its

target or the affinity of the toxicant-target interaction, thereby conferring resistance at the level of the whole organism.

Despite the diversity of both chemical structures and arthropod species in the instances of resistance the number of identified resistance mechanisms is small (Georghiou, 1986). The resistance mechanisms include reduced cuticular penetration of toxicants enhanced metabolism by cytochrome P_{450}-dependent monooxygenases, hydrolases, or glutathion-S-transferases, reduced sensitivity of mutant acetylcholinesterases to organophosphate and carbamate insecticides and of other neuronal targets for pyrethroids, to DDT and analogues, and chlorinated cyclodienes (Oppenoorth, 1985). All these mechanisms are non-specific in that they generally confer cross-resistance to other structurally related toxicants and, in several cases, to chemically unrelated compounds as well. Since these mechanisms span all three pharmacokinetic levels of insect-insecticide interactions, the occurrence of multiple resistance mechanisms acting at different levels has the potential to produce highly synergistic interactions that can result in virtual immunity in some insect populations (Welling and Paterson, 1985).

2.8.1.1 Reduced cuticular penetration

Reduced penetration is both the least significant as a single resistance mechanism and the least understood of these mechanisms (Soderlund and Bloomquist, 1990). Plapp and Hoyer (1968) studied the reduced penetration of dieldrin and DDT in a strain of housefly, and named the gene *organotin*-R with the mutant gene symbol *"tin."*

Apparently this gene introduces a general penetration barrier to many chemicals as judged by its cross-resistance characteristics. These workers suggested that the gene works as an intensifier for the already existing resistance factors. The reduction in the rate of cuticular penetration gives the metabolic systems the opportunity to degrade the insecticide to innocuous materials. Thus it may be expected that a reduced penetration factor would be more significant in resistance to the readily metabolised insecticides such as malathion (Benezet and Forgash, 1972; Matsumura and Brown, 1963) than to the more stable chemicals such as dieldrin. Reduction in penetration may be viewed as a reduced penetration constant *P* as shown below:

$$C_i = C_o (1 - e^{-PAt})$$

where C_i is the concentration of insecticide inside the insect, C_o is the original concentration outside, *P* is the penetration constant, *A* is the area of contact, and *t* is the time (Matsumura, 1983). In such a relationship reduced *P* merely indicates the slowdown of penetration, and in due course the same amount of pesticide may enter into the insect body.

2.8.1.2 Enhanced detoxification

2.8.1.2.1 Cytochrome P_{450} – dependent monooxygenases

The monooxygenase system has very broad substrate specificity, which may be catalysing hydroxylations at both aromatic and aliphatic carbon atoms, expoxidation of double bonds, desulphuration of phosphorothionates and oxidation of sulphides.

Cytochrome P_{450} – dependent monooxygenases preferentially metabolise lipophilic substrates to products with increased water solubility or with functional groups that enable conjugation reactions, thereby promoting excretion (Soderlund and Bloomquist, 1990).

The oxidised cytochrome P_{450} bind both substrates and oxygen and undergo a reduction-oxidation cycle, releasing oxidised substrate and water as the products. The reduction of oxidised cytochrome P_{450} forms by NADPH is catalysed by the enzyme NADPH- cytochrome P_{450} reductase.

Enhanced oxidative metabolism has been implicated as a major mechanism of resistance for all insecticide classes except the chlorinated cyclodienes. Much of the evidence is based mainly on the ability of piperonyl butoxide (PBO) and related compounds, which are inhibitors of cytochrome P_{450}-dependent monooxygenases, to reduce the magnitude of resistance observed when they are used as synergists with insecticides in bioassays (Soderlund and Bloomquist, 1990).

Several molecular mechanisms might account for the enhanced rates of insecticide oxidation observed in enzyme preparations from resistant insect strains. First, resistant insects might possess generally higher levels of all components of the monooxygenase system. Second, rapid oxidation may result from overexpression of a single constitutive form of cytochrome P_{450} having high catalytic activity toward the insecticide substrate. Third, resistant insects may have alterations in cytochrome P_{450} gene regulation so that a form of cytochrome P_{450} that is observed only upon induction in susceptible insects is instead constitutively expressed in resistant insects. Finally,

resistant insects may possess a mutant form of cytochrome P_{450} with unique properties and high catalytic activity toward the insecticide substrate (Hodgson, 1985). Qualitative differences in the cytochrome P_{450} of resistant and susceptible insects have been inferred from the unique spectral properties of cytochrome P_{450}- carbon monoxide and other cytochrome P_{450}- ligand complexes in microsomes prepared from resistant flies (Hodgson, 1985). The ability to detect electrophoretically distinct forms of cytochrome P_{450} in the fruit fly has permitted an analysis of the separate expression and regulation of those forms in susceptible and resistant strains of this species (Waters and Nix, 1988).

2.8.1.2.2 Hydrolases

Hydrolases that are capable of cleaving carboxylester and phosphorotriester bonds play a significant role in the metabolism of organophosphate and pyrethroid insecticides (Dauterman, 1985). Among OP compounds two distinct hydrolytic pathways are found: (1) cleavage of carboxylester groups in malathion, phenthoate and related compounds and (2) cleavage of the phosphate ester bond of a wide variety of compounds (Dauterman, 1985). Insect hydrolases exist in multiple forms, which can be resolved by electrophoresis and detected using simple spectrophotomeric assays with substrates such as the acetate and butyrate esters of *p*-nitrophenol and the acetate esters of α-and β-naphthol (αNA and βNA) (Maa and Terriere, 1983).

The involvement of hydrolytic mechanisms in resistance has been recognised or inferred in three ways: detection of high levels of insecticide hydrolysis products in metabolism studies with resistant insects; synergism of insecticide toxicity in resistant insects by nontoxic OP esterase inhibitors such as TPP (*O,O,O*-triphenyl phosphate), DEF (*S,S,S*-tributyl phosphorotrithioate, a cotton defoliant), or IBP (*O,O*-bis[1-methylethyl] *S*-phenylmethyl phosphorothioate), which is a fungicide (Soderlund and Bloomquist, 1990).

Biochemical studies in both *Anopheles stephensi* (Hemingway, 1982, 1984) and *An. arabiensis* (Hemingway, 1983) showed that the toxicity of malathion to resistant strains was greatly increased by the use of esterase-inhibiting synergists, thus implicating a hydrolytic resistance mechanism. Although susceptibility and resistant strains did not differ in their ability to hydrolyse αNA and βNA (Hemingway, 1982, 1983), subsequent studies demonstrated increases in malathion-specific hydrolysis in the resistant strains (Hemingway, 1985; Scott and Georghiou, 1986). A similar situation was also found in malathion-resistant *Culex tarsalis*, which exhibited extensive malathion hydrolysis *in vivo* and *in vitro* but negligible increases in the *in vitro* hydrolysis of αNA (Ziegler *et al.*, 1987).

The carboxylesterase-based resistance mechanism has been reported from more than thirty different medical, veterinary and agricultural insect pests. It is the primary mechanism for organophosphorus insecticide resistance as well as a secondary mechanism for carbamate resistance in mosquitoes (Peiris and Hemingway, 1993). In some other insect species however, pyrethroid-resistance is also conferred

(Devonshire and Moores, 1982). The majority of OPs are esters of phosphoric acid and can be hydrolysed by carboxylesterases (Hemingway and Karunaratne, 1998), thus rendering them ineffective against the target vectors.

2.8.1.3 Reduced neuronal sensitivity

2.8.1.3.1 Altered acetylcholinesterase

Acetylcholinesterase (AchE) is a serine esterase, which hydrolyses the neurotransmitter acetylcholine. Once these insecticides inhibit AchE, paralysis or death occurs. Organophosphate and carbamate insecticides exert their neurotoxic effects by inhibiting the enzyme acetylcholinesterase (AchE), thereby prolonging the residence time of acetylcholine at cholinergic synapses and producing hyperexcitation of cholinergic pathways (Hama, 1983, Oppenoorth, 1985).

2.8.1.3.2 Reduced neuronal sensitivity to DDT and pyrethroids

DDT and pyrethroids are capable of disrupting the normal function of many enzymes, neuroreceptors and ion channels *in vitro*, but the toxic actions of these compounds in insects and at the level of isolated nerves are best explained by their action on the voltage-sensitive sodium channel of nerve membranes (Soderlund and Bloomquist, 1989). Consequently, the sodium channel has been considered to be the principal site of action of DDT and pyrethroids.

2.8.1.4 Knockdown resistance (kdr)

Plapp and Hoyer (1968) first reported evidence for a *kdr*-like mechanism of resistance in mosquitoes and identified a recessive gene unrelated to DDT-ase in DDT-resistant *Culex tarsalis* that conferred cross-resistance to pyrethrins/piperonyl butoxide (PBO) combination. Subsequent studies have identified similar mechanisms in *Aedes aegypti* (Chadwick *et al.*, 1977, 1984), *C. quinquefasciatus* (Halliday and Georghiou, 1985; Priester and Georghiou, 1978, 1980) and *An. stephensi* (Omer *et al.*, 1980). Although no subsequent studies with the *C. tarsalis* strain used by Plapp and Hoyer have been reported, additional characterisation of the latter three species has provided substantial evidence supporting a *kdr*-like nerve insensitivity mechanism. A failure of bioresmethrin to control *A. aegypti* in Bangkok, Thailand, in 1975 led to the characterisation of DDT and pyrethroid resistance in this species (Chadwick *et al.*, 1977). The field-collected (BKK) strain was highly resistant to DDT and moderately resistant to several pyrethroids, and neither PBO nor a DDT-ase inhibitor was able to reduce the level of resistance observed. Further, mass selection of this strain with permethrin produced a substrain (BKPM) having greater resistance to several pyrethroids and DDT (Chadwick *et al.*, 1984). A single-family sib selection of the BKPM strain with permethrin for three generations produced a strain (BKPM3) exhibiting apparent homogeneity for permethrin resistance (Malcolm and Wood, 1982a). Comparisons of the penetration and metabolism of [^{3}H][1R,*trans*]-permethrin in adult susceptibility and BKPM mosquitoes failed to identify differences in rates of penetration or metabolism between strains (Brealey *et al.*, 1984). Moreover, these studies showed that BKPM insects required a much higher internal concentration of insecticide to produce toxic effects, thus indirectly providing evidence for a nerve

insensitivity mechanism. Genetic studies with the BKPM3 strain identified a single pyrethroid resistant factor (R^{py}) on chromosome III (Malcolm and Wood, 1982b) and DDT resistant factor (R^{DDT} and R^{DDT2}) on chromosomes II and III (Malcolm, 1983a). Detailed mapping studies showed that R^{DDT} conferred a DDT-ase dependent resistance mechanism, whereas R^{DDT2} and R^{py} were allelic and conferred the observed non-metabolic DDT-pyrethroid cross-resistance (Malcolm, 1983b). When isolated in a susceptible genetic background, R^{DDT2}/R^{py} conferred approximately 10-fold resistance to permethrin. Unlike the *kdr* factor in houseflies, R^{py} was incompletely dominant rather than recessive in inheritance (Malcolm and Wood, 1982b). Although direct demonstration of reduced neuronal sensitivity is missing for the BKK, BKPM, and BKPM3 strains, the weight of evidence from bioassays and metabolic and genetic experiments strongly implicates a *kdr*-like mechanism in this species.

Selection of field-collected *C. quinquefasciatus* with [1R, *trans*]-permethrin produced a strain having >4000-fold resistance to the selection compound and high levels of resistance to a variety of other pyrethroids and to DDT (Priester and Georghiou, 1978, 1980). Substantial resistance remained in the presence of synergists capable of inhibiting monooxygenases, hydrolases, or DDT-ases, thereby implicating a nonmetabolic mechanism as a major resistance factor in this strain. Genetic studies suggest that nonmetabolic resistance to both permethrin and DDT is inherited similarly, but allelism has not been shown in mapping studies (Halliday and Georghiou, 1985). A larval neuromuscular preparation from this strain was >1000-fold less sensitive to [1R, trans]-permethrin than an equivalent preparation from susceptible insects, thus providing evidence of reduced neuronal sensitivity in this species (Salgado, *et al.*, 1983b).

Cross-resistance between DDT and pyrethroids was also documented in a field-collected colony of *An. stephensi* that was selected first with DDT and then with DDT plus PBO and chlorfenethol, a DDT-ase inhibitor (Omer, *et al.*, 1980). Larval neuromuscular preparations from this strain were resistant to the actions of [1R, *cis*]-permethrin, but the magnitude of resistance was much lower than that found for *C. quinquefasciatus* (Omer *et al.*, 1980; Salgado *et al.*, 1983b).

Dieldrin resistance involving a specific resistance mechanism [γ-amino butyric acid (GABA) receptors], was recorded among *An. gambiae* s.l. populations in Africa (Chandre *et al.*, 1999c).

Resistance of *An. gambiae* s.s. to pyrethroids was first observed in Côte d'Ivoire by Elissa *et al.* (1993), who reported a significantly decreased mortality for permethrin and a lower susceptibility to the knockdown effect of deltamethrin and λ-cyhalothrin. More recently, knockdown resistance (*kdr*) to pyrethroids and DDT was observed in several countries from West Africa (Chandre *et al.*, 1999a). Investigations revealed that pyrethroid and DDT resistance was associated with a single point mutation on the gene coding for the sodium channel, resulting in the change of one amino acid (Martinez-Torres *et al.*, 1998). A PCR amplification of specific alleles (PASA) as a diagnostic test has been developed to identify the mutation on a single mosquito and to score for the genotype (Chandre *et al.*, 1999b).

2.8.1.5 Glutathione S-transferase-based resistance in Anopheles gambiae

Glutathione S-transferases (GSTs) are ubiquitous enzymes found in complex multigenic families in most eukaryotic organisms. They primarily conjugate reduced glutathione (GSH) to a range of xenobiotics, aiding in their detoxification and excretion. A number of these GSTs are able to utilise organophosphates in conjugation reactions, others dehydrochlorinate DDT to the non-neurotoxic metabolite DDE using GSH as a co-factor rather than a conjugate (Hemingway, 1999).

The majority of reports of DDT resistance in *Anopheles* species indicate that it is GST-based (Herath *et al.*, 1988; Prapathandara *et al.*, 1995). In some cases there is cross-resistance between this DDT resistance mechanism and some organophosphates. For example, GST-based DDT resistance in *An. subpictus* acts as a secondary resistance mechanism for fenithrothion and is maintained in linkage disequilibrium with a monooxygenase-based organophosphate resistance mechanism in this species in Sri Lanka by organophosphate insecticide resistance pressure (Hemingway, 1991).

CHAPTER THREE

MATERIALS AND METHODS

This study was carried out in three phases. The first phase involved a questionnaire survey to determine the people's habits, attitudes and knowledge of insecticide use in the study area. For the second phase information obtained from the survey was used as basis to select sites for mosquito larvae collection. The mosquito larvae were reared to adults in the laboratory and female *Anopheles gambiae* s. l were used for pyrethroid bioassays. For the third phase assayed mosquitoes were subsequently used for species-specific identification and for the detection of the *kdr* mutation using molecular techniques.

3.1 Study Sites

The study was carried out in Accra, which is situated in the driest southeast coastal plains of Ghana, where the annual rainfall is a little less than 7500 mm with a mean monthly temperature of 25°C and a mean relative humidity of 65% (Dickson and Benneh, 1988).

Table 1 Specific locations where samples were collected.

Site	Location	Coordinates	
1	East Legon	N 05. 64359°	W 00. 14611°
2	East Legon	N 05. 64230°	W 00. 14674°
3	East Legon	N 05. 64623°	W 00. 15522°
4	Motorway (Dzorwulu)	N 05. 61733°	W 00. 19692°
5	Gbawe	N 05. 57172°	W 00. 28399°
6	Airport Residential Area	N 05. 60939°	W 00. 18826°
7	Roman Ridge	N 05. 61187°	W 00. 17942°
8	New Achimota	N 05. 63018°	W 00. 23526°
9	North Legon	N 05. 60305°	W 01. 17844°
10	Osu	N 05. 55574°	W 00. 18572°
11	Nima	N 05. 57908°	W 00. 20028°
12	Teshie	N 05. 60227°	W 00. 0793°
13	Abossey-Okai	N 05. 56187°	W 00. 23149°
14	Kwashieman-Odokor	N 05. 59243°	W 00. 26739°
15	Maamobi	N 05. 60559°	W 00. 18935°

3.2 Knowledge, Attitude and Perception Studies on Insecticide Usage

3.2.1 Questionnaire survey

The questionnaire survey was carried out between September 1999 and February 2000 using a prepared form (Appendix 1) to obtain data on insecticide usage and pattern, insecticides of choice (aerosol or coil), the frequency and mode of insecticide application, the number of individuals occupying a room or making up a household in relation to reported incidences of malaria and socio-economic status. Respondents were also asked to describe the effectiveness of their insecticide(s) of choice, whether they used bednets or not, whether bednets were impregnated and if they were, the type of insecticide used in impregnation.

Four socio-economic categories, upper, upper-middle, lower-middle and lower classes were designated based on the quality of residential facility and environmental sanitation. The respondents answered the questionnaires in the presence of the administrator. The data obtained were analysed using the SPSS statistical software version 10.0 for Windows. The various statistical methods employed can be found in relevant sections below.

3.3 Insecticide Susceptibility Studies

3.3.1 Field collection and rearing of mosquitoes

Mosquito larvae were collected with copper ladles from breeding puddles at specific locations and were transported to the laboratory in 2-litre bottles that were loosely covered to allow ventilation Plastic containers (h x w x l = 5cm x 27cm x 36cm) with large surface area and water depth of no more than 2cm. were used to culture the larvae.

The larvae were reared to adults at 25°C±2°C and 55% - 68% relative humidity. The relative humidity was maintained using Defensor 300™1 humidifier. A 12h: 12h light and dark cycle was also maintained.

Under these conditions, all larvae pupated synchronously over one or two days although this was only occasionally achieved. If more than 95% of larvae had not pupated within four days, suboptimal conditions were suspected and conditions were altered where necessary. Each morning, pupae were harvested and placed in cages until adults emerged. The adults were fed only on 10% sugar solution for two days and used for bioassay.

3.3.2 Morphological identification of life stages of *Anopheles gambiae* s. l.

Adult and larval *An. gambiae* s.l. were morphologically identified using the taxonomic key of Gillies and Meillon (1966). The larvae lack siphon, (see page 25)with the result that when they are at the water surface they lie parallel to it and are not subtended at an angle as are the Culicinae.

The respiratory trumpets of anophiline pupae are short and broad distally, thus appearing conical (see page 28) whereas in most Culicinae the trumpets are narrower and more cylindrical. The most reliable characteristic for identifying anopheline pupae is the presence of short peg-like spines situated laterally near the distal margins of abdominal segments two or three to seven. In the culicines there are no such spines (Service, 1980).

Adult *Anopheles* usually rest with the body at an angle to the surface. This is a very useful characteristic allowing adults at rest to be readily identified as *Anopheles*. Most, but not all, *Anopheles* have spotted wings, that is the dark and pale scales are arranged in small blocks or areas on the veins. The number, length and arrangement of these dark and pale areas differ considerably in different species (see page 29) and provide useful characters for species identification (Service, 1980).

Female mosquitoes have non-plumose antennae. If the adults are females and also *Anopheles* then the palps will be about as long as the proboscis and usually lie closely alongside it and may be marked, especially the apical half, with broad or narrow rings of pale scales (Service, 1980) (See page 29).

3.3.3 Bioassay experiments

The tests for susceptibility to permethrin and deltamethrin were carried out simultaneously using the WHO test kit, therefore the same controls were used for both. Two-day old non-blood fed adult females were collected with an aspirator in lots of not more than 4 and gently transferred to paper cups until each cup contained 25 of them. A pre-test holding period of 1 hour was allowed to guard against including damaged specimens in the tests. The mosquitoes were then transferred into the insecticide exposure tube in lots of not more than 10. They were then exposed to papers impregnated with permethrin and deltamethrin at diagnostic concentrations of 0.75% and 0.05% respectively for 80 minutes. At the end of the exposure period, the number of mosquitoes knocked down was counted. The holding tube was then screwed onto the exposure tube and the slide pulled out to a point beyond the filling-hole so that no part of it occluded the tube opening. The mosquitoes were then blown gently down into the holding tube. The exposure tubes were held horizontally and tapped to dislodge the knocked down insects from the slide before the latter was withdrawn. The slide was then closed and the exposure tube unscrewed. The holding tube was then set so that it stood on the slide and a pad of wet cotton wool was placed on the screen. Mortalities were recorded 24 hours post exposure. Mosquitoes that were unable to fly were also counted as dead. The survivors were immobilised at –20°C for 5 minutes. Mosquitoes exposed to untreated papers impregnated with Dow Corning 556 silicone fluid served as controls. Each assay was replicated five times.

Each impregnated paper was used not more than 3 times and up to 7 days after removal from the package. The papers were left in the tubes after each test and the open end wrapped with aluminium foil.

Survivors and susceptible mosquitoes were preserved separately on silica gel in 2.5 ml Eppendorf tubes until ready to use.

3.3.3.1 Determination of insecticide susceptibility levels

The numbers of the susceptible and the survivors of the tests and the controls were used in Abbott's formula to determine the insecticide susceptibility levels when control mortalities were between 5% and 20%. The Abbot's formula is expressed as follows:

$$\text{Susceptibility} = \frac{(100\text{-}\%\ \text{test mortality})}{(100 - \%\ \text{control mortality})} \times 100$$

3.4 Molecular Studies

3.4.1 Biological material

Single *An. gambiae* mosquitoes were used for the study.

3.4.2 Standard solutions

The standard solutions used for the studies were as follows:

Bender buffer	0.1M NaCl, 0.2M sucrose, 0.1M Tris-HCl pH 7.5, 0.05M EDTA, pH 9.1, 0.5% SDS. Sterilised and stored at 4°C.
0.5M EDTA (pH 8.0)	186.1gl^{-1} in water, pH adjusted with NaOH pellets. Sterilised and stored at room temperature.
EtBr (10mg ml^{-1})	1g EtBr dissolved completely in 100ml sddH_2O and stored in dark at 4°C.
KAc (5M)	49.75gm dissolved in 100 ml of sddH_2O.
KAc (5M K, 8M Ac)	60ml 5M KAc and 11.5ml glacial acetic acid in 28.5ml *sdd*H_2O.

NaOH (10N)	40gm NaOH dissolved in 100ml *sdd*H_2O.
RNAse	10mg ml^{-1} in water. Heated to 65°C to inactivate any DNAse and sterilised by filtration. Stored at -20°C.
TAE (10x) buffer	242g Tris base, 57.1 ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) in 1 litre water.
Taq DNA polymerase dilution buffer	10mM Tris-HCl (pH 7.5), 10mM DTT, 10 mg ml^{-1} BSA, 10% glycerol. Stored at -20°C.
TE (desired pH)	10mM Tris-HCl (desired pH), 1mM EDTA (pH 8.0).
1M Tris (desired pH)	121.1gl^{-1} of Tris base in water. pH adjusted to desired value with concentrated HCl. Sterilised by autoclaving and stored at room temperature.
Phenol	(water saturated phenol, 0.01% hydroxyquinoline, 0.01M Tris-HCl pH 8.0)

3.4.3 Isolation of Genomic DNA from single anopheline mosquitoes

The DNA was extracted from adult female *An. gambiae* s. l. mosquitoes using the modified protocol of Collins (1987). For this method each mosquito was homogenised in 100µl Bender buffer in 1.5ml Eppendorf tube and incubated in a hot water bath at 65°C for 30 minutes and thereafter centrifuged at 14,000 rpm for 15 minutes. The supernatant (≈100µl) was recovered into a fresh tube and 15µl of 8M-potassium acetate was added, vortexed well and allowed to incubate at –20°C for 15 min. It was then centrifuged for 15 min. at 14,000 rpm, and the supernatant recovered into a fresh tube. Then 100µl phenol-chloroform mixture was added and mixed by inversion for three or four times and centrifuged at 14,000 rpm for 5 min. The aqueous phase was transferred into a new tube and 200µl of pre-chilled absolute ethanol was added, mixed by inversion and kept for 15 min. at –20°C and then centrifuged for 15 min. at 14,000 rpm. The pellet was washed in 150µl of 70% ethanol, centrifuged for 10 min. at 14, 000 rpm. The alcohol was poured off and the pellet allowed air-dried. The pellet was dissolved in 20 µl Tris-EDTA + RNAse (50 µg/ml), and kept at 4°C overnight and thereafter kept at –20°C until ready to use.

3.4.4 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method of nucleic acid synthesis by which a target DNA fragment is exponentially replicated (Saiki *et al.*, 1985; Mullis *et al.*, 1986; Mullis and Faloona, 1987). It uses a thermostable DNA polymerase enzyme isolated from the hot-springs bacterium *Thermus aquaticus* and two oligonucleotide

primers, which flank the target DNA sequence to be amplified. The reaction involves repeated cycles of heat denaturing of the DNA template, annealing of the primers to their complementary sequences at a lower temperature, and extension of the annealed primers with the DNA polymerase. The primers hybridise to the opposite strands of the target DNA and are oriented (3' ends pointing towards each other) so that DNA synthesis by the polymerase proceeds across the region between the primers. The extension products are complementary to and capable of binding primers, therefore successive cycles of amplification result in the doubling of the target DNA synthesised in the previous cycle.

3.4.4.1 Identification of member species of Anopheles gambiae complex

The method of Scott *et al.* (1993), using species-specific oligo primers was used for the identification for *An. gambiae* s.l. This involved using universal primer UN as forward primer in a cocktail reaction that contained all the reverse primers, GA, for *Anopheles gambiae* s.s., ME for *An. melas* and *An. merus*, AR for *An. arabiensis* and QD for *An quadriannulatus*. The lengths of the sequences amplified between UN and each of the four species-specific primers are 153bp for *An. quadriannulatus*, 315bp for *An. arabiensis,* 390bp for *An. gambiae*, and 464bp or 466bp for *An. melas/An. merus* respectively. Laboratory strain of *An. arabiensis*, obtained from Wageningen Agricultural University, The Netherlands, was used as positive control for all the reactions.

The sequence details of the oligo primers for *Anopheles gambiae* species complex identification is as follows:

Name of primer	sequence	T_m (°C)
UN	5' GTG TGC CCC TTC CTC GAT GT 3'	58.3
GA	5' CTG GTT TGG TCG GCA CGT TT 3'	59.3
ME	5' TGA CCA ACC CAC TCC CTT GA 3'	57.2
AR	5' AAG TGT CCT TCT CCA TCC TA 3'	47.4
QD	5' CAG ACC AAG ATG GTT AGT AT 3'	42.7

The reaction conditions that were varied during the optimisation stage were (1) units of Sigma *Taq*, (2) concentration of the PCR primers, (3) concentration of $MgCl_2$ and (4) amount of extracted mosquito DNA. Finally, the reaction mix contained 1x buffer C [300mM Tris-HCl, 75mM $(NH_4)_2SO_4$, 2.5mM $MgCl_2$, pH 8.5)] and 0.25µl of 20mM dNTP mix. For the species complex identification, 0.25µM each of the primers GA, UN, ME, AR and QD were used. The PCR reaction conditions were set at 94°C for 30 sec., at 48°C for 30sec and at 72°C for 2min for 35 cycles using Hybaid PCR Express* (ThermoHybaid Ltd., U.K), for the thermal cycling. There was a final extension step at 72°C for 10 min. The quantity of *Taq* was 0.625unit/25µl per reaction and 50ng of the extracted DNA was used as template. The volume was made up to 25 µl with sterile double distilled water (*sdd*H_2O).

3.4.4.2 PCR detection of kdr alleles in An. gambiae s.s.

The method of Martinez-Torres *et al.* (1997) was used. The *kdr* primers were Agd1 and Agd4 (*Oligos Etc.* Inc., USA) and Agd2 and Agd3 (Oswel, UK) were used. The sequence details of these primers are shown below:

Name of primer	sequence	T_m (°C)
Agd1 (Forward)	5' ATA GAT TCC CCG ACC ATG 3'	64.5
Agd2 (Reverse)	5' AGA CAA GGA TGA TGA ACC 3'	45.6
Agd3 (Reverse)	5' AAT TTG CAT TAC TTA CGA CA 3'	45.2
Agd4 (Reverse)	5' CTG TAG TGA TAG GAA ATT TA 3'	60.0

The reaction mix contained 0.5µM each of Agd1, Agd2, primers and 0.25µM each of Agd3 and Agd4 primers; 300mM Tris-HCl, 75mM $(NH_4)_2SO_4$, 2.5mM MgCl, pH 8.5 and 0.25µM of each dNTP and 50ng of DNA prepared as in Section 3.5.4 above in a final volume of 25µl. The PCR reaction conditions were 94°C for 30 sec., 52°C for 30 sec. and 72°C for 2 min. for 40 cycles with a final extension step of 72°C for 10 minutes.

Agd1 and Agd2 amplification resulted in a 293bp fragment, which is the internal control for the reaction. Agd1 and Agd3 resulted in a 195bp fragment in resistant mosquitoes whilst Agd2 and Agd4 produced a 137bp fragment in susceptible mosquitoes. Thus for each reaction 2 fragments are observed.

Two laboratory strains of *An. gambiae* s.s. "Kisumu", homozygous susceptible and *An. gambiae* s.s. VKPR homozygous resistance for *kdr* that were used as positive controls were obtained from the OCCGE, Institut Pierre Richet in Côte d'Ivoire.

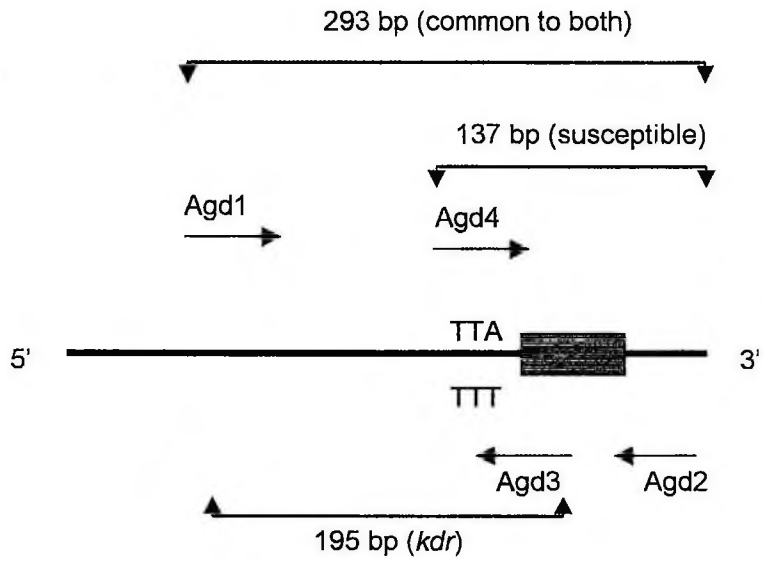

Fig. 6 Schematic representation of the identification of *kdr* by PCR-based diagnostic test. The positions of the triplets encoding leucine (TTA) in susceptible mosquitoes and phenylalanine (TTT) in *kdr* individuals are indicated above and below the thick line representing the IIS4-IIS6 region of the sodium channel. The position of intron 2 is represented by a shaded box (After Martinez-Torres *et al.*, 1998).

3.4.4.3 Detection and analysis of PCR products

The lengths of PCR products were estimated by comparison with molecular weight markers of known size on an agarose gel. In principle the migratory rates of DNA molecules on agarose gels are inversely proportional to the logarithms of the molecular weights and is expressed as below:

$$D = a - b (\log M)$$

Where D is the distance, M, the molecular weight of DNA, and a and b are constants (Mullis and Faloona, 1987).

After each reaction, 5µl of the product was run alongside a 100 base-pair molecular-weight marker in a 1.5% agarose gel (from Promega Corporation, USA) that contained 0.8% ethidium bromide. DNA bands on the gel were visualised under ultraviolet trans-illumination. By comparing the product sizes with those of the 100 base-pair molecular-weight marker, the fragment sizes were determined.

CHAPTER FOUR

RESULTS

4.1 Questionnaire-Survey

4.1.1 Study sites

The exact location of each sampling site was determined using a portable global positioning system (GPS). The coordinates and location of the ten study sites are shown in Table 1.

4.1.2 Respondents

A total of 171 respondents were involved in the study. Their socio-economic classes were distributed as follows: 31 (18.2%) were in the upper class, 54 (31.8%) in the upper-middle class, 64 (37.6%) in the lower-middle class and 21 (12.4%) in the lower class. One respondent did not provide information on socio-economic status. Twenty-six (15%) respondents had had basic education while 61 (35.7%) had had tertiary education. Respondents with secondary education were 70 (40.9%). The number of respondents who did not disclose their educational background was 14 (8.4%). The proportions of males and females were 91 (53.2%) and 80 (46.8%) respectively.

There were, on the average, five persons per household. The number of persons per household in the upper class ranged from two to ten. In the upper-middle and lower-middle classes the range was from five to ten whilst in the lower class the range was from seven to ten. In all socio-economic classes the mean was five persons per household.

4.1.3 Choice of insecticides

The number of respondents who used aerosols was 92 (54.0%) and were distributed as follows: 18 (19.6%) in the upper class, 33 (35.9%) in the upper-middle class, 32 (34.8%) in the lower-middle class and 9 (9.8%) in the lower class. Fourteen respondents (8.2%) used coils only and had the following distribution, 4 (28.6%) in the upper class, 3 (21.4%) in the upper-middle class, 5 (35.7%) in the lower-middle class and 2 (14.3%) in the lower class. A total of 60 (35%) respondents used both coils and aerosols. Out of this number 7 (11.7%) were in the upper class, 18 (30%) were in the upper-middle class, 26 (43.3%) were in the lower-middle class, whilst 9 (15%) were in the lower class. A Chi square test revealed that aerosols were preferred to coils ($p = 0.005$).

4.1.3.1 Aerosols

Six different aerosols were commonly used by the respondents. These were by the trade names, "Raid", "Elf", "Mobil", "Shelltox", "Goiltox", and "Killit".

"Raid", was preferred by 41 (44.6%) of aerosol users, whilst 20 (21.7%) used "Elf" Twelve (13%) used "Mobil" and 10 (10.9%) preferred "Shelltox". Only one respondent (1.1%) used "Killit" and this was in the upper class. The distribution of "Raid" users according to socio-economic status was as follows: four (9.8%) in the upper class, 10 (24.4%) in the upper-middle class, 20 (48.8%) in the lower-middle class and 7 (17.1%) in the lower class. "Elf" users were distributed as follows: five (25%) were in the upper class, 7 (35%) were in the upper-middle and 8 (40%) in the lower-middle. None of the respondents in the lower class used "Elf". Two (16.7%) of Mobil users were in the upper class, 3 (25%) were in the upper-middle class, 4 (33%) were in the lower-middle class and 3 (25%) were in the lower class. Of the total number of "Shelltox" users, 2 (20%) each were in the upper, lower-middle and lower classes whilst 4 (40%) were in the lower-middle class. The "Goiltox" users were distributed as follows: one (12.5%) each in the upper and lower classes, 4 (50%) in the upper-middle class and 2 (25%) were in the lower-middle class. Test of homogeneity of variances showed significant differences between choice of aerosols ($p = 0.026$). Symmetric measures based on normal approximation showed strong correlation between choice of aerosols and socio-economic classes (Spearman correlation, $p = 0.007$).

4.1.3.2 Coils

The common coils identified were "Raid", "Good-Night", "Cock", "Elf", "Antelope", "Safe-Nite", "Tiger" and "Peacock". The number of coil users who preferred "Raid" was 40 (54.1%), 18 (24.3%) used "Good-Night", whilst 16 (21.6%) respondents used the other coils (Fig. 7). The distribution with respect to socio-economic status was as

follows: 14.9% (11) in upper class, 28.3% (21) in upper-middle class, 41.9% (31) in lower-middle class and 14.9% (11) in lower class (Fig. 8). There was a significant difference between choice of "Raid" and the other coils ($p = 0.005$).

4.1.4 Frequency of insecticide application

Forty-three (25.1%) respondents applied aerosols once in a week, whilst 113 (41%) sprayed between twice and four times in a week with 35 (20.5%) spraying five times or more in a week. Twenty-eight respondents (16.4%) used coils daily. This usage was the same as that of respondents who burnt coils once in a week. Sixteen (9.4%) of the respondents burnt coils three to six times in a week. There was no significant difference between frequencies of application of aerosols in all socio-economic classes ($p > 0.05$). There was also no significant difference between frequency of coil usage and socio-economic classes ($p > 0.05$).

4.1.5 Mode of insecticide application

Seventy-six (44.4%) respondents sprayed everywhere in the room, but did not specify how long the doors and windows were shut. The number of those who sprayed everywhere for 30 minutes or less was 48 (28.1%), whilst 22 (12.9%) sprayed everywhere for more than 30 minutes and 23 (13.5%) of them did not spray at all. Two of the respondents (0.01%) did not provide any information on mode of insecticide application.

Sixty-five (38%) respondents provided information on the mode of application of coils. Thirty-nine (60%) of these burnt the coil near the window, 20 (30.8%) burnt the coils close to the centre of the room whilst 6 (9.2%) burnt the coil outside the room. Test of homogeneity of variances showed a significant difference between modes of application of coils ($p < 0.05$). Multiple comparisons revealed significant differences at 0.05 level in mode of application in upper-middle, lower-middle and lower classes ($p < 0.05$) but not in upper class ($p > 0.05$). There was no significant difference between modes of application of aerosols amongst the socio-economic classes ($p > 0.05$).

4.1.6 Perception on the effectiveness of insecticides

The detailed results are illustrated in Fig. 9. Out of the 171 respondents, 25 (14.7%) claimed that the aerosols were very effective; 68 (39.9%) said aerosols were just effective whilst 57 (33.7%) said they were quite effective or not effective (standard deviation = 2.60 ± 1.17). In contrast, only one (0.6%) coil user claimed that the coils were effective. Five (2.9%) respondents said coils were not effective (standard deviation = 4.16 ± 1.16).

Analysis of variance (ANOVA) showed that there was a significant difference between perceptions on effectiveness of aerosol use in the various socio-economic classes ($p = 0.009$). Multiple comparisons showed that significant differences existed between upper ($p = 0.006$), upper-middle ($p = 0.003$) and lower-middle ($p = 0.003$) classes.

4.1.7 Reported incidence of malaria

A total of 128 (74.9%) of the respondents had had malaria in the year prior to the survey (1999). Out of this number, 23 (18%) were in the upper class, 41 (32%) were in the upper-middle class, 50 (39.1%) were in the lower-middle class and 14 (10.9%) were in the lower class (Fig. 10).

No significant difference however was found between the number of persons in a household or sharing the same room, their socio-economic status and incidence of malaria ($p > 0.05$).

4.1.8 Usage of anti-mosquito screen

One hundred and thirty-one (77.1%) respondents used anti-mosquito screens. Twenty-one (13.45%) were in the upper class. Four (2.9%) respondents from the upper class had air-conditioned bedrooms and did not use anti-mosquito screens. Forty-two (32.1%) respondents who used anti-mosquito screens were in the upper-middle class, 52 (39.7%) were in lower-middle classes, whilst 16 (12.2%) were in the lower class (Fig. 11). Eighteen (11.8%) respondents did not use anti-mosquito screen. Chi-square test revealed a significant difference between usage of anti-mosquito screens and socio-economic status ($p < 0.05$).

4.1.9 Use of bednet and other ITMs

Only 10 (5.8%) out of 171 respondents had ever used bednets and none of them used impregnated bednets (Fig. 12). There were four (40%) bednet users in each of upper and lower middle classes, two (20%) in the lower class and none in the upper-middle class. Chi-square test revealed significant difference between usage and non-usage of bednets ($p < 0.05$).

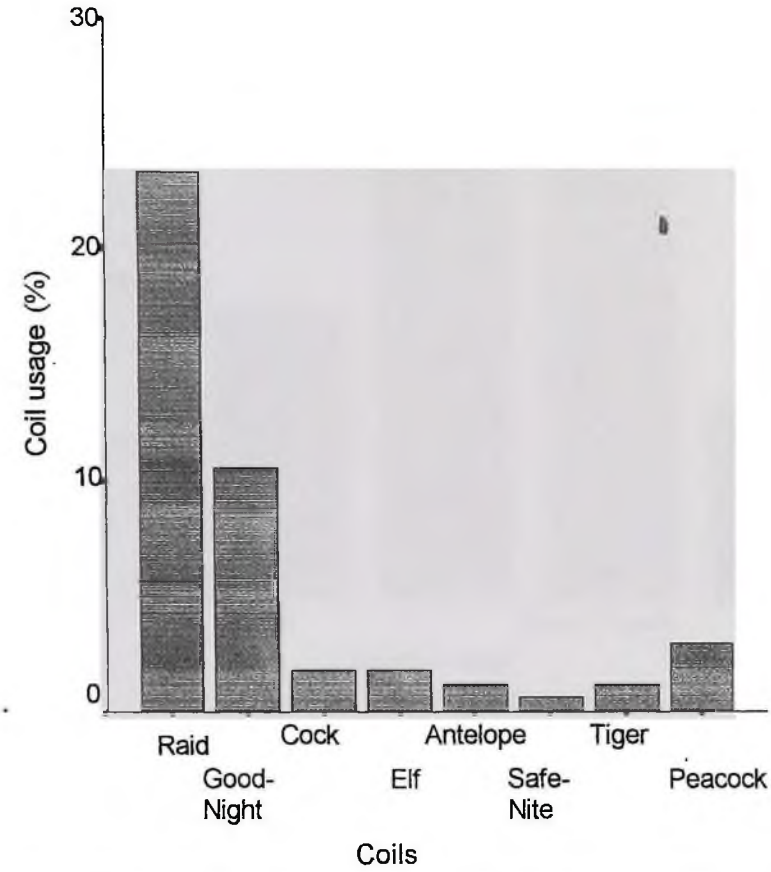

Fig. 7 Graph showing the types and preference for mosquito coils and the extent of their usage in the study population.

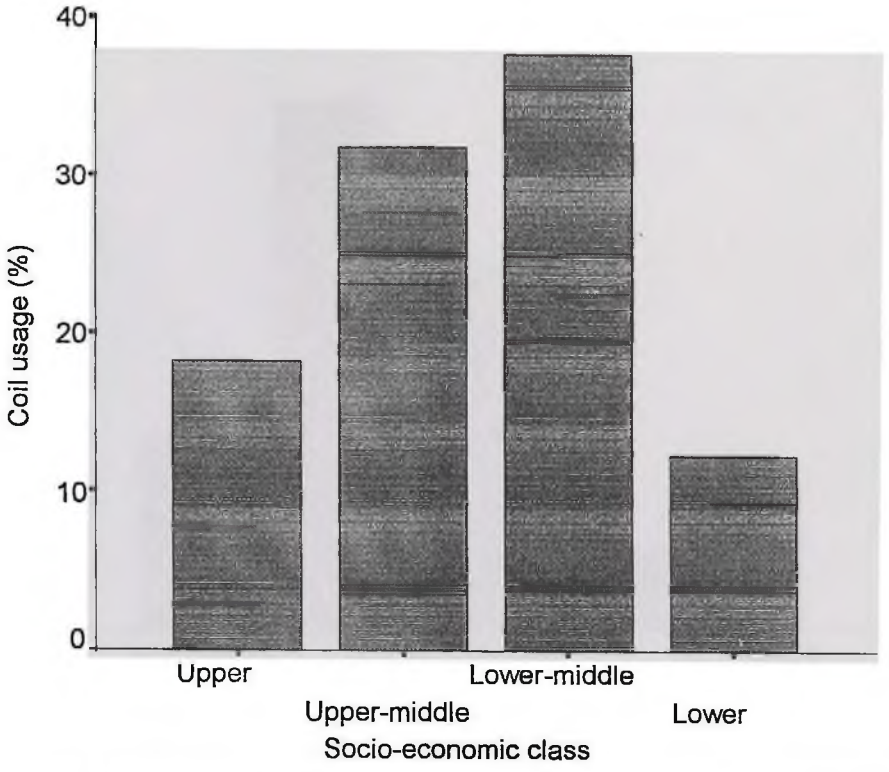

Fig. 8 Graph showing coil usage by respondents in the different socio-economic classes.

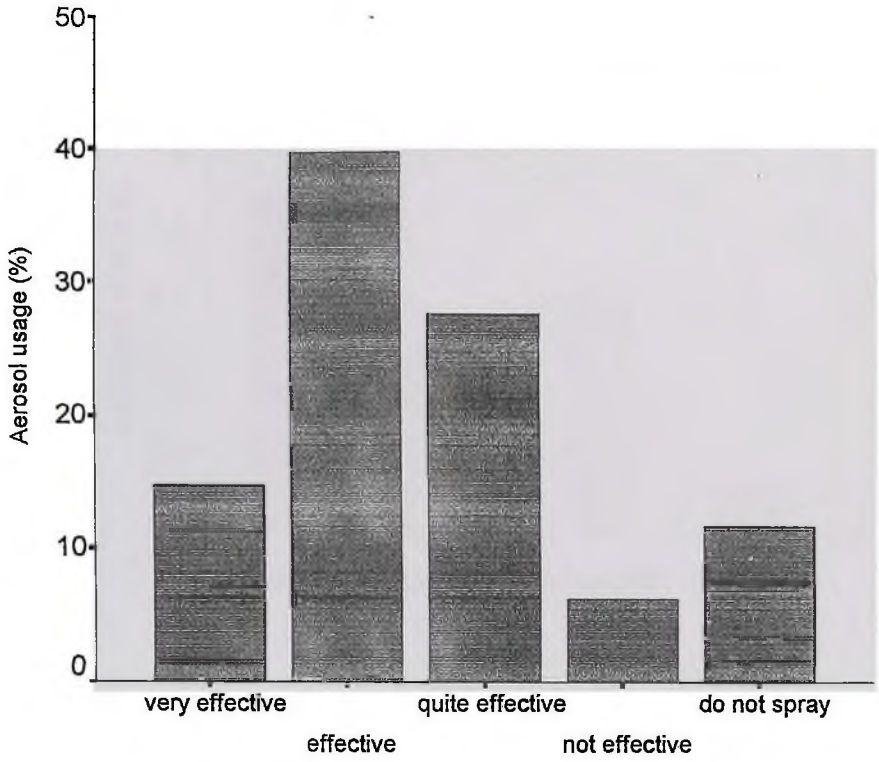

Fig. 9 Graph of the perception of the effectiveness of aerosol use.

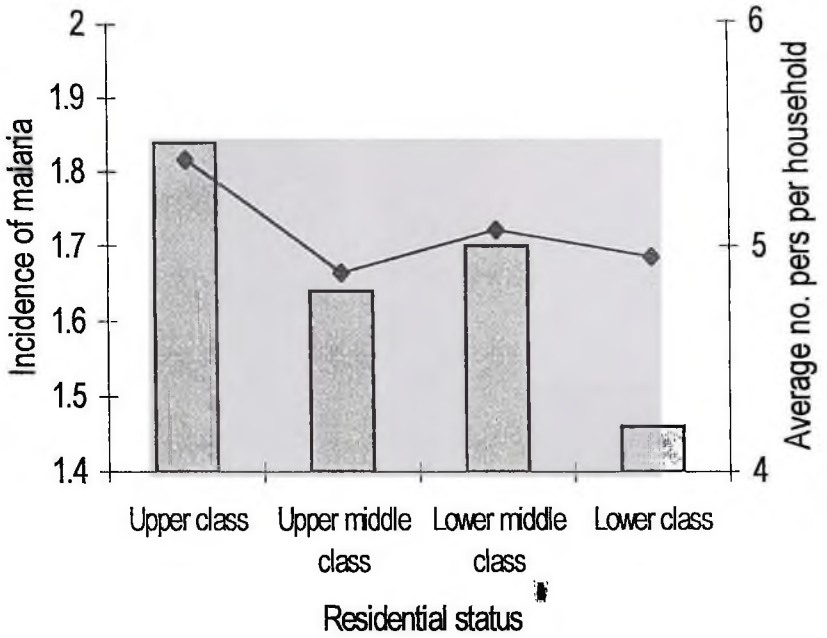

Fig. 10 Graph showing the incidence of malaria, residential status and average number of persons/ household. The bars represent the incidence of malaria, whilst the line represents the average number of persons per household.

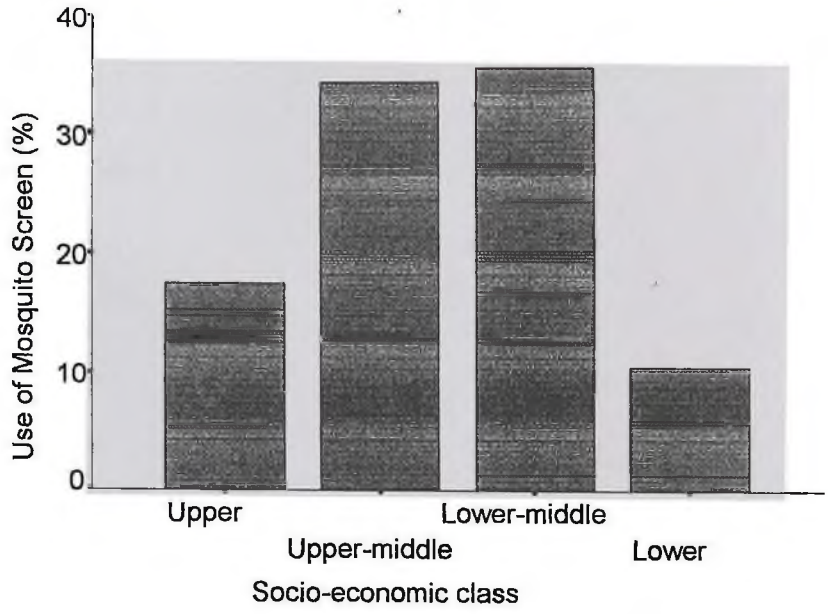

Fig. 11 Graph showing the use of mosquito screen by the respondents in the different socio-economic classes.

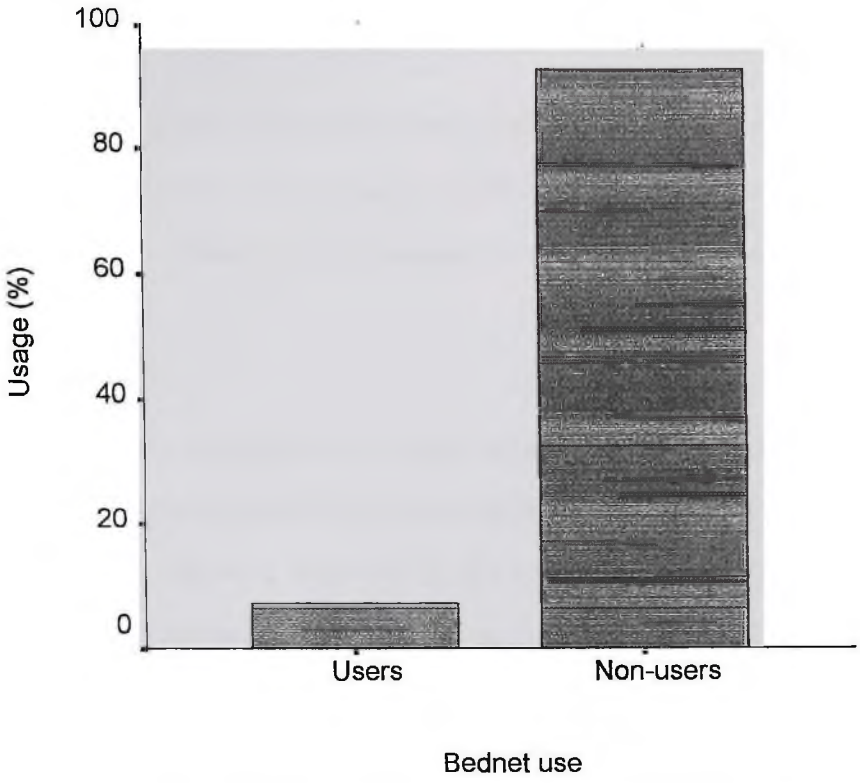

Fig. 12 Graph showing bednet use in the study area

4.2 Rearing of Mosquitoes

A common error in larval culture was overcrowding. This resulted in wide distribution of rate of development. Even a single day of overcrowding had noticeable effects on development, so utmost attention was paid to the larval conditions to minimise this problem.

The most delicate and troublesome stages were the early instars. During these stages, uncontrolled microbial growth often resulted in foul water, scum formation, and consequently larval death. Unfortunately, the addition of fishmeal as food for optimal larval growth contributed to these problems. To avoid this, mostly third and fourth-instar larvae were collected in the field. These stages were able to limit microbial growth quite efficiently when exposed to higher food concentrations since they filter and forage a larger volume of water.

4.3 Bioassays

A total 100 mosquitoes were used in the four control replicates. The control mortalities were in the range 8% - 16% and therefore were considered satisfactory.

4.3.1 Deltamethrin

The detailed results are shown in Table 2. A total of 100 mosquitoes were assayed with 0.05% deltamethrin. At the end of the exposure time of 1hr 20min., a total of 62 (62%) mosquitoes had been knocked down. The corrected mortalities for the four replicates were 68.2%, 68.2% 77.3% and 77.3%.

4.3.2 Permethrin

The detailed results are shown in Table 3. A total of 95 mosquitoes were assayed with 0.75% permethrin. The number of mosquitoes that were knocked down at the end of the exposure period was 40 (42.1%). The corrected mortalities for the four replicates were 63.6%, 63.6% 77.3% and 83%.

Table 2 Detailed results obtained for tests with 0.05% deltamethrin.

Test	Test Replicate 1	Test Replicate 2	Test Replicate 3	Test Replicate 4
No. of mosquitoes in control	25	25	25	25
No. of control mortalities	3	2	4	3
No. of mosquitoes exposed	25	25	25	25
No. of mosquitoes knocked down at end of exposure period	15	15	18	14
No. of mosquitoes dead at end of holding period	18	20	20	18
Mortality observed (%)	72	80	80	72
Mortality corrected (%)	68.2	77.3	77.3	68.2

Table 3 Detailed results obtained for tests with 0.75% permethrin

Test	Test Replicate 1	Test Replicate 2	Test Replicate 3	Test Replicate 4
No. of mosquitoes used in control	25	25	25	25
No. of control mortalities	3	2	4	3
No. of mosquitoes exposed	20	25	25	25
No. of mosquitoes knocked down at end of exposure period	10	8	10	12
No. of mosquitoes dead at end of holding period	17	20	17	17
Mortality observed (%)	85	80	68	68
Mortality corrected (%)	83	77.3	63.6	63.6

4.4 The Identification of Members of the *Anopheles gambiae* complex

The results of the polymerase chain reaction (PCR) for species identification revealed that all the 295 female mosquitoes used in the bioassays, including those in the controls were *Anopheles gambiae* s. s. For an example electrophoregram of the results obtained see Fig. 13.

4.5 Insecticide Susceptibility Tests and Detection of *kdr* Mutations

Out of the total of 195 mosquitoes used in the PCR to detect knockdown resistance (*kdr*) mutations, 147 (75%) were susceptible, while survivors were 48 (25%). Fifty-two (26.7%) of the 195 mosquitoes possessed the *kdr* mutation. Thirteen (8.8%) of the dead mosquitoes had *kdr* mutation whilst 134 (91.2%) did not. Thirty-nine (81.3%) out of the 48 mosquitoes that survived had *kdr* mutations whilst 9 (18.7%) did not. Chi-square tests revealed highly significant difference between wildtype and *kdr* mutation mosquitoes (Pearson Chi-square value = 97, p = 0.00). For an example electrophoregram of the results obtained see Fig. 14.

Table 4 Relationship between insecticide susceptibility and *kdr* mutations.

	kdr	wildtype	Total
Susceptible	13	134	147
Survivors	39	9	48
Total	52	143	195

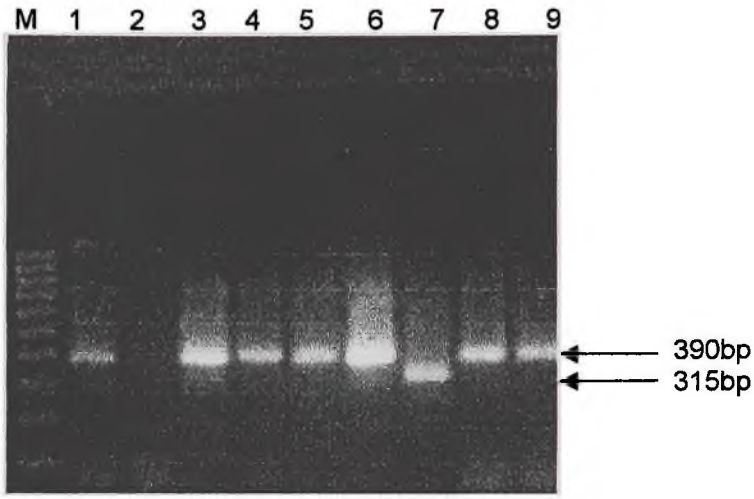

Fig. 13 An example of gel electrophoregram obtained for identification of the *Anopheles gambiae* species complex: Lane M = 100bp molecular weight marker, lanes 1, 3, 4, 5, 6, 8 and 9 = *An. gambiae* s.s, lane 2 = negative control and lane 7 = *An. arabiensis* (the positive control from Wageningen University, the Netherlands).

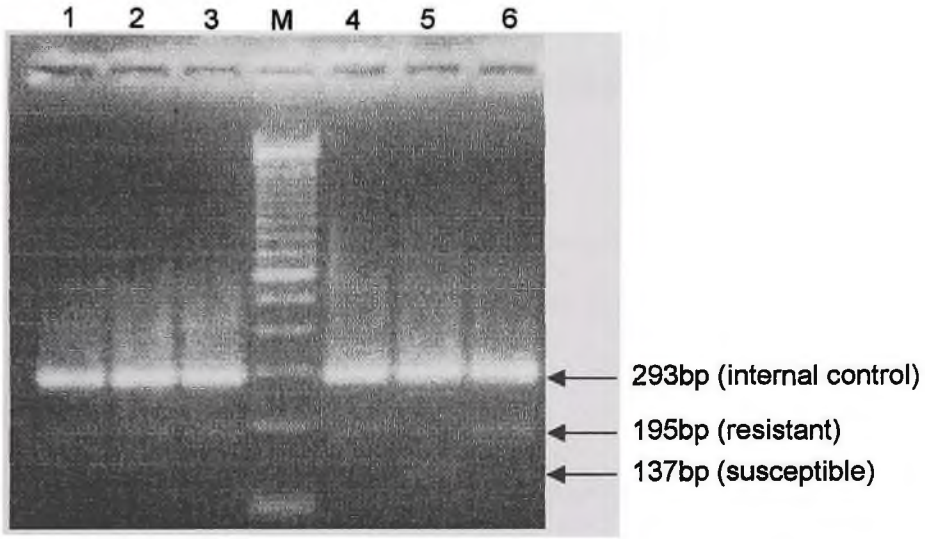

Fig. 14 An example of electrophoregram obtained for the detection of the kdr mutation. Lane M = 100bp molecular weight marker, lanes 1, 2, 3 and 4 are wild-type resistant mosquitoes. Lane 5 and 6 are positive controls representing Kisumu (susceptible) and VKPR (resistant) respectively.

CHAPTER FIVE

DISCUSSION

The GPS information obtained during the questionnaire-survey provided useful guide for the selection of sites for mosquito larvae samples collection (see Appendix III) so that the locations were fairly distributed.

This study has shown that aerosols under different brand names are used as household insecticides but all contained synthetic pyrethroids. These pyrethroids are tetramethrin, diallethrin, permethrin, cypermethrin, phenothrin and deltamethrin. None of the aerosols contained all these active substances, but each had at least two of these pyrethroids. Each aerosol contained a synergist, probably piperonyl butoxide (PBO) because PBO is a well-known synergist of pyrethroids (Kakko *et al.*, 2000). The pyrethroids share similar modes of action and are considered as axonic poisons. They apparently work by keeping open the sodium channels in neuronal membranes. Pyrethroids affect both the peripheral and central nervous systems of the insect. They initially produce repetitive discharges and eventually cause paralysis. Such effects are caused by their action on the sodium channel, a tiny hole through which sodium ions are permitted to enter the axon to cause excitation (Ware, 1980).

Insect mixed function oxidases (MFOs) detoxify many insecticides, especially synthetic pyrethroids making them resistant. PBO inhibits the action of MFOs and restores the toxic action of the insecticide. The synergists are usually emulsifiable concentrates and cannot be incorporated in coils (Bloomquist and Miller, 1986). The

mosquito coils, under different brand names were also all pyrethroids but none contained a synergist.

The general perception of the study population was that mosquito bites could be prevented to a large extent by spraying human habitations with insecticides, burning of mosquito coils and use of bednets. The observation that aerosols were preferred to coils by all socio-economic classes is interesting because the expectation was that people of lower class with little income would prefer mosquito coils. The fact that 8.2% of the respondents used coils only indicates their low usage as household insecticide. Most aerosols users also used coils as supplementary insecticides. According to most respondents smoke from mosquito coils irritates the eyes and induces nasal congestion. A similar observation on the health impact of coils has been reported by Ahorlu *et al.* (1997). Furthermore, the respondents claimed that the coils were not as effective as the aerosols and they were likely to go off, in which case the mosquitoes become more aggressive in their nuisance and biting behaviours. Although the use of coil was similar for all socio-economic classes, in the upper class, coils were burnt usually at dusk outside the room to repel mosquitoes.

Bednet usage was found to be very low and impregnated bednets were not used in the study population and this is likely to be the situation nationwide. Gyapong *et al.* (1996) carried out a study in the Kassena Nankana district of the Upper East Region of Ghana and found that prior to the introduction of insecticide impregnated bednets in this district only 4% of the people had owned a bednet. Furthermore, a study by Ahorlu *et al.* (1997) indicated that bednet usage was very low in the Dodowa and Prampram

communities, both in the Dangme West District of the Greater Accra Region of Ghana which colloborate the findings reported in this study. Among respondents from the lower class, cost of bednets could be the most important factor for their low usage whilst among the more affluent classes complaints that bednets reduce ventilation in already humid environment could account for the low patronage. There was also the perception of respondents, irrespective of socio-economic status, that bednets could act as traps especially if holes developed in them, causing greater nuisance than without them.

Insecticide-treated bednets have been shown to reduce overall child mortality significantly in the Gambia (Alonso *et al.*, 1991, 1993; D'Alessandro *et al.*, 1995), Ghana (Binka *et al.,* 1996) and Kenya (Nevil *et al.*, 1996) due to effective personal protection against malaria vector *Anopheles* mosquitoes, thus their usage must be encouraged. The incidence of malaria in the study population was quite high since 40% of respondents had reported of malaria at least once in the year prior to the study. Ghana has adopted the roll back malaria (RBM) programme and the large-scale usage of impregnated bednets constitutes one of its main strategies. Thus the observation that ITMs are not used means that the Ministry of Health as part of its malaria control programme, should adopt an intensive and sustained campaign to educate the public on the benefits of insecticide treated materials (ITMs) in reducing malaria burden in order to gain their acceptance.

The bioassay results have shown increased tolerance in mosquitoes to permethrin and deltamethrin in the study area since 81.3% of the mosquitoes that survived the

bioassays possessed the *kdr* mutation by the PCR diagnostic assay and therefore were truly resistant.

That there was reduced susceptibility by the mosquitoes to the two insecticides is further supported by the fact that it was found necessary to extend the exposure time of 1 hour by 20 minutes in order to obtain 42.1% and 62% knockdown for permethrin and deltamethrin respectively. These figures compare with other pyrethroid resistance in *Anopheles gambiae* sensu lato studies in Botswana, Cameroon, Senegal, Burkina Faso, Côte d'Ivoire and Benin where the exposure time was 1 hour (Chandre *et al.*, 1999c). Knockdown time has long been accepted as an indicator of early detection of susceptibility (Privora, 1975; Kang, *et al.*, 1995; Martinez-Torres, *et al.*, 1997). It also provides initial information on the possible involvement of the *kdr* gene.

The threat of pyrethroid resistance evolving in *Anopheles* mosquitoes and its impact on the large-scale use of impregnated bednets has long been recognised (Curtis *et al.*, 1990, 1998). Previous use of permethrin-impregnated bednets and curtains in four Kenyan villages for one year, 1990-91, raised the permethrin LT_{50} of *An. gambiae* to 2.4-fold above its baseline value, designated permethrin tolerance (PT) at 0.25% permethrin (*Anopheles gambiae* Genome database, 1998). During 1992-93, with ongoing use of permethrin-impregnated nets and curtains, PT regressed slightly compared with the contemporary susceptibility level of *An. gambiae* from non-intervention villages, to 1.8-fold in 1992 and only 1.6-fold in 1993. Thus the selection pressure of impregnated nets for PT in *An. gambiae* appears to be minimal in the study villages. However, the impact of permethrin was demonstrated by a significantly lower parous-rate of *An. gambiae* females in the intervention (63-66%) than in non-

intervention (79%) villages, and by reduced malaria transmission (*Anopheles gambiae* database Genome, 1998). This gives an indication that although resistance may exist, bednet usage provides appreciable protection against malaria. In the long term, however, it is necessary to monitor the changes in tolerance levels of *An. gambiae* to the pyrethroids that are currently being used for ITMs. In addition to monitoring, other alternative insecticides to permethrin and deltamethrin must be studied for their suitability for vector control. Luo Dapeng *et al.* (1994) have reported successful malaria control in China using nets treated with α-cypermethrin at a much lower dose (20mg a.i.m^{-2}) than required for permethrin (500mg a.i.m^{-2}). However, it is important to determine whether this insecticide is as effective against Afrotropical vectors of malaria as it was against Chinese vector mosquitoes.

Marbiah *et al.* (1994) compared the effectiveness of permethrin with deltamethrin as well as with another pyrethroid, λ-cyhalothrin used for bednet impregnation in Sierra Leone, and found that λ-cyhalothrin is effective at 10mgm^{-2} and this dosage is much cheaper than the recommended dosage for permethrin (Fielden, 1996). Maxwell *et al.* (1999) compared bednets treated either with α-cypermethrin or λ-cyhalothrin and found out that they had similar effectiveness by various entomological criteria. However, λ-cyhalothrin was associated with significantly more reports of nasal irritation than α-cypermethrin. The acceptability and side-effects of these other pyrethroids as alternatives to permethrin and deltamethrin for bednet impregnation needs to be studied in Ghana.

Presently pyrethroids seem to be the insecticides of choice both for house spraying and treated materials, such as bednets and curtains, therefore resistance to these

compounds should be of particular concern. Thirteen (8.8%) of the dead mosquitoes had *kdr* mutations and this can be explained by the fact that in the bioassay, mosquitoes were counted as "dead" if they could not fly.

According to Ranson *et al.* (2000), a leucine-phenylalanine substitution at position 1014 of the voltage-gate sodium channel is associated with *kdr*-resistance to permethrin and DDT in many insect species, including *An. gambiae* from West Africa. Thus the nine mosquitoes that survived and yet did not possess the *kdr* gene mutation may have other mechanisms of resistance, including a possibility of a *kdr*-type resistance at a different locus or metabolic resistance. This is because the position of mutation may be different in different regions. It is also possible that more than one mutation may be found in one area. For example, the diagnostic PCR developed by Martinez-Torres *et al.* (1998) to detect *kdr* alleles in field populations of *An. gambiae* will not detect the Kenyan allele, hence sole reliance on this assay may lead to underestimation of the prevalence of pyrethroid resistance in species from different geographical and ecological zones. Therefore, developing PCR-based tests for all *kdr*-type of resistance is imperative for effective monitoring of pyrethroid resistance in the study area.

The biochemical/physiological resistance mechanisms involving esterases, glutathione-S-transferases, acetylcholinesterase, cytochromes P_{-450} in *An. gambiae* s.l. also need to be investigated. In *An. gambiae,* resistance to pyrethroid insecticides has been shown to involve increased levels of cytochrome P_{-450s} (Ranson *et al.*, 2000).

Insecticide resistance in *Anopheles* vector mosquitoes poses a great challenge to malaria control initiatives, particularly in tropical Africa, since insecticides play a key role in vector control. Incidentally studies in this area have been conducted in neighbouring countries like Côte d'Ivoire, Burkina Faso and Benin but not in Ghana.

This study has provided preliminary information on resistance status in *An. gambiae* in parts of Accra. This is the first time that *An. gambiae* s.s. has been shown to exhibit pyrethroid insecticide resistance in Ghana and this raises several important issues. Firstly, identification of members of the *An. gambiae* complex in other ecotypic regions of Ghana is imperative for devising effective control strategies. Secondly, *An. gambiae* remains the most important vector for malaria in West Africa. Although the use of pyrethroids in the Gambia for the treatment of bednets has not led to reduced susceptibility in the vector, the biological phenomenon of resistance development is dynamic and manifestation of mutations may take a longer period. It is therefore of utmost importance to extend this project to cover all ten regions of the country, to establish the relationship between insecticide use and levels of pyrethroid resistance, the type of *kdr* mutations in *Anopheles gambiae* s.s in Ghana in organised malaria vector control. Thirdly, the susceptibility status of the other members of the complex needs to be investigated to provide baseline information in the planning of malaria control strategies.

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APPENDICES

Appendix I:

SAMPLE OF QUESTIONNAIRE FOR INSECTICIDE USAGE IN ACCRA

Please tick √ where necessary

Date: Day……..Month……..Year……

Area:……

Location:……

Name of respondent:……

Educational background: (optional) Basic ☐ Secondary ☐ Tertiary ☐

Gender: Male ☐ Female ☐

Marital status: Married ☐ Single ☐ Divorced ☐

No. of persons occupying room/ house……

Insecticide usage:

Aerosols

Type	Active ingredients
Mobil	……
Raid	……
Shelltox	……
Goiltox	……
Elf	……
Other (specify)	……

Coils

Type	Active ingredients
☐ Mobil	.....
☐ Raid	.....
☐ Shelltox	.....
☐ Goiltox	.....
☐ Elf	.....
☐ Other (specify)	.....

☐ Other toxicants .....

Frequency and mode of application

How often do you apply insecticide or toxicant?

Aerosol		Coil	
Once a week	☐	Once a week	☐
Twice a week	☐	Twice a week	☐
Daily	☐	Daily	☐
Other (specify)	☐	Other (specify)	☐
.....		.....	

Mode of application.....

.....

Observation on effectiveness of insecticide.....

.....

Incidence of malaria

Gender	Age	Frequency
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....
.....	.....	.....

Was malaria severe? Yes ☐ No ☐

Do you have any knowledge of mosquito breeding?.....

.....

.....

Do you use bednet? Yes ☐ No ☐

Is bednet impregnated? Yes ☐ No ☐

Do you know the insecticide used for impregnation?.....

Any other information on mosquitoes and malaria.....

.....

.....

Appendix II: Molecular Techniques

Gel loading buffers

5x Orange G	20% (w/v) Ficoll, 25mM EDTA, 2.5% (w/v) orange G stored at -4°C.
Bromophenol blue	0.25% bromophenol blue, 40% sucrose in water. Stored at -4°C

DNA molecular weight size marker

A100bp ladder molecular weight size marker was used throughout the study. The first band size is 100bp, the subsequent ones measure 200, 300, 400, 500, 600, 700, 800, 900 and 1000bp.

Casting gels

1. The casting tray was placed on a level surface and the running plate kept inside the casting tray.
2. Preparation of agarose solution:

0.4-g agarose was weighed out in 40-ml Tris-EDTA buffer and melted in a microwave oven for 2 minutes. The molten agarose was allowed to cool to about 60°C and 2µl-ethidium bromide added. This was mixed thoroughly and the conical flask placed in a hot water bath at 50°C until gel was ready to be poured. Gloves were worn since ethidium bromide is carcinogenic. To avoid warping the unit agarose was not poured at temperature hotter than 50°C.

3. The agarose was poured into the casting tray. If agarose seeped under the running plate, it was minimised by pushing down the handles of the running plate.

4. A comb of desired thickness was place across the rim of the casting tray to form sample wells. The height of the teeth of the comb was adjusted so that they penetrated the gel but left about 1 mm of gel between the teeth and the running plate.

5. The gel was allowed to set for at least 30 minutes.

6. The comb was carefully removed.

7. The running plate was lifted out of the casting tray and any gel adhering to the underside of the plate was peeled off. The plate and the gel were transferred to the main unit.

Running the gel

1. DNA sample was carefully loaded after mixing with 1/3 volume of the loading dye, using a Gilson pipette.
2. The lid was placed on the unit so that the cathode (black cord) was nearest the sample wells, since nucleic acid samples migrate towards the anode (red cord).
3. Electrodes were connected to the gel and electrophoresis conducted towards the anode (+) at 70V for 1.5hr.
4. DNA bands were visualised under UV light box inside a dark room. DNA is normally visualised by fluorescence of bound ethidium bromide.
   (i) The gel was removed from the main unit and transferred to a black Perspex sheet.
   (ii) The DNA bands were observed fluorescing under short wavelength UV light in a dark room.

NOTE: Goggles were worn to protect the eyes from UV radiation damage. Gloves were always used to handle gels or buffers containing ethidium bromide, which is a powerful mutagen.

5. Photographs were taken of the gels using type 667 film on a Polaroid camera fitted with an orange filter for permanent record of result.

Gel concentrations for efficient separation

Agarose (%, w/v)	**Size of Linear DNA (kb)**
0.3	5 – 60
0.6	1 – 20
0.8	0.5 - 10
1.0	0.4 – 8
1.2	0.4 – 6
1.5	0.2 – 4
1.8	0.2 - 3

Notes on the master mix

The master mix buffer is often stored as a 10X stock solution (100-mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$) which is diluted to 1X for use. Both the master mix buffer and the purified water can be stored at room temperature. Store deoxynucleotides, primers and *Taq* DNA polymerase enzyme at -20°C.

Although 100µl of master mix per reaction is generally used, it is possible to use as little as 25 or 50µl to save on cost of reagents. Regardless of the total volume, be certain to keep the final concentrations of reagents constant.

Master mix reagents can be obtained from a variety of companies. Often the initial concentration of the reagent will differ depending on which company produced it. It is easy to figure out how much stock reagent to use by following a simple formula:

(Initial concentration) X (volume needed) = (final concentration) X (volume of sample)

Factors affecting the PCR

Denaturing temperature and time

The specific complementary association due to hydrogen bonding of single-stranded nucleic acids is referred to as "annealing". Two complementary sequences will form hydrogen bonds between their complementary bases (G to C, and A to T or U) and form a stable double-stranded, anti-parallel "hybrid molecule. If the nucleic acid is heated in buffers of ionic strength lower than 150mM NaCl, the melting temperature is generally less than 100°C-, which is why PCR works with denaturing temperatures of 91-97°C (Rybicki, 1996).

Taq polymerase is given as having half-life of 30 minutes at 95°C, which is partly why one should not do more than about 30 amplification cycles: however, it is possible to reduce the denaturation temperature after about 10 rounds of amplification, as the mean length of target DNA is decreased. For templates of 300bp or less, denaturation temperature may be reduced to as low as 88°C for 50% (G+C) templates (Yap and McGee, 1991), which means one may do as many as 40 cycles without much decrease in enzyme efficiency.

"Time and temperature" is the main reason for denaturation/ loss of activity of *Taq*: thus, if one reduces this, one will increase the number of cycles that are possible, whether the temperature is reduced or not. Normally the denaturation time is 1 min at 94°C. It is possible for short template sequences, to reduce this to 30 sec or less.

Increase in denaturation temperature and decrease in time may also work. Innis and Gelfand (1990) recommend 96°C for 15 sec.

Cycling number

The number of amplification cycles necessary to produce a band visible on a gel depends largely on the starting concentration of the target DNA: Innis and Gelfand (1990) recommend from 40-45 cycles to amplify 50 target molecules, and 25-30 to amplify 3x105 molecules to the same concentration. This non-proportionality is due to the so-called plateau effect, which is the attenuation in the exponential rate of product accumulation in late stages of a PCR, when product reaches 0.3-1.0nM. This may be caused by degradation of reactants (dNTPs, enzymes); reactant depletion (primers, dNTPs-former a problem for with short products, latter for long products); end-product inhibition (pyrophosphate formation); competition for reactants by non-specific products; competition for primer binding by re-annealing of concentrated (10nM) product (Innis and Gelfand, 1990).

If desired product is not made in 30 cycles, a small sample (1µl) of the amplified mix is taken and re-amplified 20-30x in a new reaction mix rather than extending the run to more cycles: in some cases where template concentration is limiting, this can give good product where extension of cycling to 40x or more does not.

Primers

A primer is a short segment of nucleotides, which is complementary to a section of the DNA, which is to be amplified in the PCR reaction. Primers are annealed to the denatured DNA template to provide an initiation site for the elongation of the new DNA molecule. Primers can either be specific to a particular DNA nucleotide sequence or they can be "universal." Universal primers are complementary to nucleotide sequences, which are very common in a particular set of DNA molecules. Thus, they are able to bind to a wide variety of DNA templates.

Sometimes primer units are listed in optical density reading (OD). If this is a problem you will need to convert to molarity using the following equations: Change optical density reading of primer to molarity (µM units)-

1. N = No. of primer bases
2. σ 260 =~ 10,000 X N/ m X cm
3. Molecular weight =~ 330 X N
4. OD_{260} / σ 260 X 10^6 = Concentration (µM)

For example- primer is 20 bases long/ OD_{260} = 10.

1. N = 20
2. σ 260 =~ 10,000 X 20/m X cm = 20,000/m X cm
3. Molecular weight =~ 330 X 20 = 6,600
4. 10 OD_{260}/20,000 $m^{-1}cm^{-1}$ X 10^6 = 50µM

Agarose gel electrophoresis of DNA

Agarose gel electrophoresis is a powerful and versatile tool in the investigation and characterisation of DNA molecules. It is rapid, precise and relatively inexpensive and requires only small amount of DNA. Agarose is a polysaccharide extracted from various red algae. It is a polymer of repeating disaccharide units composed of β-D-galactopyranose and 3, 6 anhydro-L-galactose joined in a 1-3- β-glycosidic linkage.

Interchain hydrogen bonds are presumed to form the cross-links that lead to polymerisation. When DNA is subjected to agarose gel electrophoresis, it is forced to migrate through the interstices of this network toward the anode (due to its negatively charged phosphate residues) with a migration velocity determined by the molecular size of DNA, conformation of DNA, pore size of the gel (determined by the agarose concentration), voltage gradient applied to the gel and the electrophoretic buffer (Priefer, 1984).

Ethidium bromide is used in the detection of the DNA bands on the gel. This dye intercalates between the bases of the DNA molecule and gives DNA an orange fluorescence when irradiated with a short wavelength UV light source. Ethidium bromide slows the migration of DNA by about 15 percent (Jeyaseelan, 1987).

Appendix III: Map of Sampling Sites Produced from GPS Data