MOLECULAR ANALYSIS OF MECHANISMS AND IDENTIFICATION OF FACTORS OF PYRETHROID RESISTANCE IN *ANOPHELES GAMBIAE SENSU LATO* IN SOUTHWESTERN NIGERIA AND SOUTHERN BENIN REPUBLIC

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ABSTRACT

The development of resistance to insecticides by *Anopheles* mosquitoes continues to threaten the success of malaria control programmes in West Africa. Local data on mechanisms and factors causing resistance in the region are scanty. This study was designed to investigate the environmental factors and mechanisms implicated in resistance to pyrethroids by *Anopheles gambiae* in southwestern Nigeria and southern Benin Republic.

Larvae of *Anopheles* mosquito were collected in 2007 from 19 localities in the six states of southwestern Nigeria and 18 localities in the seven divisions of southern Benin and reared to adults. These were identified morphologically and with Polymerase Chain Reactions (PCR). They were also bioassayed for susceptibility to pyrethroids. Molecular characterisation of pyrethroid resistant phenotypes was carried out using PCR and microarray analyses of the expressed genes. Dissolved Oxygen (DO) and pH were determined using a digital multipurpose meter while physical appearances of breeding sites were assessed visually. Xenobiotic factors such as Spilled Engine Oil (SEO) and agricultural pesticides that might contribute to the emergence of resistance in *Anopheles* populations were examined through bioassay. Associations between pyrethroid resistance with environmental factors and molecular profiles of *Anopheles* were evaluated using Chi square.

A. gambiae complex genotyped in Nigeria comprised of 73.6 % *A. arabiensis* and 26.3 % *A. gambiae sensu stricto*; while those genotyped in Benin were 92.9 % *A. gambiae s.s.* and 7.0 % *A. melas*. Pyrethroid resistance in Nigeria and Benin were recorded in 68.4 % and 94.4 % of the localities examined respectively. Breeding sites contaminated with SEO (B-SEO) or Pesticide Residues (B-PR) had low DO (B-SEO = 13.4 ± 1.5 mg/l, B-PR=12.2 ± 1.7 mg/l), the Non-contaminated Breeding sites (B-NC) had higher levels of DO (B-NC=33.1 ± 2.3) and mainly produced pyrethroid-susceptible *Anopheles* ($p<0.05$). Significant variations in pH were not recorded. Differences in habitation by resistant-*Anopheles* in breeding sites contaminated with SEO or pesticide residues were observed. *A. gambiae* found around the two agricultural sites (Houeyiho, Benin and Ajibode, Nigeria) exposed to synthetic pesticides showed significant levels of pyrethroid resistance with mortality rates of 70.0 % and 89.7% respectively. *A. gambiae* larvae survived at

SEO concentrations below 11.8x10-3 µL/cm^2. Ninety six percent of larval mortality resulted from direct cuticle contact with SEO whereas only four percentage mortality was from larval suffocation. A cross resistance phenomenon was recorded with SEO and pyrethroids. *A. gambiae* showed the presence of elevated frequencies of knock down resistance West (*kdr*-W) mutations in Benin samples (*kdr*-W ranged from 0.6 to 0.9) and absence of *kdr*-W in Nigeria samples. Two detoxification genes (*CYP6P3* and *CYP6M2*) were up-regulated in resistant-*Anopheles*. Additional detoxification genes specific to agricultural and SEO sites were also over-expressed in the resistant populations.

There was an association between residual synthetic pesticides, spilled engine oil and emergence of pyrethroid-resistance in *A. gambiae* in Nigeria and Benin Republic. The diversified profile of identified metabolic genes reflected the influence of a range of xenobiotics on selection of resistance in mosquitoes.

Keywords: *Anopheles*, Pyrethroid resistance, Xenobiotics.

Word count: 487

DEDICATION

This project is dedicated to GOD almighty, who has being my source of inspiration and sustenance. To my home institution, colleagues, family members and friends for their constant support in the course of this programme.

CERTIFICATION

This is to certify that this work was carried out by MR. Jean Rousseau DJOUAKA-FOLEFACK in Cell Biology and Genetics Unit of Department of Zoology, University of Ibadan, Ibadan, Nigeria.

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CHAPTER ONE

INTRODUCTION

Malaria is the most important parasitic disease in the world (Holt *et al.*, 2002). It is thought to be responsible for 500 million cases of illness and up to 2.7 million deaths annually, more than 90% of which occur in sub-Saharan Africa (Holt *et al.,* 2002). Malaria morbidity and mortality in tropical Africa remain disproportionately high (Figure 1.1), compare to other malaria-endemic areas of the World (Gallup *et al.*, 2001). This is partly due to three efficient vectors of the subgenus Cellia: *A. gambiae*, *A. arabiensis*, and *A. funestus*. These species occur together geographically across sub-Saharan Africa and can inhabit the same villages, shelter in the same houses, and feed on the same individuals (Sharakhov *et al.*, 2002).

Till very recent, the focus of malaria control programmes in Africa was on the management of sick children through early treatment with effective antimalarial drugs (Sirima *et al.,* 2003). However, this cannot be the final strategy considering the life cycle of the parasite and the various hosts this parasite passes through (Miller *et al.*, 2002). With the fast spread of resistance of *Plasmodium species* to easily available drugs coupled with the increasing price of effective anti-malaria treatments and the absence of vaccines, National malaria control programs (NMCP) are becoming increasingly reliant on strategies targeting the mosquito vectors (Toure *et al.*, 2001).

The control of malaria vectors aim at reducing insect vector densities, their longevities or limiting their contacts with humans and therefore reducing diseases transmission, morbidity and mortality (Toure *et al.,* 2001). This can be done through various methods including: chemical (using insecticides based vector control tools such as LLINs and larviciding), physical (destruction of breeding sites and environmental sanitation), or with biological (using predators or other natural parasites of the vector) (Lawler and Lanzaro, 2005) methods. Considering the distribution of malaria disease in sub-Saharan Africa (Snow *et al.,* 1999) and taking into consideration the bio-ecology of vectors (Depinay *et al.,* 2004), *Anopheles* control strategies mainly used are based on synthetic insecticides (adulticides and larvicides) with focus on indoor residual spraying (IRS), insecticide treated nets (ITNs) of long lasting impregnated nets (LLINs) type and breeding sites treatment. ITNs constitute effective protective barriers for controlling

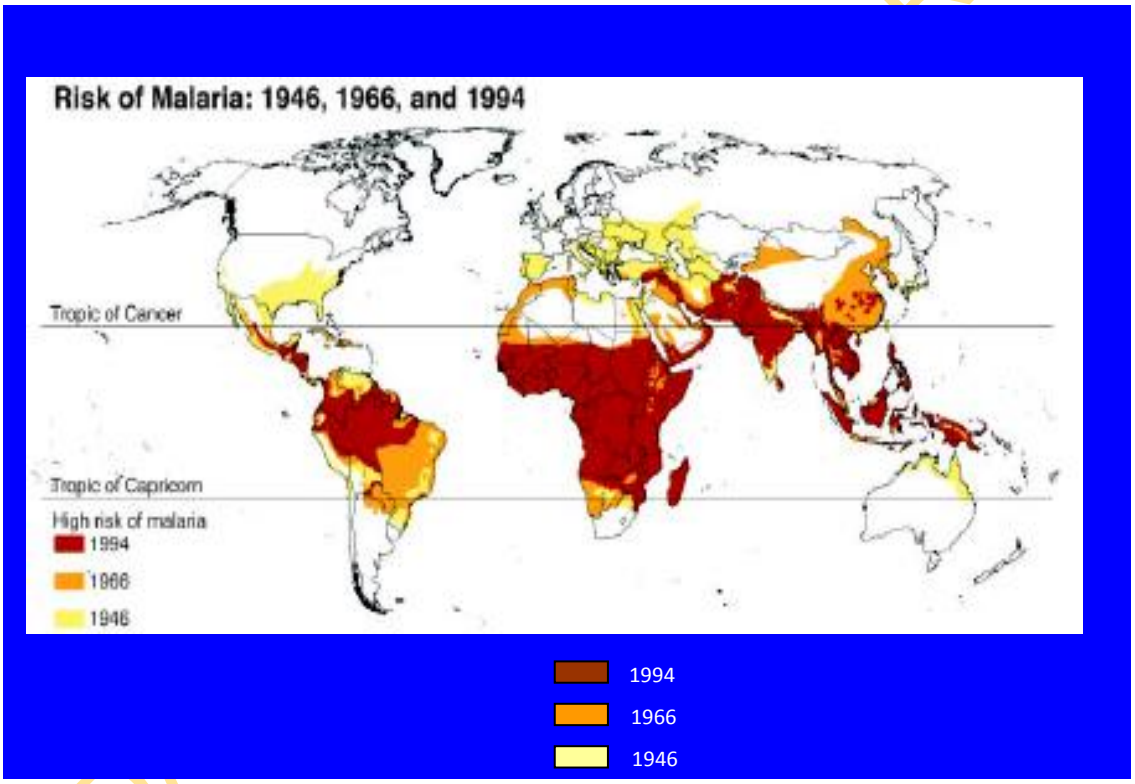

Fig.1.1 Trends of malaria in the world

Source: Gallup *et al. (*2001)

malaria vectors and have been found to significantly reduce malaria transmission rate (Carnevale et *al.*, 1988). IRS have shown effectiveness in localities under malaria endemic conditions; this strategy significantly reduces the densities of malaria vectors, rapidly stops the transmission of the disease by destroying adult female *Anopheles* perpetuating the transmission in endemic areas (Brooke *et al.*, 2000). Breeding site treatments destroy mosquito at the larval stage and thereby, reduce the overall densities of *Anopheles* in the communities. Unfortunately the emergence of mosquito populations capable of withstanding insecticide exposure is threatening these control measures (Hemingway and Ranson, 2000).

The emergence of new populations of *Anopheles* that are resistant to the type of insecticides used in public health is increasingly growing (Akogbeto *et al.*, 1999; Brooke *et al.*, 2001; Awolola *et al.*, 2002). This resistance phenomenon affects mainly the major vectors of malaria: *A. gambiae s.l.* and *A. funestus s.l.* The resistance of *A. gambiae* to pyrethroids is now well established in West, Central, East and South Africa. In South Africa, resistance to pyrethroids has been detected in *A. funestus* (Coetzee *et al.*, 1999). In West Africa, *A. gambiae* resistance to the four major classes of insecticides available for public health has been reported (Elissa *et al.*, 1993; Akogbeto *et al.*, 1999; Chandre *et al.*, 1999; Awolola *et al.*, 2002; Diabate *et al.*, 2002; Fanello *et al.*, 2003). Pyrethroids are the only insecticide licensed for impregnation of nets and house spraying, hence resistance to this class of insecticides is of concern, particularly as there has been a substantial increase (>60% coverage) in the number of people using bednets in Africa (WHO Report, 2005).

Insecticide resistance can occur via target site insensitivity and/or metabolic detoxification. Target site resistance to pyrethroids and DDT in *A. gambiae* is due to a substitution at a single codon in the sodium channel gene, and is referred to as knock-down resistance (*kdr*). Two *kdr* alleles occur in *A. gambiae;* a leucine to phenylalanine substitution, known as West *kdr* (Martinez-Torres *et al.*, 1998) and a leucine to serine substitution known as East *kdr* (Ranson *et al.*, 2000). Metabolic resistance is predominantly caused by elevated activities of one or more members of three large multigene enzyme families; cytochrome P450 monooxygenase (P450s), glutathione S-transferases (GSTs) and carboxylesterases (COEs). In *A. gambiae* these gene families have 111, 31 and 51 members respectively (Ranson *et al.*, 2002).

The emergence of resistance to pyrethroids in a number of African mosquito populations and its potential implications on the efficacy of current vector control tools highlights the importance of research into the effect of resistance on malaria transmission including the underlying mechanisms causing the resistance (Hemingway and Ranson, 2000). Long lasting impregnated nets used in most countries for malaria vector control are treated with pyrethroid insecticides hence, the emergence of pyrethroid resistance in *Anopheles* species is likely to threaten the efficacy this major malaria preventive tool . Results of studies from various regions of Africa suggest the need for close monitoring of resistance, the identification of factors selecting for resistance and the analysis of mechanisms of resistance using advanced and highly sensitive molecular tools.

Several human practices have been identified as factors implicated in the emergence of insecticide resistance in malaria vectors; misuse and overuse of agricultural pesticides by farmers have probably led to widespread insecticide resistance in mosquito vector populations (Akogbeto *et al.*, 2006; Chouaibou *et al.*, 2008). Molecular tools currently available have been helpful in identifying few mechanisms and pathways responsible for resistance (Martinez Torres *et al.*, 1998; Ranson *et al.*, 2000). However, these tools need to be more sensitive for further investigations on the various mechanisms of insecticide resistance.

The type of responses and the mechanisms of resistance developed by mosquito populations when subjected to selection pressures from human activities or from environmental changes have always been highly complex and difficult to predict. These adaptive mechanisms coupled with series of responses to environmental stimulis need to be further analysed using *Anopheles* samples from various sites subjected to a wide range of stimulis. The nature of mosquito habitats and the presence of different xenobiotics in their breeding sites could have a profound influence on mosquito development and the selection of different resistance mechanisms in malaria vectors at larval stage and adult stages.

1.1 Aim of this study.

This study aims at identifying factors responsible for the emergence of insecticide resistance and to analyse the mechanisms of pyrethroid resistance developed by *A. gambiae* in the southwestern Nigeria and southern Benin Republic.

1.2 Objectives of this study

1- Identify factors contributing to the emergence of pyrethroid resistance in *A. gambiae* species from southwestern Nigeria and southern Benin Republic.

2- Analyse the implication of target site mutation (*kdr*) in the selection for resistance in *A. gambiae sl.* in the southwestern Nigeria and southern Benin Republic

3- Analyse the implication of metabolic genes (Detox-genes) in the selection for resistance in *A. gambiae sl.*in the southwestern Nigeria and southern Benin Republic.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria disease and transmission of malaria parasites

Malaria remains a major killer, particularly in sub-Saharan Africa, with more than 1 million deaths among children every year. The reported case of malaria around the world is put at 243 million, with an estimated death of 863,000 (WHO, 2009). Eighty-nine percent of these deaths occurred in Africa (World Malaria Report, 2009). As at 2008, half of the people around the world are reported to be at risk of malaria. Out of the 109 countries where malaria is reported to be endemic, 45 are within the WHO Africa region. Nigeria accounted for one fourth of all estimated malaria cases in the WHO African region in 2006 (World Malaria Report, 2009).

In Nigeria, malaria accounts for much of the disease burden with about 97% of the approximately 150 million people at risk. It accounts for 25% of all infant related mortality, 30% of child related mortality and 11% of maternal mortality (WHO Statistics, 2007; World Malaria Report, 2009). A large percentage of the population affected with this disease live in extreme poverty in rural areas with few having access to good healthcare facilities (Otubanjo and Mafe, 2002; Amexo *et al.*, 2004; Obrist *et al.*, 2007).

The human malaria parasite in Nigeria includes: *Plasmodium falciparum, Plasmodium ovale* and *Plasmodium malariae*. However, *P. falciparum* is responsible for more than 95% of all malaria cases transmitted. Malaria parasites are usually transmitted through the bites of infected female mosquitoes of the genus *Anopheles*. This species is widely distributed across the different ecological zones in Nigeria where suitable sub Saharan climatic conditions exist (Molineaux and Gramiccia, 1980; Kiszewski, 2004).

Studies on malaria transmission conducted in Northern Nigeria have identified eleven species of *Anopheles mosquitoes*: *A. gambiae sensu stricto, A. arabiensis, A. funestus, A. rufipes, A. pharoensis, A. wellcomei, A. squamosus, A. coustani, A. maculipalpis, A. nili and A. pretoriensis* of which 2 species*; A. gambiae* and *A. funestus* were regarded as main vectors (Bruce – Chwatt, 1951; Hanney, 1960; Service 1965; Boreham *et al.*, 1979; Molineaux and Grammicia 1980; Rishikesh *et al.*, 1985).

Most of the studies in Southern Nigeria have focused on the bionomics and the characterization of breeding sites of malaria vectors such as *A. gambiae s.s., A. arabiensis,*

A. melas, A. funestus, A. nili, A. pharoensis, A. coustani and A. moucheti (Barber and Olinger, 1931; Muirhead-Thomson, 1947; Mattingly, 1949; Service, 1961; Nwoke *et al.*, 1993). Reviewed studies on malaria vectors characterisation in Nigeria relied mainly on the use of morphological keys of identification (Okorie, 1973; Mafiana *et al.*,1998; Aigbodion and Odiachi 2003). However, the advent of molecular and immuno diagnostic tools such as PCR, RT-PCR, micro-array and enzyme link imunosorbant assay (ELISA) have alleviated the difficulties associated with identifying morphological indistinguishable members belonging to species complexes and the incrimination of *Anopheles* species that are involved in malaria transmission (Service, 1993). Despite the development of molecular techniques, only few studies have utilized them in malaria vector research in Nigeria (Awolola *et al.*, 2003; Onyabe and Conn, 2003; Okwa *et al.*, 2007; Oyewole *et al.*, 2007).

The life cycle of malaria parasites is complex and passes through two hosts: Human, and *Anopheles species*. In humans the cycle involves trophozoites and merozoites production followed by differentiation into gametocytes; in the invertebrate hosts, the parasites are ingested by mosquitoes at the gametocytes stage during blood feeding. These gametocytes ex-flagellate and mate to produce zygotes. From the zygotes develops the ookinetes, the oocystes and finally the sporozoite stage. At this last step, the parasites migrate massively to the salivary gland of *Anopheles* where they are ready for inoculation during another blood feeding of the mosquito (Fig. 2.1) (Bruce *et al.*, 1980).

Malaria control is based on prompt diagnosis, appropriate drug treatment, protection of high-risk groups, and control of the mosquito vector. Optimism that mosquito-borne diseases such as malaria, dengue, and filariasis can be effectively controlled or even eradicated with inexpensive drugs, vaccines, or insecticides has been sorely tested (WHO, 1961) . Today, the efficacy of drugs is becoming debatable, vaccine development is slow, and mosquitoes are becoming resistant to insecticides, including those used to treat bed nets (Scoot *et al.*, 2002).

Current malaria control initiatives encourage the use of insecticide treated nets/long lasting impregnated nets (ITN's/LLINs), indoor residual spraying (IRS) and larviciding in an integrated vector management (IVM) strategy (Beier *et al.*, 2008). Pyrethroids treated bednets, offer personal protection from mosquito bites and have been

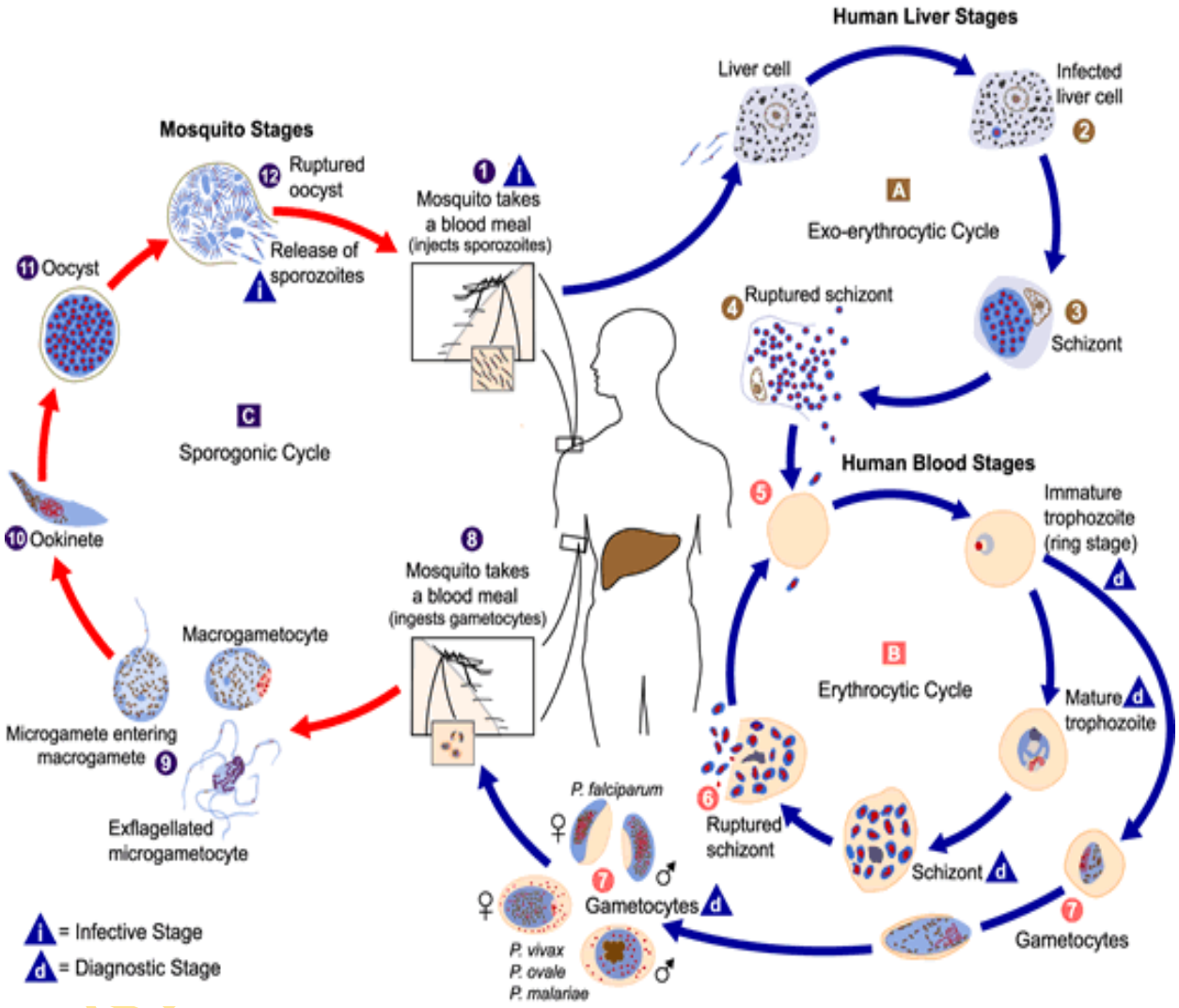

Fig. 2.1 The life Cycle of malaria parasite

Source: NIH /CDC (2004)

found to significantly reduce malaria transmission (Trigg and Wernsdorfer, 1999; Maxwell, 2002). Despite the encouraging results recorded from Insecticide Treated Nets (ITNs), resistance of the major vector to pyrethroid insecticides have emerged and is now widespread in West Africa (Chandre *et al.*, 1999; Hemingway and Ranson, 2000; Nwane *et al.*, 2009). Insecticide resistance is reported to threaten most malaria control intervention tools; for example in Benin, reduced efficacy of ITN and IRS has been associated with insecticide resistance (Nguessan *et al.*, 2007).

2.2 The biology and the ecology of malaria vectors

Like all mosquitoes, *Anophelines* go through four stages in their life cycle: egg, larva, pupa, and adult (Fig.2.2). The first three stages are aquatic and last 5-14 days, depending on the species and the ambient temperature. The adult stage is when the female *Anopheles* mosquito acts as malaria vector. The adult females can live up to a month (or more in captivity) but most probably do not `live more than 2-3 weeks in nature (Bruce Chwatt, 1993).

2.2.1 Eggs and larvae

Adult females lay 50-200 eggs per oviposition. Eggs are laid singly and directly on water and are unique in having floats on either side. Eggs are not resistant to drying and hatch within 2-3 days (Fig.2.2) (Bruce Chwatt, 1993).

Mosquito larvae have a well-developed head with mouth spikes (brushes) used for feeding, a large thorax and a segmented abdomen. *Anopheles* larvae lack a respiratory siphon and for this reason position themselves so that their body is parallel to the surface of the water. Larvae breathe through spiracles located on the 8th abdominal segment and therefore must come to the surface frequently. The larvae spend most of their time feeding on algae, bacteria, and other microorganisms in the surface microlayer. They dive below the surface only when disturbed. Larvae swim either by jerky movements of the entire body or through propulsion with the mouth brushes.

Larvae develop through 4 stages, or instars, after which they metamorphose into pupae. At the end of each instar, the larvae moult, shedding their exoskeleton, or skin, to allow for further growth. The larvae occur in a wide range of habitats but most species prefer clean, unpolluted water (Fig.2.2) (Bruce Chwatt, 1993).

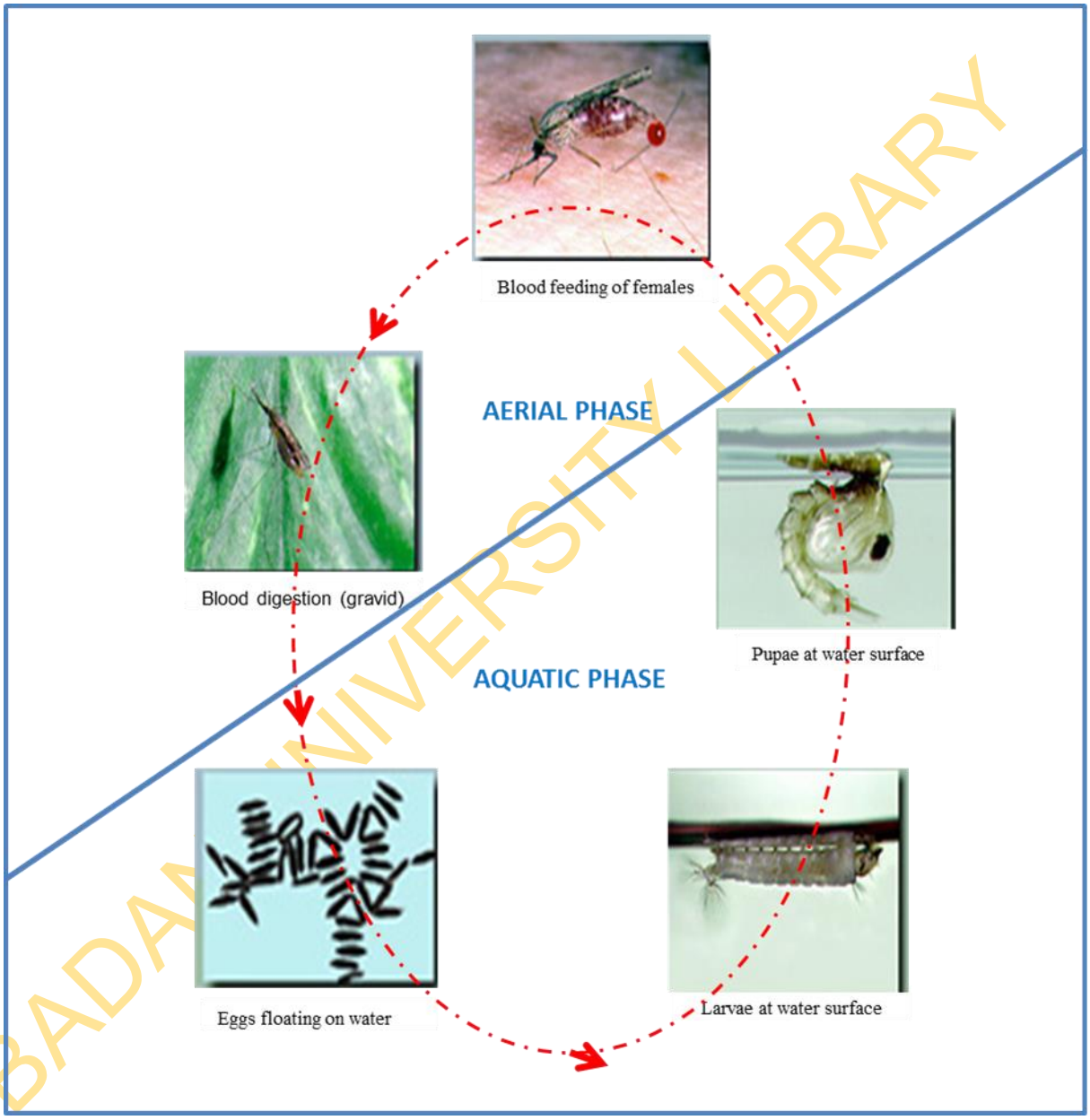

Fig. 2.2 The biological Cycle of *Anopheles species*

Source: Modified after CDC pictures, NIH /CDC (2004)

2.2.2 Pupae and adults

The pupa is comma shaped when viewed from the side. The head and thorax are merged into a cephalothorax with the abdomen curving around underneath. As with the larvae, pupae must come to the surface frequently to breathe, which they do through a pair of respiratory trumpets on the cephalothorax. After a few days as a pupa, the dorsal surface of the cephalothorax splits and the adult mosquito emerges (Fig.2.2) (Bruce Chwatt, 1993).

Adult *Anopheles* have slender bodies with 3 sections: head, thorax and abdomen. The head is specialized for acquiring sensory information and for feeding. The head contains the eyes and a pair of long, many-segmented antennae. The antennae are important for detecting host odours as well as odours of breeding sites where females lay eggs. The head also has an elongate, forward-projecting proboscis used for feeding, and two sensory palps. The thorax is specialized for locomotion. Three pairs of legs and a pair of wings are attached to the thorax. The abdomen is specialized for food digestion and egg development. This segmented body part expands considerably when a female takes a blood meal. The blood is digested over time serving as a source of protein for the production of eggs, which gradually fill the abdomen.

Anopheles mosquitoes can be distinguished from other mosquitoes by the palps, which are as long as the proboscis, and by the presence of discrete blocks of black and white scales on the wings. Adult *Anopheles* can also be identified by their typical resting position: males and females rest with their abdomens sticking up in the air rather than parallel to the surface on which they are resting. The duration from egg to adult varies considerably among species and is strongly influenced by ambient temperature. Mosquitoes can develop from egg to adult in as little as 5 days but usually take 10-14 days in tropical conditions (Fig.2.2) (Bruce Chwatt, 1993).

2.2.3 Mating and blood-feeding of adult *Anopheles*

Adult mosquitoes usually mate within a few days (2-3 days) after emerging from the pupal stage. In most species, the males form large swarms, usually around dusk, and the females fly into the swarms to mate. Males live for about a week, feeding on nectar and other sources of sugar. Females will also feed on sugar sources for energy but usually require a blood meal for the development of eggs. After obtaining a full blood meal, the

female will rest for a few days while the blood is digested and eggs are developed. This process depends on temperature but usually takes 2-3 days in tropical conditions (NIH /CDC, 2004). During blood digestion, female's abdomen undergo series of changes from unfed (tiny abdomen) to blood-fed (red abdomen) then semi-gravid (half red and half whitish) to gravid (whitish); then the female lays eggs and resumes host seeking. This marks the end of a gonotrophic cycle and the beginning of a new one. The cycle repeats itself until the female dies. Females can survive up to a month (or longer in captivity) but most probably do not live longer than 1-2 weeks in nature. Their chances of survival depend on temperature and humidity, but also their ability to successfully obtain a blood meal while avoiding host defences.

2.2.4 The ecology of breeding sites

Gravid *Anopheles* lay their eggs in different types of breeding sites depending on the species (Savage, 1990). Most *Anopheles* species prefer clean water and edges of streams, while others thrive in irrigation areas, rice fields, grassy ditches and reservoirs. Some species require extensive vegetative cover, for oviposition while some will prefer water bodies with dark or light bottomed pools. Others will prefer swamps and other permanent water bodies laden with dissolved organic matter (Mc Crae, 1983; 1984; Huang *et al*., 2005). Many of these sites develop into zones of transmission due to the concomitant increase of human populations moving to these areas. Ecological disturbance as a direct result of human activity may also increase the number of breeding sites. Road building and maintenance projects often impede drainage of runoff from rainfall. Clogged drainage ditches along roads left by logging and construction activities are ideal places for floodwater mosquitoes. Around the house, objects such as empty cans, discarded tyres, potted plants, and similar objects used as a result of human activities are often responsible for the collection of rainwater which allows mosquitoes to breed (Kitron, 1989, Tadei 1998).

2.3 Factors involved in malaria transmission

Knowledge of the biology and behaviour of *Anopheles* mosquitoes can help comprehend how malaria is transmitted and can aid in designing appropriate control strategies. Factors that affect a mosquito's ability to transmit malaria include its innate

susceptibility to *Plasmodium*, its host choice and its longevity (MacDonald, 1957; Bruce-Chwatt, 1993).

2.3.1 Preferred sources for blood meals

One important behavioural factor is the degree to which an *Anopheles* species prefers to feed on humans (anthropophily) or animals such as cattle or pigs (zoophily). Anthrophilic *Anopheles* are more likely to transmit the malaria parasites from one person to another. Most *Anopheles* mosquitoes are not exclusively anthropophilic or zoophilic. The primary malaria vectors in Africa, *A. gambiae* and *A. funestus*, are strongly anthropophilic and, consequently, are two of the most efficient malaria vectors in the world (MacDonald, 1957).

2.3.2 Life Span

Once ingested by a mosquito, malaria parasites must undergo development within the mosquito before they are infectious to humans. The time required for development of the parasite in the mosquito ranges from 10-21 days (extrinsic incubation period), depending on the parasite species and the temperature. If a mosquito does not survive longer than the extrinsic incubation period, then she will not be able to transmit any malaria parasites (MacDonald, 1957).

2.3.3 Patterns of feeding and resting

Anopheles mosquitoes are crepuscular (active at dusk or dawn) or nocturnal (active at night). Some *Anopheles* mosquitoes feed indoors (endophagic), while others feed outdoors (exophagic). After blood feeding, some *Anopheles* mosquitoes prefer to rest indoors (endophilic) while others prefer to rest outdoors (exophilic). Biting by nocturnal, endophagic *Anopheles* can be markedly reduced through the use of insecticide-treated bed nets (ITNs) or through improved housing construction (e.g. window and door screens) whereas, exophagic vectors are best controlled through breeding sites destruction. Endophagic *Anopheles* have an increase contact with humans and consequently are likely to be able to transmit more cases of malaria (MacDonald, 1957).

2.4 Major vectors of malaria in sub-Saharan Africa

There are several species of malaria vectors in Africa. Two of these species have been reported to be widely distributed and being able to efficiently transmit the malaria

parasite: *A. gambiae sl.* and the *A. funestus sl.* Both species belong to a complex comprising of morphologically indistinguishable species.

2.4.1 The *A. gambiae* complex

A. gambiae is the principal vector of malaria in tropical Africa. It has the capacity to colonise sunlit, temporary small water bodies that are scattered, around human dwellings (Minakawa, 1999; Gimnig, 2001). The *A. gambiae* complex was initially considered to be of a single species until much later when it was confirmed using molecular tools to be made up of six named species: *A. gambiae* (*sensu stricto*), *A. arabiensis*, *A. merus*, *A. melas*, *A. bwambe*, and *A. quadriannulatus* (Hunt *et al.*, 1998). All members of *A. gambiae* complex are morphologically identical but have few molecular differences. *A. gambiae ss.* and *A. arabiensis* are the most widespread of these groups with *A. arabiensis* broadly distributed in arid regions (Coetzee *et al.*, 2004). Both species occur in sympatry and are often found breeding in temporary stagnant water often associated with human activities (Coetzee *et al*., 2004). Another member of the group, *A. quadriannulatus* is known to have a restricted distribution which is limited to south-east Africa and Ethiopia (Fettene and Temu, 2003), *A. merus* and *A. melas* are salt water species, and their breeding is confined respectively to coastal regions of Africa (Moreno *et al*., 2004).

2.4.2 *A. funestus* group

Members of the *A. funestus* group are widespread throughout sub-Saharan Africa and Madagascar (Gillies and De Meillion, 1968, Mouchet *et al*., 1998). Species of this group include *A. funestus ss.*, *A. parensis*, *A. aruni*, *A. vaneedeni*, and *A. rivulorum.* Of these species, *A. rivulorum* has few morphological features which can be used for identification at adult stage (Gillies and Coetzee, 1987). The vectorial capacities within the group members vary significantly; for example, *A. funestus s.s* has been reported to have the highest vectorial capacity in the *funestus* group (Gillies and De Meillion, 1968). Other members of the *funestus* complex have very low vectorial capacities. An isolated conducted on malaria transmission in Tanzania reported the presence of *P. falciparum* sporozoite in the salivary glands of *A. rivulorum* (Wilkes *et al*., 1996). Working in the laboratory, an experimental transmission was obtained with *Anopheles vaneedeni* (De Meillon *et al*., 1977). Cohuet *et al*. (2003) described a new taxon closely related to *A.*

rivulorum, based on biological, morphological and genetic characters. The species, provisionally called '*A. rivulorum-like'*, is present in Burkina Faso (Hacket *et al*., 2000) and Cameroon, and is clearly different from the *A. rivulorum* of South Africa (Gillies and Coetzee, 1987). This new taxon does not seem to play any role in malaria transmission. While members of the *A. gambiae* complex are well characterised, the species composition of members of the *A. funestus* group however, is still being determined (Mouchet *et. al.*, 1993). In Nigeria and Benin Republic, data on *A. funestus* group are almost inexistent. Few unpublished reports exist on the description of this species and its implication on malaria transmission in both countries. The absence of extensive information on *A. funestus s.l.* probably results from the difficulties for colonising this species in the laboratory.

2.5 Vector Identification

In the epidemiology of any vector borne diseases, it is essential to identify and incriminate the responsible vector species. Identification of the vectors therefore becomes an important component of a vector borne disease control programme. Most arthropods of medical importance are readily identified through taxonomic keys with morphological characteristics that are species specific (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). However there is a limitation to the use of morphological characteristics in distinguishing related organisms sharing similar morphological features. There are a number of biological species sharing morphologically identical taxon but reproductively isolated. These populations are known as cryptic species, sibling species or isomorphic species such as members of *A. funestus* complex or *A. gambiae* complex (Hunt, 1998). Vector identification has helped to quantify the role of several cryptic species belonging to major group in disease transmission (Coetzee *et al.*, 2004). The occurrence of species complexes is often accompanied by different genetic variations presenting different behaviours and vectoral capacities. As a result, control managers lacking adequate techniques may spend scarce resources to control non-vector species (Coetzee, 2004). In addition, proper species identification allows appropriate decision making (Weeto, 2004). Several features were used for identifying *Anophele* species: (i) the horizontal position of larvae at the surface of water, (ii) the grey-darkish dots on wings and (iii) the triangular segmentation of the posterior part of the abodomin (Gillies and De Meillon, 1968).

2.5.1 Application of molecular techniques in the identification of sibling species

Different molecular techniques are available for species identification. These techniques include: DNA and RNA probes Restriction Fragment Length Polymorphism (RFLPs) described by Fanello *et al.*(2002) and Garros *et al.* (2004), Random amplified polymorphic DNA (RAPD – PCR), Single strand conformational polymorphism (SSCP) by Koekemoer *et al.* (1999). These techniques have been extensively used to characterize the major vectors of malaria. Other techniques used are crossing experiments, mitotic and meiotic karyotypes, polythene chromosomes, electrophoretic banding pattern and advanced molecular techniques such as the use of micro-array chips.

2.5.1.1 The Polymerase Chain Reaction Assay (PCR)

The use of PCR has created a revolution in diagnostic research by providing new ways of studying parasites, vectors and their hosts (Greenwood, 2002). The technique involves repeated amplification of small fragments of DNA present in a test sample. The detection of nucleotide changes involves the use of specific primers. This technique allows a rapid and efficient analysis of a large numbers of samples collected during field studies. The purpose of a PCR is to make huge copies of a gene. PCR has a major advantage because it utilises DNA which is relatively robust and can be transported from field and stored in the laboratory for long periods (up to 20 years) and with less storage requirements (Long *et al.*, 1995; Li *et al.*, 1997).

Using DNA and RNA based techniques; members of mosquito complexes can be distinguished from a well preserved fragment of the insect body. Several PCR protocols have been developed to distinguish members of the *A. gambiae* complex (Collins *et. al.*, 1987; Scott *et. al.*, 1993; Hill and Crompton, 1994), *A. funestus* group (Koekemoer *et al.*, 2002), and the sibling species or molecular forms of *A. gambiae s.s* (Favia *et al.*, 1997).There are three major steps in a PCR which are repeated for 25 or 40 cycles depending on the protocols. These are: denaturation for opening of DNA strands, annealing for specific binding of primers and extension for amplification of the products (Fig. 2.3). They are carried out in an automated thermocycler, which can heat and cool the tubes with the reaction mixture within the programed timing.

The denaturation temperature in cycling reaction for *A. gambiae* and *A. funestus* speciation is 94º C and 99ºC respectively; during this step, both strand of the DNA are

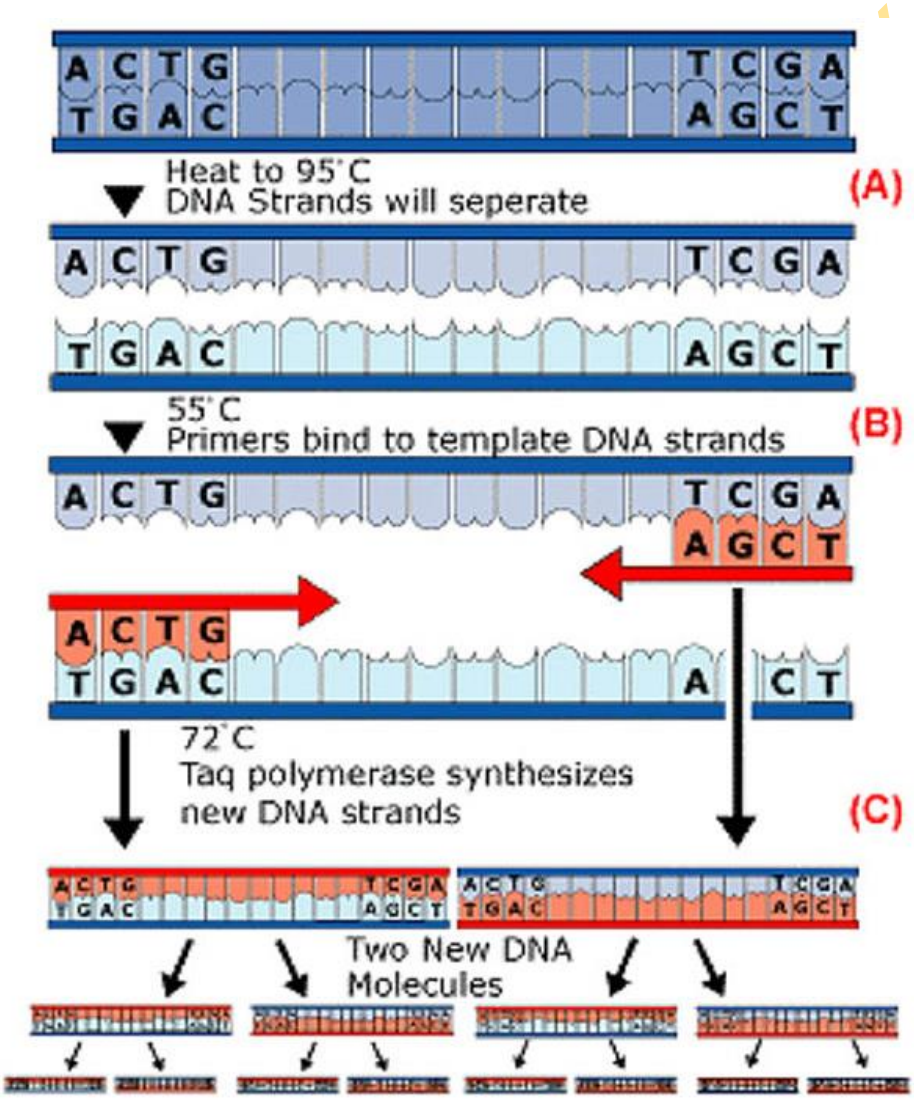

Fig. 2.3 The exponential amplification of the gene in Polymerase Chain Reaction (PCR); (A) represents the denaturation phase, (B) the annealing or hybridization and (C) the amplification or extension phase.

Source: Oceanexplorer (2011)

disrupted leading to two single stranded DNA. The annealing (hybridization) temperature in a cycling reaction for species specific PCR assay for *A. gambiae* and *A. funestus* is 50º C and 45ºC respectively. At this relatively low temperature, several reactions are possible.

The primers through the Brownian motion jiggle around in the reaction mixture for identifying the matching DNA sequence in the single stranded DNA template. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template until the matching sequence is identified. At the identification of the matching sequence, the primers fit properly and more stable bonds that last a little bit longer are formed between the single stranded DNA template and the primer. Once this initial hybridization is successful the polymerase enzyme can attach and starts copying the template (extension phase).

During the extension phase, the temperature is slightly increased from 45ºC to 72ºC. This is the ideal temperature for polymerase reactions. The primers having few bases built in, form stronger hydrogen bonds with templates. Primers that are not properly matched become loose (primer-dimers) and do not add to the extension of the fragment. The complementary bases (dNTPs) to the template are coupled to the primer by the polymerase enzyme which adds dNTP's from 5' to 3', reading the template from 3' to 5' side.

2.5.1.2 Restriction fragment length polymorphism

Detection of nucleotide changes by this method involves subsequent analysis of the PCR products by sequence-specific analytical methods such as digestion with restriction enzymes or hybridization with probes directed at specific nucleotides. This method is particularly employed in the identification of the 2 molecular forms of the *A. gambiae s.s*. Here, a digesting enzyme (HhaI) is added to the PCR reactions following the protocol described by Scott *et al.* (1993). Since the restriction site for the HhaI enzyme (GCGC) lies within the *A. gambiae* fragment amplified in the assay, it is therefore possible to differentiate between the two molecular forms (Fanello *et al.,* 2002).

The technique here described by Fanello *et al.,* (2002) has been well standardized and is gradually becoming a routine laboratory technique in several countries for characterising molecular forms of *A. gambiae ss* (*A. gambiae* "M" form and *A. gambiae* "S").

2.5.1.3 Cytogenetic techniques for *A. gambiae* speciation

As *A. gambiae* has tracked humans across temporally and spatially diverse habitats it appears to have been forced to undergo extensive ecological adaptation, which in turn is driving population divergence (Bradley, 2010). The first evidence for ecological adaptation of *A. gambiae* came via the examination of chromosomal inversions, which occur when a segment of a chromosome breaks off, flips 180 degrees, and becomes reinserted into same position (Hoffmann and Rieseberg, 2008). This event causes gene order within the inversion to be reversed relative to that of an ancestral or standard chromosome. Due to this rearrangement, when homologous chromosomes attempt to pair in a heterokaryotype (an individual heterozygous for an inverted and standard chromosome) a characteristic loop is created due to the inability of the two chromosomes to linearly align. In the ovarian nurse cells of semi-gravid *A. gambiae* females, giant polytene chromosomes are formed (Fig. 2.4). By viewing the characteristic banding pattern and/or loops of these chromosomes under a microscope, one can determine which inversions are present (della Torre, 1997). Coluzzi (1985) believed that genes within the inversions were responsible for speciation and, used inversion frequency data to name five non-Linnaean chromosomal forms – Bamako, Guinea-Bissua, Forest, Mopti, and Savanna, each of which he predicted to be in the early stages of speciation (Coluzzi *et al.*, 1985; Toure *et al.*, 1998; Powell *et al.*, 1999).

2.6 Malaria vector control strategies

Vector control aims to decrease contacts between humans and vectors of human diseases. Control of mosquitoes may prevent malaria as well as several other mosquito-borne diseases. Socio-economic improvements of households combined with vector reduction efforts and effective treatments have led to the elimination of malaria diseases in several North American countries (Schofield and White, 1984). Vector control strategies for controlling malaria are mainly focussed on: insecticide-treated bed nets, indoor residual spraying and larviciding (larval reduction).

2.6.1 Insecticide-treated bed nets

Insecticide-treated bed nets (ITNs) such as long lasting insecticide trateted nets (LLINs) are a form of personal protection that has repeatedly been shown to reduce severe disease and mortality due to malaria in endemic regions (Fig.2.5). ITNs have been shown

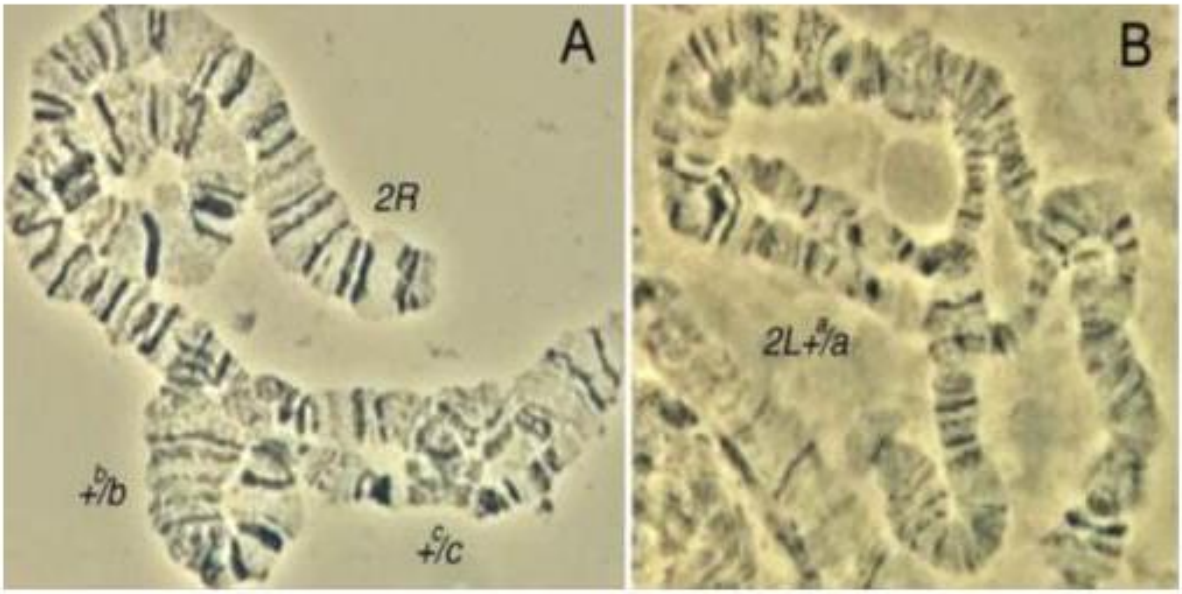

Fig. 2.4 Chromosomal inversions in *A. gambiae* females; this mosquito is heterokaryotypic for inversions 2Rb and 2Rc (A) as well as the 2La inversion (B)

Source: Sharakhov and Sharakhova (2008).

Fig. 2.5 The use of insecticide-treated bed nets (ITNs) for malaria vector control

Source: WHO (2003).

to reduce malaria mortalities by about 20% and morbidity to 50% (UNICEF, 2003). Untreated bed nets form a protective barrier around persons using them. The application of a residual insecticide greatly enhances the protective efficacy of bed nets. Besides their lethal activities, some insecticides have repellent properties that reduce the number of mosquitoes that enters the house and attempt to feed. There are several types of nets available in the market; these nets may vary by size, material, and/or treatment.

Most ITNs are made of polyester (permaNet®) but nets are also available in cotton, or polyethylene (Olysetnet®). Currently, only pyrethroid insecticides are approved for use on ITNs (WHO, 2006). These insecticides have very low mammalian toxicity but are highly toxic to insects and have a rapid knock-down effect, even at very low doses. Pyrethroids have a relatively high residual effect: they do not rapidly break down unless washed or exposed to sunlight. Previously, nets had to be retreated at intervals of 6-12 months and more frequently if the nets were washed. Nets are retreated by simply dipping them in a mixture of water and insecticide and allowing them to dry in a shady place. The need for frequent retreatment, the lack of understanding of the importance of bednets, and the additional cost for insecticides resulted in very low retreatment rates in most African countries and constituted the major barrier to full implementation of ITNs in endemic countries (Binka et *al.*, 1998). This condition has led to the development of long lasting insecticides treated nets (LLINs) (WHO, 2006). More recently, several companies have developed long-lasting insecticide-treated nets (LLINs) that retain lethal concentrations of insecticide for at least 3 years (WHO, 2006).

Although ITNs have shown their effectiveness for malaria control, some key strategic questions remain unanswered: should ITNs be sold or provided free of charge to the groups most at risk? Should ITNs be provided to the entire households or exclusively to children and pregnant women? (Maxwell et *al.*, 2006)

2.6.2 Bednets ownership patterns in Nigeria

In 2004, a survey conducted in 5 sentinel sites: Kano and Maiduguri in the North, Nsukka, in the East and Ibadan and Lagos in the southwestern Nigeria showed that the percentage of households owning a bednet in Nigerian was very low in 2000, but significantly increase from 0% in several localities in 2000 to more than 10% in 2004 (NetMark, 2004).

In 2004, Ibadan remained lowest at 10% and Maiduguri highest at 51%. Rural ownership has increased more quickly than urban ownership. In 2000, the percent of households that owned a net was 13% in urban areas and 11% in rural areas. In 2004, 23% of urban and 29% of rural households owned at least one net (NetMark, 2004). Ibadan had the lowest proportion of untreated nets (6%) and Nsukka the highest (20%). The data on baby nets were not available in 2000. In 2004 baby net ownership is relatively high: 40% of households owned a baby net with a built-in frame in the 5 sentinel sites (NetMark, 2004). Baby net ownership ranged from 15% in Kano to 52% in the city of Lagos. There was a small rise in the percent of children under five sleeping under a hanging net the prior night, from 8.8% in 2000 to 10.3% in 2004. The proportion ranged from a low of 3% in Ibadan site to a high of 17% in Maiduguri site. The total number of women of reproductive age in all households sampled was 2,528; of these, 703 were from net owning households. The total number of pregnant women in the households sampled was 249 and, of these, 76 were from net-owning households (NetMark, 2004). Recently, LLINs are massively being introduced in Nigeria through the Global Funds initiatives (USAID, 2010)

2.6.3 Bednets ownership patterns in Benin Republic

In Benin Republic, the national malaria control program (NMCP) places emphasis on the use of LLINs for the prevention of malaria among children under five and pregnant women. A survey conducted in 2006 by the Ministry of Health showed that more than half of all households (56%) owned at least one mosquito net of any type (treated or untreated nets). The coverage rate in rural areas was 50% and 66% in urban settings. Twenty five percent of net owners reported owning at least one ITN and only 20% of children under five and pregnant women said that they had slept under an ITN the previous night. Based upon these data, the NMCP is supporting a four-pronged approach to net distribution in Benin, which includes free distribution through health centers during ante natal care (ANC) and immunization clinic visits, distribution of highly-subsidized ITNs through community-based channels, free distribution through mass campaigns, and the sale of ITNs in the commercial market (USAID, 2010). At the health center level, ITNs are distributed through antenatal kits that include antimalarial drugs, and other drugs, at a cost of about $1 per kit. These kits are subsidized by USAID, UNICEF and the World Bank. In

the local market, prices of treated nets (LLINs) range from $7 to $12. At the hospital settings, these long lasting treated nets are subsidized by USAID at a cost price of $2 each (USAID, 2010).

2.6.4 Indoor residual spraying

Many endophilic malaria vectors are particularly susceptible to control through indoor residual spraying (IRS). This strategy involves coating the walls and other surfaces of a house with a residual insecticide. For several months, the residual insecticide will kill mosquitoes and other insects that come in contact with these surfaces (WHO, 2006). Contrary to ITNs, IRS does not directly prevent people from being bitten by mosquitoes. Rather, it kills mosquitoes after they have fed, if they come to rest on the sprayed surface. IRS thus prevents transmission of infection to other persons. IRS is an efficient approach to the control of malaria transmission as chances of killing an *Anopheles* mosquito is repeated every time the mosquito rests on the treated wall and before it reaches the age of transmitting mature sporozoites (Curtis *et al*., 2000). Historically the best vector control results have been achieved by IRS, e.g. the reduction of the incidence of malaria in India from about 75 million cases per year in the 1930s to about 110 000 in the 1960s (a reduction of 99.8%), the near eradication of previously holoendemic malaria from Zanzibar, in the 1960s, and the management of malaria catastrophe in South Africa in the late 90s (Brooke *et al*., 2000).

Results obtained so far with insecticide-treated nets (ITNs) have not matched these achievements. However, the use of ITNs requires less equipment and labour, and may be more feasible in many circumstances (Curtis *et al*., 1998; Kroeger *et al*., 2002). IRS with DDT and dieldrin was the primary malaria control method used during the global malaria eradication campaign from 1955 to 1969 (MacDonald, 1957). The campaign did not achieve its stated objective but it did eliminate malaria from several areas and sharply reduced the burden of malaria disease in others (MacDonald, 1957).

Resistance to DDT and dieldrin coupled with their negative impact on the environment resulted to the introduction of new insecticides which unfortunately were more expensive and could not sustainable in poor endemic countries. The negative publicity of DDT and dieldrin insecticides used in IRS campaigns accounted negatively for the up scaling of this malaria control strategy. However, the recent success of IRS in

reducing malaria cases in South Africa (more than 80% reduction of malaria prevalence) has revived interest in this malaria prevention tool and has also reignited the necessity of using DDT in some countries for malaria vector control (WHO, 2009).

2.6.5 Breeding sites reduction

Breeding sites reduction is the method of choice for mosquito control when the mosquito species targeted are concentrated in a small number of discrete habitats. The larval habitats may be destroyed by filling depressions that collect water, by draining swamps, or by ditching marshy areas to remove standing water. Container-breeding mosquitoes are particularly susceptible to source reduction as people can be educated to remove or cover standing water in cans, cups, and rain barrels around houses. Mosquitoes that breed in irrigation water can be controlled through careful water management. The use of larvicides in malaria control requires several prerequisites: the knowledge of laying behaviors of *Anopheles* in the locality, the survey, mapping and monitoring of breeding sites and finally the composition and chemical activity of the larvicide to be used. Larvicides could be biological or synthetic. Biological larvicides include *Bacillus thuriengiensis*, *Bacillus sphaericus*, larvivours species of fish etc.

The control of *A. culicifacies* larvae using larvivorous fish is reported to be working well in Karnatakastate, India (Yapabandara *et al*., 2002). However, it does not seem to be applicable to those *Anopheline* mosquitoes which typically breed in small puddles that frequently alternate between dryness and being re-filled with rainwater, and in situations where there are many such sites within mosquito-flight range of a village. This applies to many *A. gambiae* rural breeding sites (Curtis *et al*., 2003). Several trials to combat mosquito larvae with bacillus have been successfully conducted at low scale (Hougard *et al*., 1983; Becker *et al*., 1994; Hougard and Back, 2003). These bacteria release toxins which are ingested by larvae and have a cytotoxic activity in the midgut cells of insect larvae.

2.6.6 Less implemented vector control strategies

Other vector control strategies with less implementation in communities include: (i) fogging or outdoor spraying which is primarily reserved for emergency situations such as halting epidemics or rapidly reducing adult mosquito populations when they have become severe pests; (ii) the use of repellents such as DEET (Fradin and Day, 2002),

wearing light-coloured clothes, long pants and long-sleeved shirts, (NH-USA, 2009); (iii) the spray of petroleum products in mosquito breeding sites, (Burton, 1967; Thevagasayam et *al.*, 1979); (iv) the genetic modification of malaria vectors aims to develop mosquitoes that are refractory to the parasite. This approach is still several years from applications in the field (Lorena, 2003).

2.6.6.1 The development of genetically modified *Anopheles* (GMA) for malaria control

Curtis (1968) proposed the basic concept of genetic control of vector borne diseases since 1968, but major advances in the molecular manipulation of *Drosophila melanogaster* during the 1980s encouraged re-evaluation of this idea. The *Anopheles* genome sequence provides an architectural scaffold for mapping, identifying, selecting, and exploiting desirable insect vector genes. It also promotes understanding of mosquito biochemistry, physiology, and behavior as well as of malaria epidemiology, and spurs development of new public health interventions (Hemingway *et al*., 2002).

A strain of *A. stephensis* that is unable to transmit malaria in mice has already been engineered (Tu *et al*., 2001; Ito *et al*., 2002). The next big challenges are: driving of refractory genotypes in wild strains (Tu *et al*., 2001), studying the bio-ecology of engineered mosquitoes (Scott *et al*., 2002) and getting information on stability of engineered genes (Tu *et al*., 2001), understanding of oxidative stress which appears to be important in refractory strains to resist parasite infections and to drive refractory gene into wild populations of *Anopheles* (Hemingway *et al*., 2002), and getting communities fully involved in the process. The use of genetically modified insect vectors in the field will require careful consideration of bio-safety, ecological, ethical, legal, and social issues to ensure public acceptance (WHO, 1991).

2.7 The use of insecticides in public health

A great variety of insecticides are used in public health for malaria vectors control. These insecticides can be grouped under 6 main families: organochlorine, organophosphate, carbamates, pyrethroids, growth regulator and bacterial toxins. Currently, a total of 12 insecticides from these families are used in public health against mosquitoes at adult stage: 7 pyrethroids, 3 organophosphate compounds, 1 carbamate and the dichloro diphenyl trichloroethane (DDT) (WHOAfro, 2003).

2.7.1 Insecticides and mode of action

Insecticides usually act on the nervous system of insects and more specifically on the transfer of nervous impulses. The insecticidal activity results in either tetanisation (organochlorine and pyrethroids) or paralysis (organophosphates and carbamates) leading to death of mosquitoes. The nervous impulse moves through sodium channels to synapses where it is transmitted to the next axon through neuro transmitters (acetylcholine). After the transfer of nervous impulses, the synthesized acetylcholine is degraded and its activity stopped by acetylcholinesterase, an enzyme whose function is to degrade acetylcholine. Insecticides are capable of disturbing this transfer of impulse by either maintained opening of the sodium channel or acting on the chlorine channel or inhibiting activities of acetylcholinesterase (Fig. 2.6)

2.7.1.1 Organochlorine

This family is subdivided into 3 subgroups based on their chemical structure and mode of action. The main members of the family are: DDT and its analogues, lindane and cyclodiene. DDT was discovered in 1939 by Paul Muler in Switzerland and tested in 1942 as an anti-mosquito spray in army camps in the USA and UK. In 1944, DDT was tested for the first time as an IRS insecticide in civilian areas at Voluntoro, Italy. This insecticide was highly successful (Singh *et al.*, 1962). In 1950, DDT water dispersible powder containing 50 to 75 % technical grade DDT was made available and its remarkable convenience in application prompted it to be an ultimate choice in anti-malaria campaigns. Organochlorine efficacy in agriculture and public health generated a great interest of WHO and led to launching of malaria eradication programme in the 50s (Mouchet, 1994). DDT has a complex chemical structure (Fig. 2.7). Its activity is focused on peripheral and central nervous system of insects (Hassal, 1990); it has a rapid knock down effect on mosquito populations (Fig. 2.6). Despite these high performances, its bioaccumulation in the environment and the appearance of cases of resistance in some regions prompted WHO to stop using and even to ban it in many countries. Lindane and cyclodiene are subgroups in the family of organochlorine. A known member of this subfamily is Dieldrine. Their activities are focused on the central nervous system, where they inhibit chlorine channels, the main receptors of gamma-aminobutyrique acid (GABA) (Fig. 2.6). This set of insecticides was also banned because of their bioaccumulation, toxicity and the

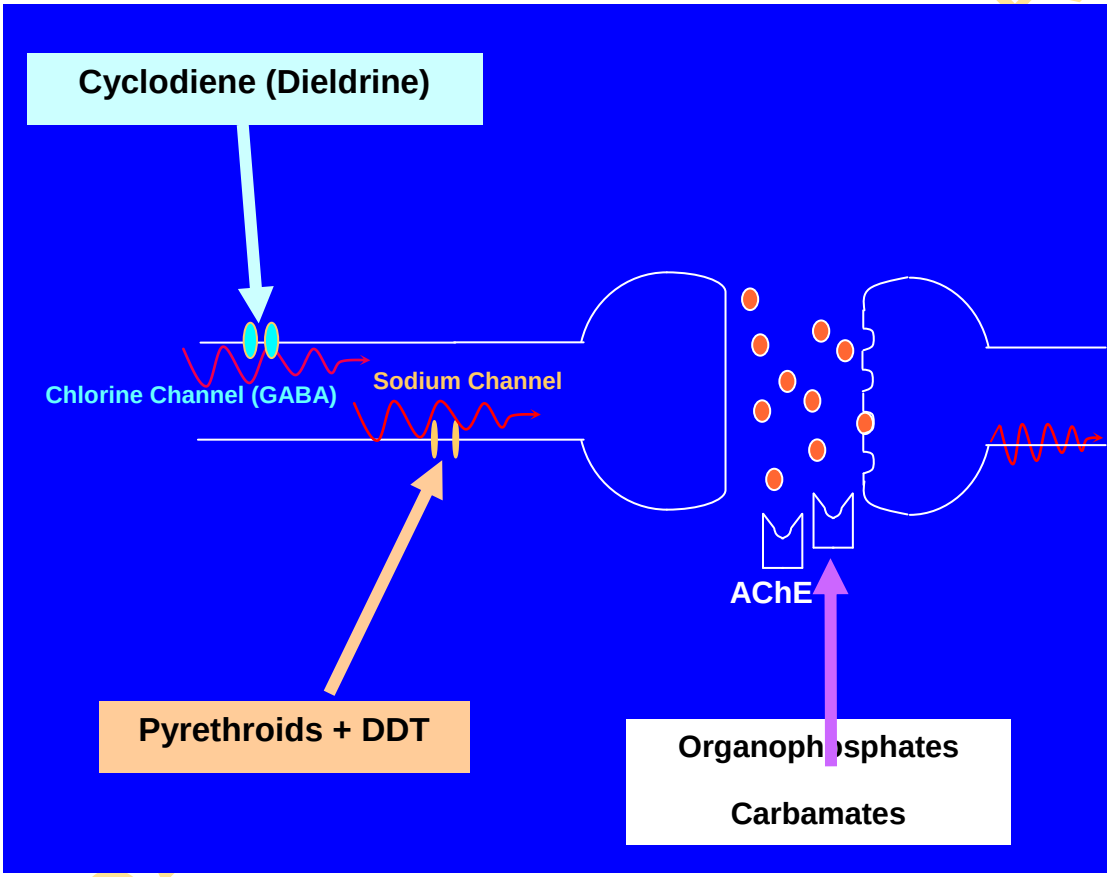

Fig. 2.6 The different targets of insecticides in *Anopheles* mosquitoes

Source: Modified after WHO picture, WHO (2006)

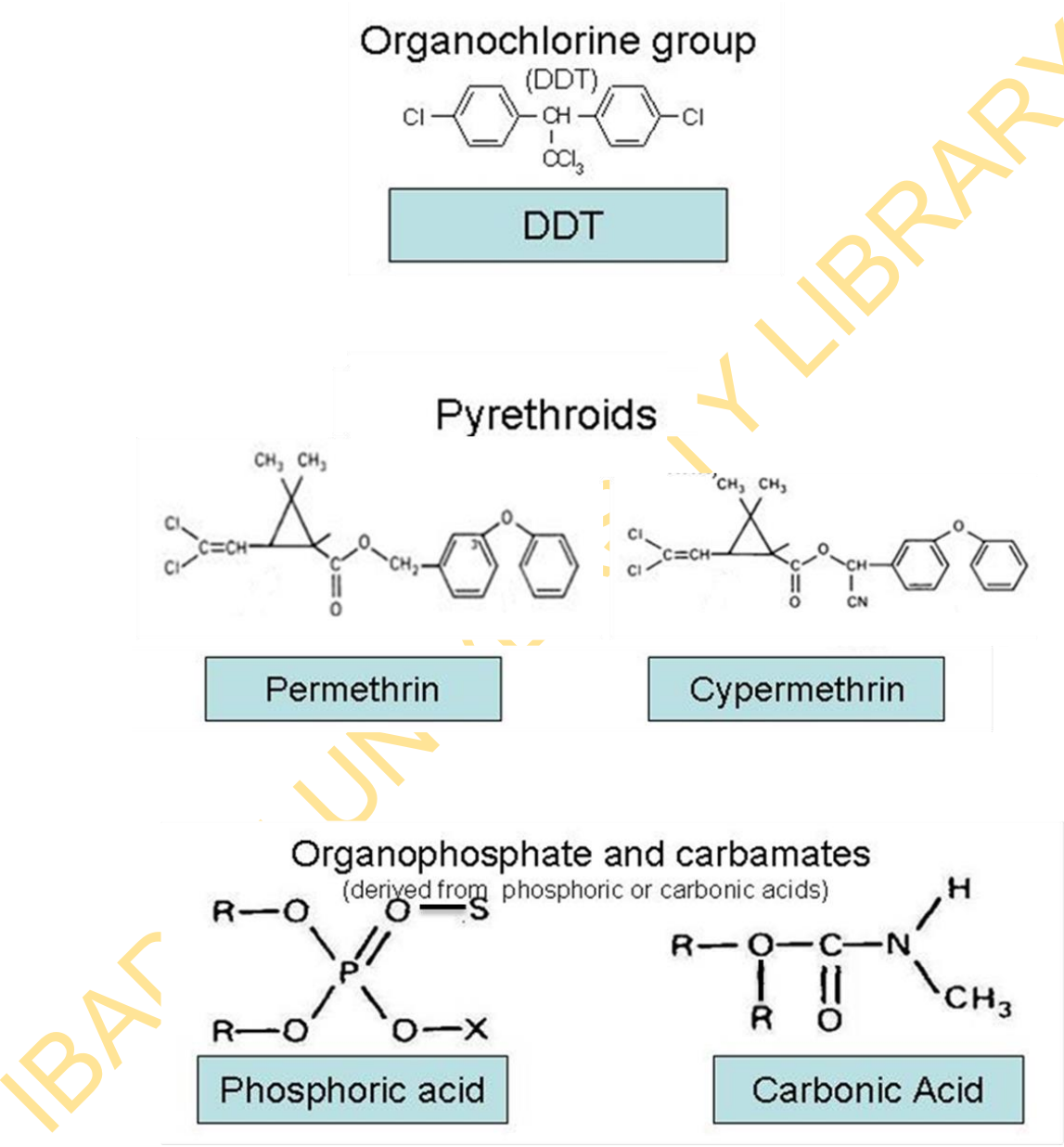

Fig. 2.7 Chemical structure of some insecticides used in public health

Source: Krieger (2001)

emergence of cases of resistance.

2.7.1.2 Organophosphate

These are derived from phosphoric acid, and replaced the organochlorine because of their relatively low toxicity. The members of this family of insecticides used in public health are: malathion and fenitrothion. When coupled with oxygen molecules, organophosphates are good inhibitors of acetylcholinesterase (Keith, 2005). This enzyme degrades activities of acetylcholine which neuromediates cholinergic synapses located in the central nervous system of insects. The fixation of organophosphates on acetylcholinesterase leads to accumulation of acetylcholine at the synaptic junction. When the level of acetylcholine becomes too high, the acetylcholine receptors are blocked. It is this blockage that leads to paralysis and death of insects (Figs. 2.6 and 2.7)

2.7.1.3 Carbamates

These are synthetically derived from eserine. They act like organophosphates by inhibiting activities of acetylcholinesterase (Keith, 2005).The family are made up of carbamate and bendiocarb. These insecticides are derived from carbonic acids. They are less used because of their cost and their toxicity on mammals (Figs. 2.6 and 2.7)

2.7.1.2.4 Pyrethroids

They are synthesized from pyrethrines which are natural extracts from C*hrysanthemum cinerariaefolium* flowers. First generations of pyrethroids were very volatile and therefore less persistent. With more studies conducted on these compounds, this instability was overcome and more stable molecules were developed (Elliot *et al.*, 1978). Pyrethroids are divided in to two groups based on their α radicals: group I: permethrin, group II: deltamethrin, lambda-cyhalothrin and cypermethrin. Pyrethroids act on sodium channel by keeping it opened and therefore accelerate the speed of nervous impulses (Figs. 2.6 and 2.7). The insect ends up dying by tetanisation.

Pyrethroids have a rapid knockdown effect coupled with high excito-repulsive action and are less toxic to mammals at operational doses (Darriet, 1984). These features explain why pyrethroids were quickly welcome and used for nets impregnation (WHO, 1984). However, the emergence of populations of insects capable to withstand lethal doses of this family of insecticides threatens the efficacy of current malaria control strategies.

2.7.2 Protective mechanisms of *Anopheles* and development of resistance to insecticides

All living organisms have natural mechanisms of protection against harmful compounds including insecticides. In *Anopheles*, 3 types of enzymatic activities are normally observed when they get in contact with insecticides: Esterase, oxydase and glutathion-s-transferase (Figs. 2.8 and 2.9). With esterase activities, ester bonds found on the insecticide are destroyed and the insecticide is converted into carboxylic acid and alcoholic compounds which can easily be metabolized by the insect. The enzyme acting during this conversion is known as carboxylesterase. Insecticides can be oxidized by oxidase enzymes to produce alcoholic compounds which can also be easily degraded by the mosquito. With glutathion S. transferase, insecticides activity is conjugated by a tripeptide known as glutathione and this conjugation leads to sequestration of insecticide and reduced activity on the target site (Chandre *et al.*, 1999). In area of high insecticides pressure and constant exposure of mosquitoes to insecticides, these protective phenomena are enhanced and, a proportion of insects in the population will gradually inherit a high capability of withstanding lethal doses of insecticides: this condition is known as resistance.

2.7.2.1 Resistance of *Anopheles* to insecticides

Resistance is defined as: the occurrence in a population of a group of individual capable of tolerating doses of chemicals which under normal condition could kill the majority of the population" (Hamon and Mouchet, 1961). Resistance in mosquito populations has a genetic backup and its spread is due to allelic selections from spontaneous mutations or migrations. Toxicity of an insecticide results from interaction between the insecticide and the biological set up of the mosquito. Various steps are necessary for this to take place: the insecticide must get in contact with the insect, enter the insect, be transformed into a metabolite and carried to the target site for expression. All these steps are governed by either one or several genes of which any structural or functional modification could lead to resistance (Soderlund and Bloomquist, 1990). These modifications can lead to changes in the behaviour of the insects like the capacity of the insects to identify and escape from areas under insecticide treatments (this is known as behavioural resistance), the ability of the insects to over-express detoxification genes for metabolising the insecticides also

1- Esterases:

$$R\text{-}COO\text{-}C_2H_5 + H_2O \longrightarrow R\text{-}COOH + C_2H_5\text{-}OH$$

2- Oxydases:

$$R\text{-}H + O_2 + 2H \longrightarrow R\text{-}OH + H_2O$$

3- Glutathion -S-transferases:

Tripetide conjugation and insecticides by glutathion

Fig.2.8 Chemical equations of enzymatic activities developed by mosquitoes to withstand insecticide lethal doses

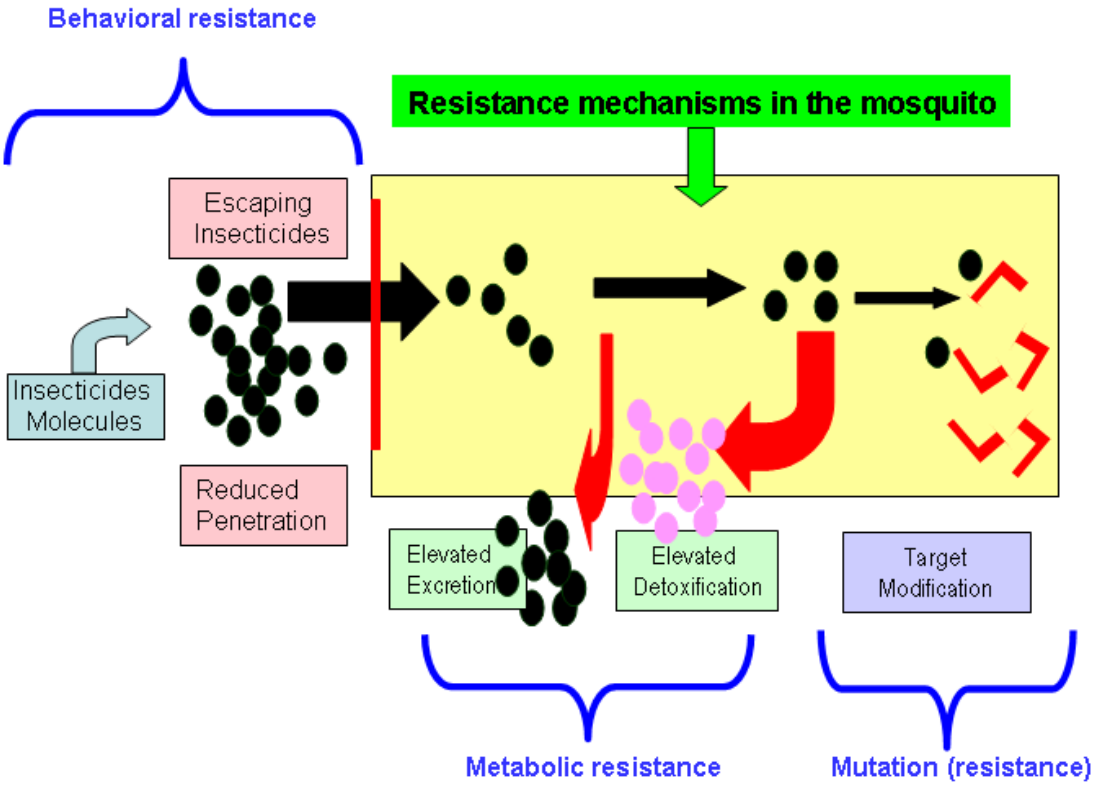

Fig.2.9 Series of mechanisms of resistance to insecticides developed by *Anopheles*

Source: Modified after WHO picture, WHO (2006)

referred as metabolic resistance and finally the modification of the insecticide target sites in the insects known as target site resistance (Fig. 2.9).

2.7.2.2 Behavioral mechanisms of resistance

Behavioral mechanisms are rarely observed in mosquito populations. The mechanism is based on the ability of insects to run away from areas which have been treated with insecticides, thereby avoiding contact with the insecticide. With the publishing of mosquito genome, investigations are currently focused on genes responsible for neurosensory perception, and chemical detection by the mosquito (Ranson *et al.,* 2002).

2.7.2.3 Metabolic mechanisms of resistance

Three families of proteins are largely responsible for metabolizing insecticides: the cytochrome-P450s (oxidase), carboxylesterases (esterase) and the glutathione-S-transferases (GST). A recent analysis of the *A. gambiae* genome identified 111 genes putatively encoding P450s, 51 genes encoding for carboxylesterases and 31 genes for glutathione transferases (Ranson *et al.,* 2002).

2.7.2.3.1 Cytochrome P450s (monoxygenase or oxidase mechanisms)

Cytochrome P450s exist in insects in very diverse families. Certain subfamilies of P450s have been implicated in the metabolism of insecticides (Feyereisen, 1999). Elevated P450s activities have been widely implicated in resistance to pyrethroids in many species, but the lack of sensitivity of biochemical assays designed to detect increases in P450s in individual insects and the paucity of knowledge on the role of individual P450 enzymes in insecticide metabolism have prevented an accurate assessment of this mechanism (Ranson *et al.,* 2003). Recently, elevated expression of a particular P450 gene has been associated with resistance to pyrethroids in *A. gambiae* from East Africa (Nikou *et al.,* 2003), but this preliminary finding needs further verification.

Esterase

The family of carboxylesterase proteins is extensive in insects. This includes enzymes like acetylcholinesterases which is found at the nervous synapse and responsible for degrading acetylcholine. Carboxylesterase proteins do not hydrolyse organophosphates but act by sequestration because of their high affinity with this family of insecticides (Cuany *et al.,* 1993). Insensitive acetylcholinesterase (*Ace-1*) has been reported in malaria

vectors from Sri Lanka (Karunaratne *et al.*, 1999). In West Africa, Djogbenou *et al.* (2008) identified and mapped the distribution of *Ace-1* in *A. gambiae* samples from Benin and Burkina Faso. Elevated frequencies of *Ace-1* mutation are associated with resistance to organophosphate and carbamate (Djogbenou et *al.*, 2008). Depending on esterases involved, resistance can be specific to a particular insecticide or can confer broad-spectrum resistance to a number of different insecticides (Oakeshott *et al.*, 1999).

2.7.2.3.2 Glutathione-S-transferase

This compound binds on insecticides and produces less toxic products. The most significant one is the DDT-ase which degrades DDT insecticide. Glutathione transferases have been implicated in resistance to DDT in several *Anopheles* populations (Prapanthadara *et al.*, 1993; 2000). Recently, a glutathione transferase responsible for resistance to DDT in *A. gambiae* has been elucidated (Ranson *et al.*, 2001). In other insects such as *Drosophila*, glutathione transferase has also been implicated in resistance to pyrethroids (Vontas *et al.*, 2001) and to organophosphates (Huang, 1998).

2.7.2.3.3 Target site modification (knock-down mutation)

Targets sites for insecticides are either receptors or enzymes of the nervous system like acetylcholinesterase, sodium channel and the gamma acetyl-buturic-acid (GABA) receptors. Structural modifications of these targets either reduce binding affinity or change the synthesis of enzymes leading to resistance. Target modifications are powerful mechanisms of resistance in the sense that they lead to cross resistance of all families of insecticides targeting the same pathway. It is associated to point or multiple mutations on nucleotide sequences. Mutations affecting sodium channels and GABA receptors have been identified in various species of mosquitoes (Coustau and French-Constant, 1995; Martinez-Torres *et al.*, 1998). Once insecticide resistance is developed, the genes can persist in the insect population for 30 years or more, but at low levels.

The knock down resistance *(kdr*) is a target site modification generated by a mutation in the voltage-gated sodium channel of the insect's nervous system. This target is similar for both DDT and pyrethroid insecticides. This resistance mechanism has evolved at least twice in *A. gambiae* (Matinez-Torres *et al.*, 1998; Ranson *et al.*, 2000) and is now present at very high levels in some regions of Africa (Akogbeto *et al.*, 1999; Chandre *et al.*, 1999). With *kdr* bearing *A. gambiae* collected from West Africa, the point mutation on

the sodium channel leads to different amino acids synthesis: leucine is replaced by phenyl alanine (Leu-Phe). In East Africa, the same *kdr* mutation leads to the replacement of leucine by serine (Leu-Ser) (Fig. 2.10). The *kdr* has also been detected in *A. sacharovi* (Luleyap *et al.,* 2002) and *A. stephensi* (Enayati *et al.,* 2003). Once identified, the mutation can be detected using polymerase chain reaction (PCR) techniques (Matinez-Torres *et al.,* 1998). The knock down resistance mechanism has evolved at least twice in *A. gambiae* (Matinez-Torres *et al.,* 1998; Ranson *et al.,* 2000) and is now present at very high levels in some regions of Africa (Akogbeto *et al.,* 1999; Chandre *et al.,* 1999).

2.8 Detection of insecticide resistance in malaria vectors

The detection of insecticide resistance in *Anopheles* populations is highly important for health policies and decision making on the type of vector control strategy to implement in a given locality. This detection provides information on the susceptibility to insecticides of mosquito populations and the potential mechanisms of insecticide resistance involved. Four tools are routinely used for basic detection of resistance in field *Anopheles* populations: the "WHO bioassays" in tubes with adult mosquitoes, the "bottle test" with synergists, bio-chemical assays to determine elevated enzyme activities related to resistance, and polymerase chain reaction (PCR) for detection of target sites modification in the mosquito.

2.8.1 Bio-assay for phenotyping insecticide resistance in vectors

In this bio-assay, the WHO test kits used for the purpose is composed of impregnated papers prepared using silicone oil (Dow Corning 556) with technical grade insecticides. Females of *Anopheles* are exposed to different impregnated papers for one hour and the mortality recorded after 24hours monitoring in the insectary (WHO, 1986). This assay segregates resistant and susceptible phenotypes and allows the characterisation of *Anopheles* populations as resistant when the mortality rate of exposed mosquitoes is lower than 95% or susceptible when higher than 95%.

The validation of results from this bio-assay depends immensely on the total number of exposed mosquitoes. Ideally mosquitoes should all be of the same age and the average number of exposed mosquitoes higher than 100 (WHO, 1986). The main difficulty in this diagnostic technique is getting enough *Anopheles* (minimum of 100) from the same locality all aged between 2-5 days.

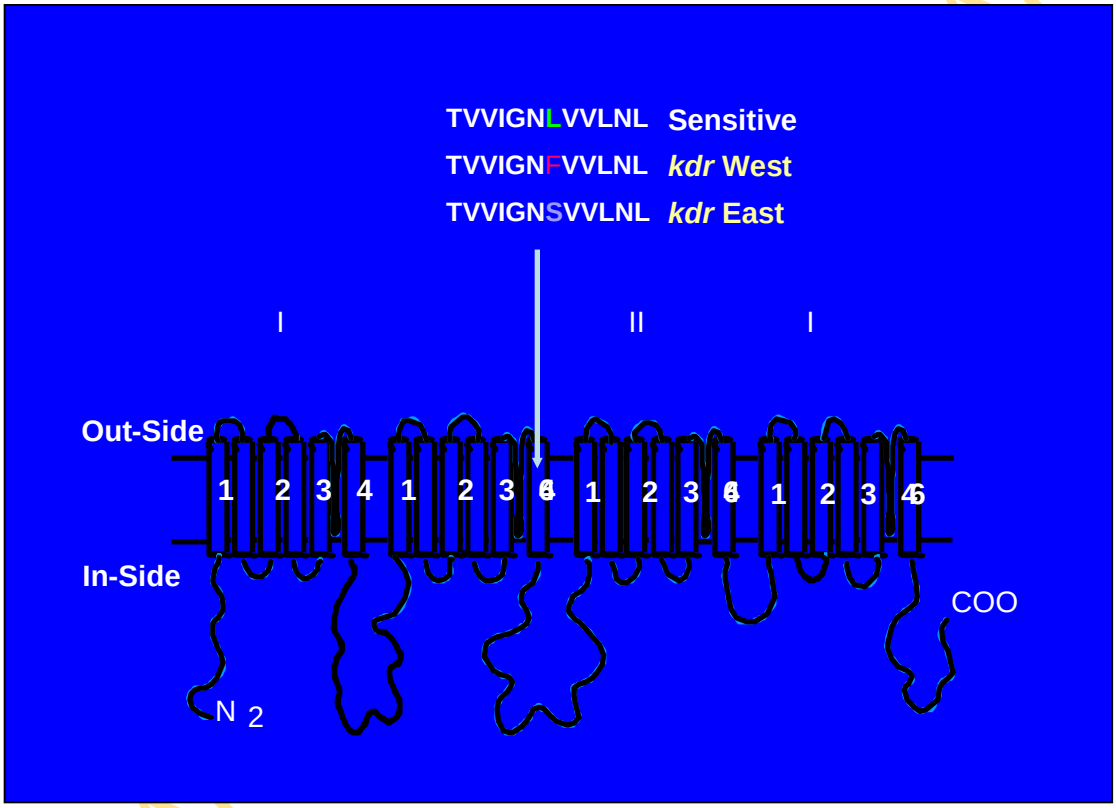

Fig. 2.10 Mutation on the sodium channel in *kdr* resistant *Anopheles* from West and East Africa

Source: Chandre (2004)

2.8.2 The bottle tests with synergists

The bottle bioassay described by Allister and Brogdon (1999) can be used to assess the biochemical mechanisms of resistance developed by mosquito populations collected in the field. The technique is based on coating of bottles with the insecticide solution to be tested and introducing female mosquitoes into these bottles. Once resistance is detected, another set of coated bottle is prepared using 2 synergists: piperonyl butoxide (PBO) and S.S.S-tributlyphosphorotrithioate (DEF). PBO is used for detecting the presence of elevated oxidase activities in the mosquito whereas DEF is for esterase (Allister and Brogdon, 1999).

2.8.3 Polymerase chain reaction (PCR) for target modification

PCR analysis provides insight information on the sequence arrangements, the presence or absence of specific nucleotides in the DNA of the field collected mosquitoes. This sequences arrangement profile is used for molecular characterisation of mechanisms of resistance in sampled *Anopheles* populations. The most common PCR for target modification is the PCR *kdr* used in knock down resistance. The technique is based on detection of single nucleotide polymorphisms following DNA extractions and using appropriate primers (Martinez- Torres *et al.*, 1998). This PCR allows determination of various resistant alleles (RR, RS, SS) and their respective frequencies in mosquito populations could be calculated. The acetylcholinesterase target site mutation (*Ace-1*) known to confer carbamate and organophosphate resistance could also be screened in field population of *A. gambiae* using PCR protocols described by Weill *et al*. (2004).

2.8.4 Microarray technique for detection of detoxification genes

This Technique provides an accurate platform for analysing series of up and down regulated genes at a single trial. Contraty to classical PCR analysis which screens for a limited number of genes at a time, the micro-array technology provides simultanous information on several gene responses (hundreds to thousands of gene responses) following a given stimulation. The microarray “detox chip” for example which is used for screening detoxifying genes expressed in *Anopheles* mosquitoes, contains fragments of 230 *A. gambiae* genes from families associated with metabolic based insecticide resistance. These gene fragments included 103 P450s, 31 COEs, 35 GSTs, 41 Redox genes, 5 ATP binding-cassette transporters, tissue-specific genes and housekeeping genes

(David *et al.*, 2005).Each gene represented on the microarray is either obtained by PCR amplification or artificially synthesized. To keep cross hybridization between closely related genes to a minimum, gene-specific segments are selected between 70 and 300 bp in length as probes which are spotted in duplicate onto gamma-amino-propyl-silane-coated glass slides (UltraGaps, Corning) by using a Biorobotics Micro- Grid II printer (BioRobotics, Cambridge, U.K.).

2.8.5 Reported cases of resistance of *A. gambiae* to insecticides in Nigeria

A limited number of extensive studies have been conducted on vector resistance to insecticides in Nigeria. Awolola (2003) studied the resistance of *A. gambiae* to insecticides in Lagos, Nigeria. The study identified the presence of resistance in some *Anopheles* populations and established the presence of M and S molecular forms existing as single or in sympatry in some localities of southwestern Nigeria. Mojca (2003) also reported on the low presence of *kdr* mutation on *Anopheles* populations from Ogun state in the South West Nigeria. Awolola *et al.* (2005) investigated the distribution of the molecular M and S forms of A. *gambiae* and the knock down resistance (*kdr*) gene associated with pyrethroid and DDT resistance in *A. gambiae s.s*. at 13 localities across Nigeria. The report showed that the overall collection was a mix of the molecular M and S forms across the mangrove (63:37%), forest (56:44%), and transitional (36:64%) ecotypes, but almost a pure collection of the S form in the Guinea and Sudan-savanna. Results of insecticide susceptibility tests showed that mosquitoes sampled at seven localities were susceptible to permethrin, deltamethrin, and DDT, but populations of *A. gambiae* resistant to these insecticides were recorded at six other localities mainly in the transitional and Guinea-savanna ecotypes.

The kdr gene was found only in the molecular S forms, including areas where both forms were both M and S forms are in sympatric. No hybrid M/S has been found during the analysis of samples from the field; this suggest as strong mating barrier between the two molecular forms of *A. gambiae ss*. The overall frequency of the *kdr* alleles in populations of *Anopheles* was low: <47% in forest, 37–48% in the transitional, and 45–53% in Guinea-savanna. The data suggest that pyrethroid resistance in *A. gambiae* in Nigeria is not as widespread when compared to neighboring West African countries (Awolola *et al.*, 2005).

2.8.6 Reported cases of resistance of *A. gambiae* to insecticides in Benin

In Benin, the first report of *Anopheles* resistance was by Akogbeto and Yakoubo (1999). This resistance resulted from the exposure to DDT of populations of *A. gambiae* from meridian regions during IRS campaign. Resistance in Benin is related to two phenomena: (i) the massive use of DDT and dieldrin for house-spraying applications in southern villages of Benin from 1953 to 1960 during WHO programmes of malaria eradication (Joncour, 1960) and (ii) the massive use of organochlorine in agricultural settings during the 1950s (WHO, 1976).

Pyrethroids were introduced for agricultural use in Benin in the 1970s, and after 30 years of continuous use, cases of resistance may be found in some populations of insects (Akogbeto *et al.*, 1999). N'Guessan (2007) analysed the operational impact of pyrethroid resistance on the efficacy of ITNs and IRS. Results from the study established a link between pyrethroid resistance caused by *kdr* and the failure of ITNs in Benin. Using biochemical assays Corbel *et al*. (2007) implicated the detoxification enzymes in conferring resistance to pyrethroids, DDT, dieldrin and carbosulfan in *A. gambiae* populations from four localities in Benin. Djogbenou *et al. (*2008) identified and mapped the distribution of *Acer-1* in *A. gambiae* samples from Benin. He reported elevated frequencies of *Acer-1* mutation associated with resistance to organophosphate and carbamate in Benin (Djogbenou et *al.*, 2008).

2.8.7 Reported cases of resistance of *A gambiae* to insecticides in other African countries

The resistance of vectors to insecticides is a real handicap to the use of insecticide-treated materials and the implememtation of IRS (N'Guessan et *al*., 2007). The first cases of resistance were mentioned in the 1950s and 1960s with identified populations of *A. gambiae* capable of withstanding lethal doses of organochlorine. In 1950-1960, this resistance phenomenon was mostly limited to dieldrin and hexachlorocyclohexane (HCH) (Coz and Hamon 1963). In Africa, the first cases of dieldrin resistance in *A. gambiae* were recorded in Burkina Faso in 1960 (Elissa et *al.*, 19993; Coz et *al.*, 1999). Ten years later, the identification of dieldrin resistance and cases of DDT resistance were reported in Togo, Senegal, Nigeria, Cameroon and Guinea (WHO, 1976). With pyrethroids, cases of resistance were published relatively late in the 1990s. The first cases of pyrethroid

resistance were reported in West African countries such as in Côte d'Ivoire (Elissa et *al.*, 1993), Benin (Akogbeto and Yakoubo, 1999), Burkina Faso (Diabate, 1999; Chandre et *al.*, 1999), Côte d'Ivoire (Chandre et *al.*, 1999) and Mali (Fanello et *al.*, 2003). Other reports followed from Kenya in East Africa (Vulule et *al.*, 1994), and Cameroon in Central Africa and in the Central African Republic (Etang *et al.,* 2003).

Two main mechanisms of resistance have so far been identified in *A. gambiae* from agricultural settings in the West Coast of Africa: the *kdr* mutation and enzymatic mechanisms of resistance. *kdr* mutations have been mostly recorded in settings where agricultural practices are associated with the use of pesticides. This mechanism is well spread in Burkina Faso (Diabate *et al.,* 2001), and in Cote d'Ivoire (Elissa *et al.,* 1993). In the northern area of Cameroon, enzymatic mechanisms of resistance have been identified in various localities (Etang *et al.,* 2003).

Studies conducted by Diabate et *al.* (2002) highlighted increased levels of resistance genes, *kdr*, in *A. gambiae* collected in cotton-growing areas and constantly subjected to insecticide treatments, as compared to the low frequency of *kdr* recorded in rural areas where farmers are restricted to food crops for local consumption with no pesticides. In Côte d'Ivoire, the *kdr* mutation identified in resistant strains of *A. gambiae* was probably selected as a result of the massive use of DDT and pyrethroids against pests in cotton fields (Chandre et *al.*, 1999; Diabate et *al.*, 2002).

2.9 Factors favouring the emergence of resistance in mosquito populations

Several environmental and human practices have been implicated in the emergence of insecticide resistance in diseases vectors. These factors include the large scale implementation of ITNs (Vulule *et al.,* 1999) and IRS (Joncour *et al.,* 1960) in communities, the agricultural pesticides (Chandre *et al.*,1999; Diabate *et al.*,2002) and the presence of pollutants (xenobiotics) in mosquito breeding habitats (Poupardin *et al.*, 2008; Muhammad *et al.,* 2009)**.** These factors either select for target sites mutations in mosquitoes or cross induce the expression of several detoxifying genes.

2.9.1 The selection of insecticide resistance by ITNs and IRS

In the Western Kenya, the permethrin tolerance (PT) of a population of the mosquito *A. gambiae* increased following the introduction of permethrin-impregnated nets for malaria control in certain villages near Kisumu (Vulule *et al.,* 1999). A similar

observation was made several years later by Aram (2004) who showed that after one year of ITN use, there was a reduction in permethrin susceptibility in *A. gambiae* from ITN villages but not from villages without ITNs. In Cote d'Ivoire, Corbel *et al.* (2003) analysed *Anopheles* mosquitoes in experimental hut trial and found that insecticide resistance was selected by carbamate impregnated bednets. The study, also demonstrated that when carbamate were combined with pyrethroids on the same net, no such selection was recorded.

2.9.2 Agricultural pesticide residues and other xenobiotics

The implication of agriculture in the selection of insecticide resistance in malaria vectors has been well documented (Chandre *et al.*, 1999; Diabate *et al.*, 1999; Akogbeto and Yacoubo, 1999). Akogbeto *et al.* (2003) later demonstrated that various insecticides are used against pests in vegetable farms and in cotton plantations. These insecticides are mainly pyrethroids, organophosphate compounds and carbamates. Insecticides are highly used in vegetable and cotton farming (Akogbeto *et al.*, 2003). Data on insecticide susceptibility tests always show elevated resistance on mosquito populations collected in or around agricultural farms under pesticides use (Mouchet *et al.*, 1988). This is because insecticide residues are left in water and soil after farms spraying. When these residues are in contact with mosquito larvae, they exercise a selection pressure on larvae and this pressure results to gradual development of insecticide resistance in mosquito populations (Akogbeto *et al* 2004.). In the Republic of Benin, insecticides were introduced in agriculture in the 1970s. It is not impossible after 30 years of use to record some cases of resistance. Several authors mainly from West Africa and Cameroon incriminate pesticides used in agriculture as a factor selecting insecticide resistance in mosquitoes (Georghiou and Lagunes 1991; Chandre *et al.*, 1999; Martin *et al.*, 2000; Diabate *et al*, 2002; N'guessan *et al.*, 2003).

Experiments conducted on larvae of *Aedes aegypti* showed that constant exposure to sub-lethal doses of xenobiotics such as the herbicide atrazine, the polycyclic aromatic hydrocarbon fluoranthene and the heavy metal copper increased their tolerance to insecticides (Poupardin *et al.*, 2008). Results of the experiement revealed a moderate increase in larval tolerance to permethrin following exposure to fluoranthene and copper while larval tolerance to temephos increased moderately after exposure to atrazine, copper

and permethrin. This study revealed the potential of xenobiotics found in polluted mosquito breeding sites to affect their tolerance to insecticides, possibly through the cross-induction of particular detoxification genes.

2.9.3 Types of mechanisms of resistance selected by ITNs, IRS, agricultural pesticide residues and xenobiotics

Surveys conducted in Kenya showed that the frequency of the *kdr* mutation prior to ITN introduction was between 3 and 4 % target communities. After ITN introduction, the *kdr* mutation increased in ITN and neighboring villages from 4% to 8% (Aram *et al.*, 2004). In addition to the *kdr* mutation, Vulule (1999) used biochemical test with synergists to establish higher oxidase levels in mosquito populations from villages with ITNs than a comparison population from villages without impregnated nets. He further speculated that the use of impregnated nets selected for higher oxidase and esterase levels in *A. gambiae* to metabolize permethrin acquired from the nets. Both oxidase and esterase mechanisms could confer cross-resistance to other pyrethroids. Evidence for selection for an insensitive acetylcholinesterase mechanism by carbamate impregnated bednets was later established by Corbel (2003).

With Agricultural pesticides, two main mechanisms of resistance have so far been identified in *A. gambiae* populations collected in or around agricultural settings in West Africa: the *kdr* mutation and enzymatic mechanisms of resistance (Elissa *et al.*, 1993; Diabate *et al.*, 2001; Etang *et al.*, 2003; Chouaibou et *al.*, 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1 Susceptibility of *Anopheles* populations to pyrethroid in studied sites

Populations of *Anopheles* were collected from various localities and analysed for their susceptibility to permethrin.

3.1.1 Description of sampling sites

Mosquito samples were collected from six states in southwestern Nigeria namely Ogun, Oyo, Lagos, Osun, Ondo and Ekiti states (fig. 3.1). In the Republic Benin, mosquito larvae were collected from 6 administrative divisions in the southern region manely the Zou, Plateau, Couffo, Atlantique, Mono and Littoral. Standing water points found in each site from these administrative divisions and states were systematically scrutinized for mosquito larvae (Fig. 3.2). Sites with breeding water containing *Anopheles* larvae were considered as sampling sites for this research.

The names of the sites were obtained from the local communities and the geographical coordinates (latitudes and longitudes) of each sampling site were determined using a geographical positioning system (GPS) device (GPS Garmin® Model 60). Each geographical coordinate as well as the name of the locality were projected on the map of Benin and Nigeria using Arch view 3.1 software.

3.1.2 Collection of *Anopheles* larvae

Contrary to other families of mosquitoes (*Culex*, *Aedes* and *Mansonia*), *Anopheles* larvae lack caudal siphon and therefore are recognized by their morphology and their horizontal position on water surface (Bruce-Chwatt, 1980) (Fig. 3.3). This regular presence of *Anopheles* larvae at the surface of water allows proper breathing of individuals. *Anopheles* larvae were scooped into large vessels, were sieved and concentrated in small buckets, washed with clean water and taken to the insectary for rearing to adult stage.

3.1.3 Rearing of larvae to adult stage in the insectary

Anopheles larvae samples collected in the field were reared in three different insectaries depending on the sites of collection and the available facilities for keeping and conveying samples from the field to the laboratory. All samples collected in the southwestern part of Nigeria were reared in the insectary of the Department of Zoology,

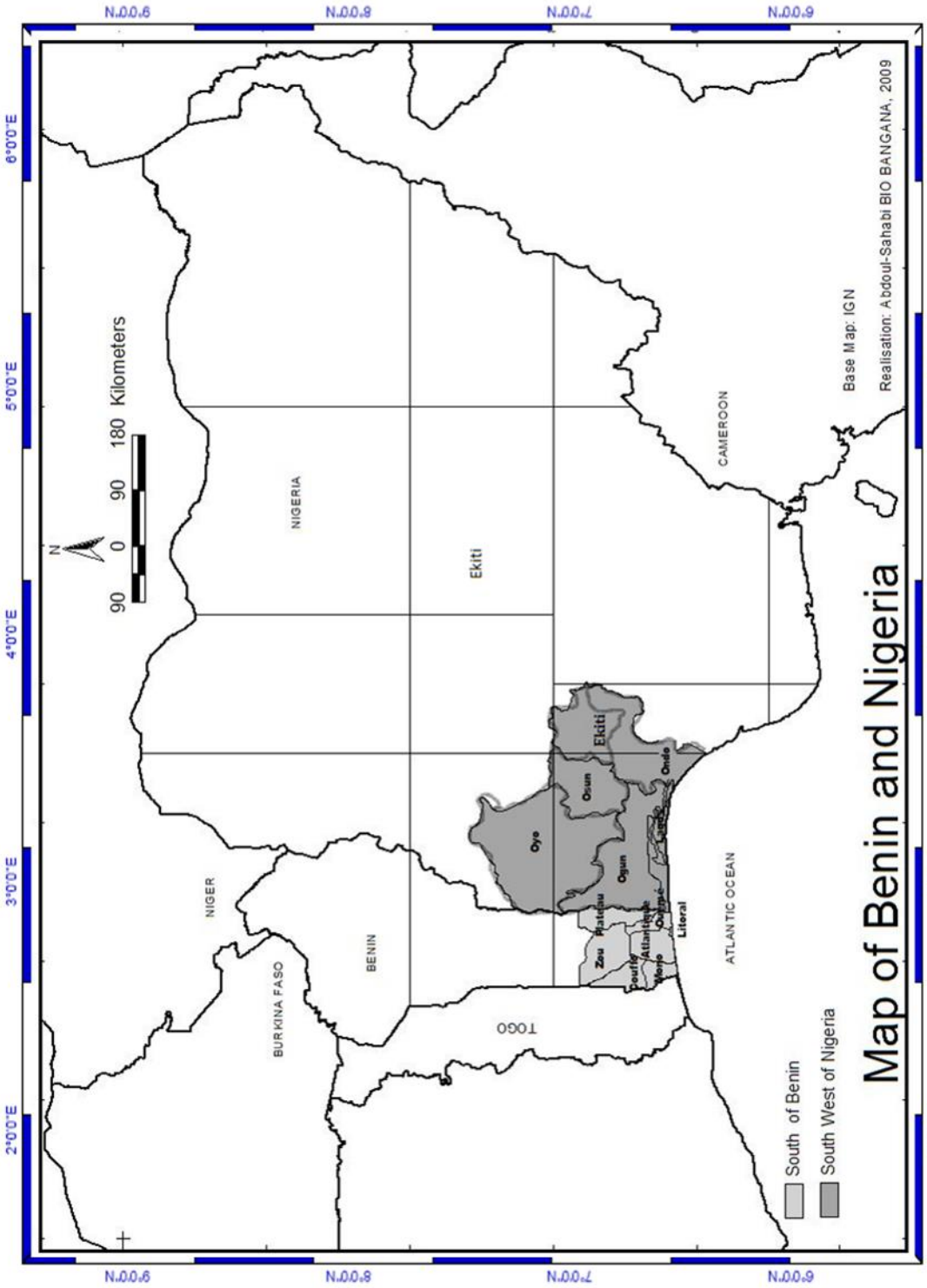

Fig. 3.1 Map of study areas (Divisions and States) in Benin and Nigeria

Fig. 3.2 Identification of breeding sites and field collection of *Anopheles* larvae

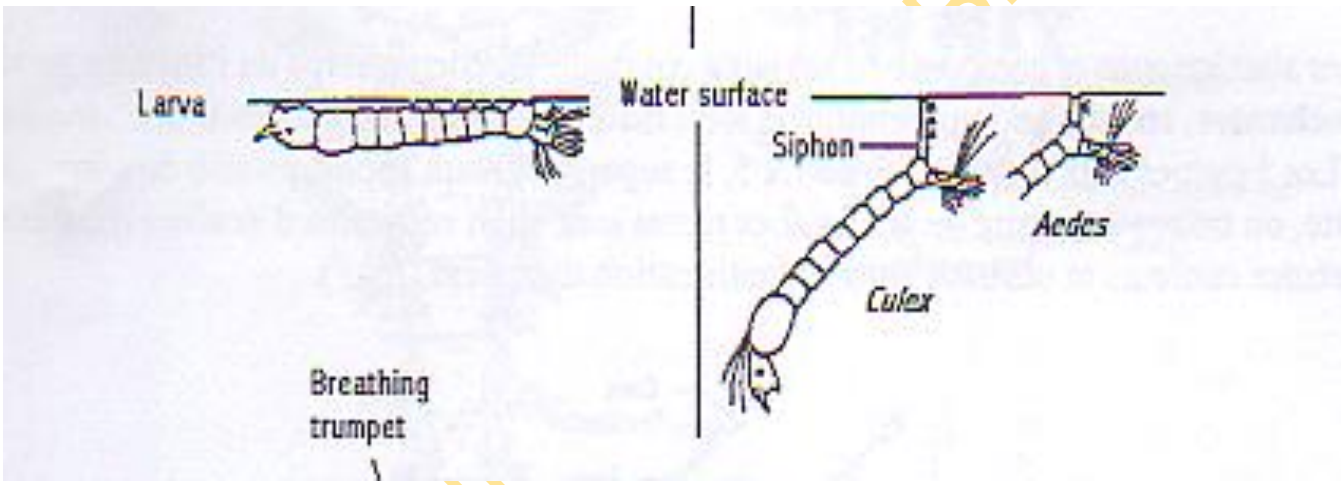

Fig. 3.3 The horizontal position of *Anopheles* larvae at the surface of water (left)

Source: Bruce-Chwatt (1980)

University of Ibadan, Nigeria. The insectaries of the Centre for Research in Entomology of Cotonou (CREC) based in Cotonou, Republic of Benin and that of the International Institute of Tropical Agriculture (IITA-Benin) for samples collected in the Republic of Benin. At the insectary, mosquito larvae were reconditioned into clean vessels of 50 cm diameter and 15 cm depth containing Well water and covered with mosquito nets. Larvae were fed through the pores of nets mesh with a powder of cat/dogs biscuits bought from local super market. At the adult stage, mosquitoes were transferred into cages and fed with 10% sucrose solutions for 2-5 days.

3.1.4 WHO susceptibility tests on adult *Anopheles* from surveyed localities

Two to five-day old *Anopheles* emerging from each identified breeding sites, were bio-assayed using discriminating doses of insecticides. Bio-assays were carried out following WHO protocols (1986). Females of *A. gambiae* were exposed for one hour to papers impregnated with diagnostic dosages of permethrin (0.75%). For each test, 5 test tubes were used: one for the control and four for exposed mosquitoes. Control tubes contained filter papers impregnated with silicon oil (insecticide carrier), while treated papers were impregnated with diagnostic doses of insecticide plus carrier. An average of 20 mosquitoes was introduced in each tube, making a total of 100 females *Anopheles* exposed to each insecticide. After one hour, mosquitoes were transferred to holding containers and provided with cotton pads with 10% sucrose solution. An average of 50 females of *Anopheles* from each locality was exposed to permethrin. Mortality rates were recorded at 24 hours (Fig. 3.4 and Appendix.3) and the susceptibility status of the population was graded as susceptible if the mortality rate is between 97% to 100%; as reduced susceptibility if the mortality rate is between 95% to 95% and as resistant if the mortality rate is below 95% according to WHO protocol (1986).

3.1.5 Mapping of permethrin susceptibility in *Anopheles* populations from studied localities

The susceptibility of malaria vectors to permethrin was mapped using data (latitude and longitude) on the geographical locations where *Anopheles* were collected. Mortality rates following exposure to permethrin of *Anopheles* populations from surveyed locality were projected onto the maps of Nigeria and Benin using Arc-view 3.1 software and then after, converted as PDF files.

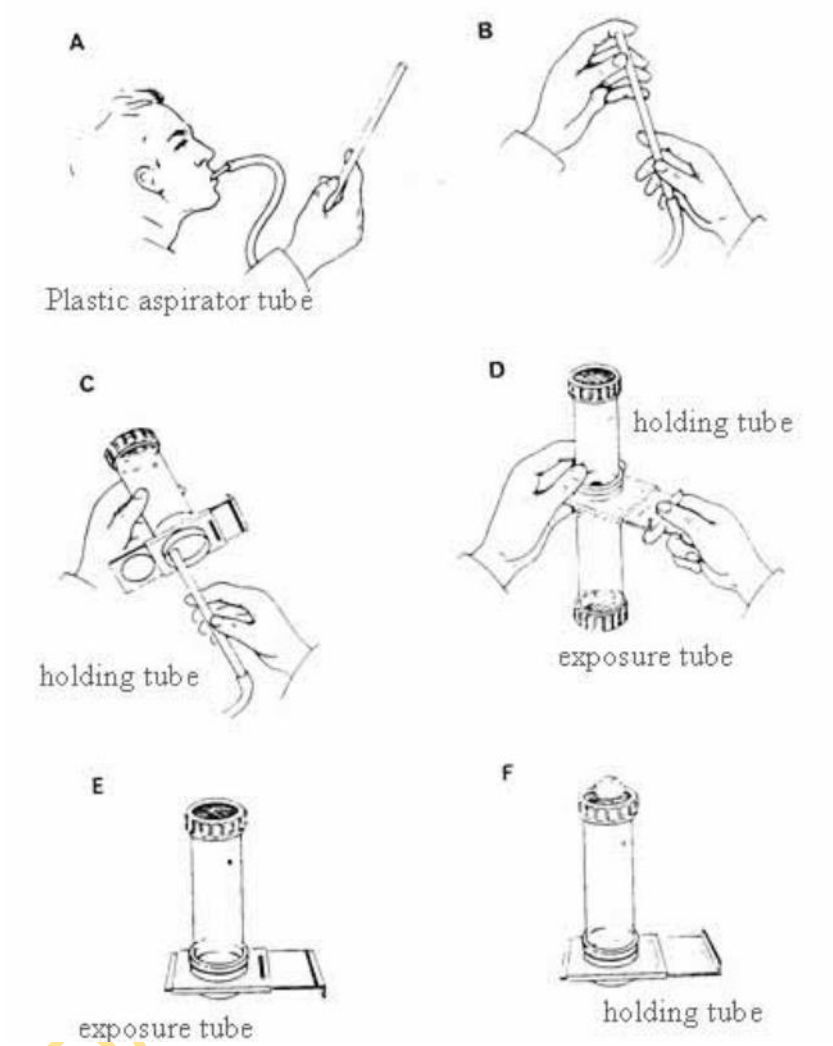

Fig. 3.4 WHO bioassay steps for analysing the susceptibility status of *Anopheles* to insecticides (WHO, 1996).

3.2 Molecular characterization of *Anopheles* populations from studied sites: PCR-species, PCR-forms, PCR-*kdr*

In each locality, an average of 30 *Anopheles* that had been exposed to permethrin was subjected to PCR Analysis. The 30 mosquitoes for PCR analysis were sorted from the group of *Anopheles* that had survived the exposure to permethrin and those that died after 24 hours post exposure to permethrin impregnated papers (making an average of 15 survivors and 15 dead for each locality). PCR-species (Koekemoer *et al.*, 1999) were conducted on *Anopheles* to identify the various members of *A. gambiae* complex encountered in each site. The next set of PCR focused on molecular forms (Favia *et al.*, 1997) and involved only *A. gambiae ss.* from the previous screening. The PCR forms sub-grouped the *A. gambiae ss.* into 2 molecular forms: *A. gambiae ss. (M)* and *A. gambiae ss. (S).* The last series of PCRs investigated the presence of the *kdr* mutation which confers a pyrethroid resistant phenotype to *Anopheles* populations. Each PCR process involved 3 main steps: DNA extraction, Polymerase chain reactions and electrophoresis (Figs. 3.5, 3.6 and 3.7).

3.2 .1 DNA extraction

DNA extraction for each adult female was carried out by adding 100 ml of lysis buffer (0.1 MNaCl, 0.2 M sucrose, 0.1 M Tris-HCl, 0.05 M EDTA, pH 9.1 and 0.5% SDS) onto the mosquito sample in an eppendoorf tube (1.5ml). A sterile laboratory pestle was used to grind and homogenize mosquito samples in each eppendoorf tube. The homogenate was incubated at 65º C for 1 h, and 15 µl of 8 M potassium acetate solution was then added and centrifuged for 10 min at 14,000 rpm. The supernatant was pipetted without disturbing the pellets and the deposit containing pellets was transferred into a new eppendoorf tube and filled up with 400µl of 100% ethanol (ice cold ethanol from -20^{o}C freezer). The ethanol suspension was well mixed and incubated at room temperature for 5 minutes then centrifuged at 14,000 rpm for 15 minutes. Ethanol is removed and the pellets are re-suspended in 200µl of 70% ethanol (ice cold ethanol from -20^{o}C freezer), centrifuged at 14,000 rpm for 20 minutes and pipetted off without disturbing the DNA pellets. After this series of ethanol washing, the DNA pellets were finally air-dried and suspended in 100µl of sterile distilled water, stored at -20^{o}C before PCR analysis (Fig. 3.5 and Appendix.4).

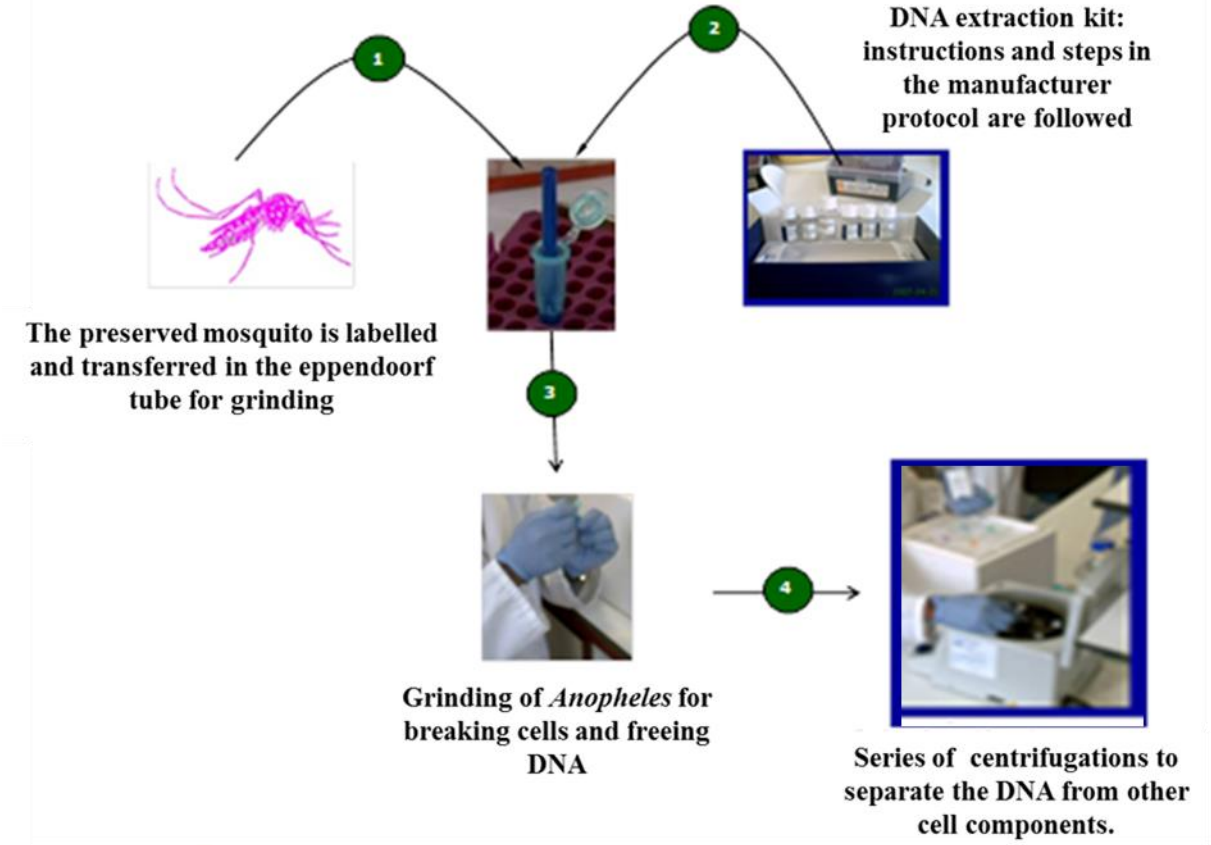

Fig. 3.5 DNA extraction for PCR analysis of collected *Anopheles* samples

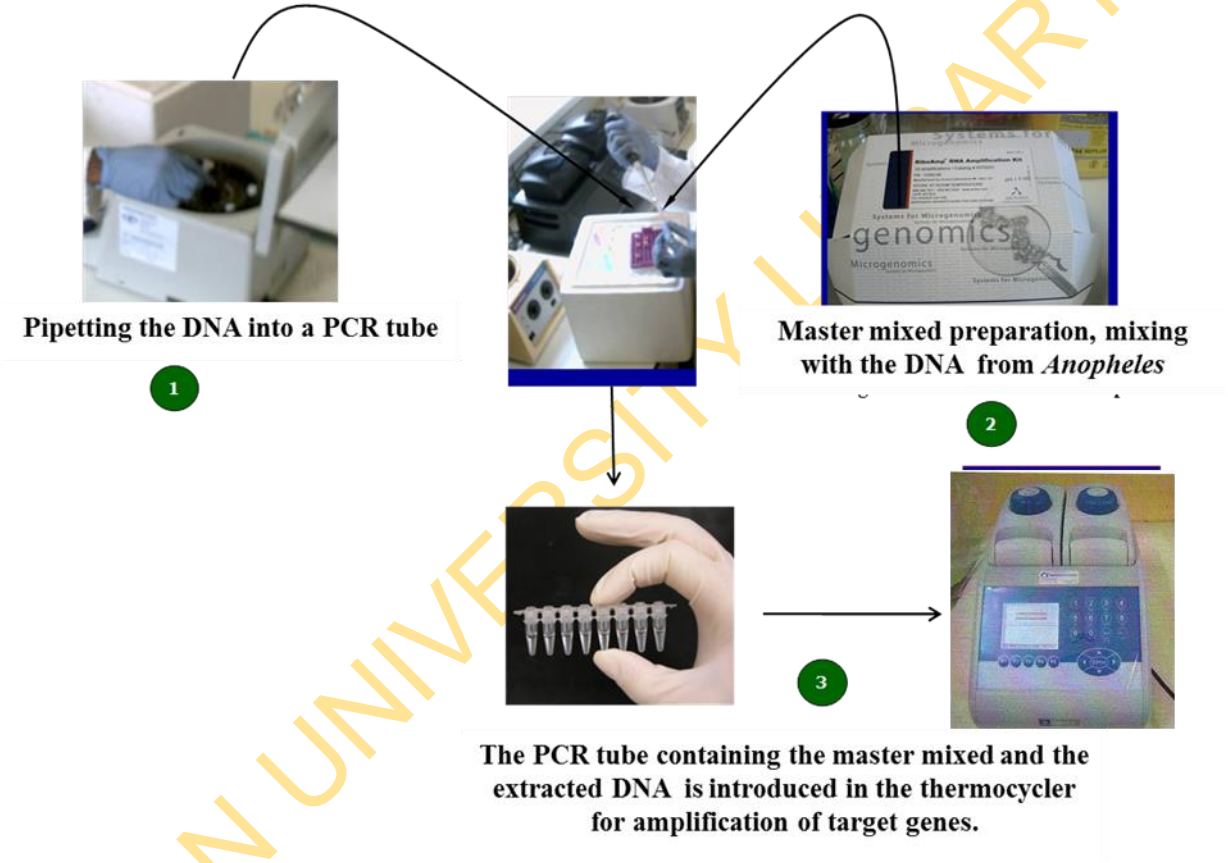

Fig. 3.6 DNA amplification (PCR reactions) of collected *Anopheles* samples

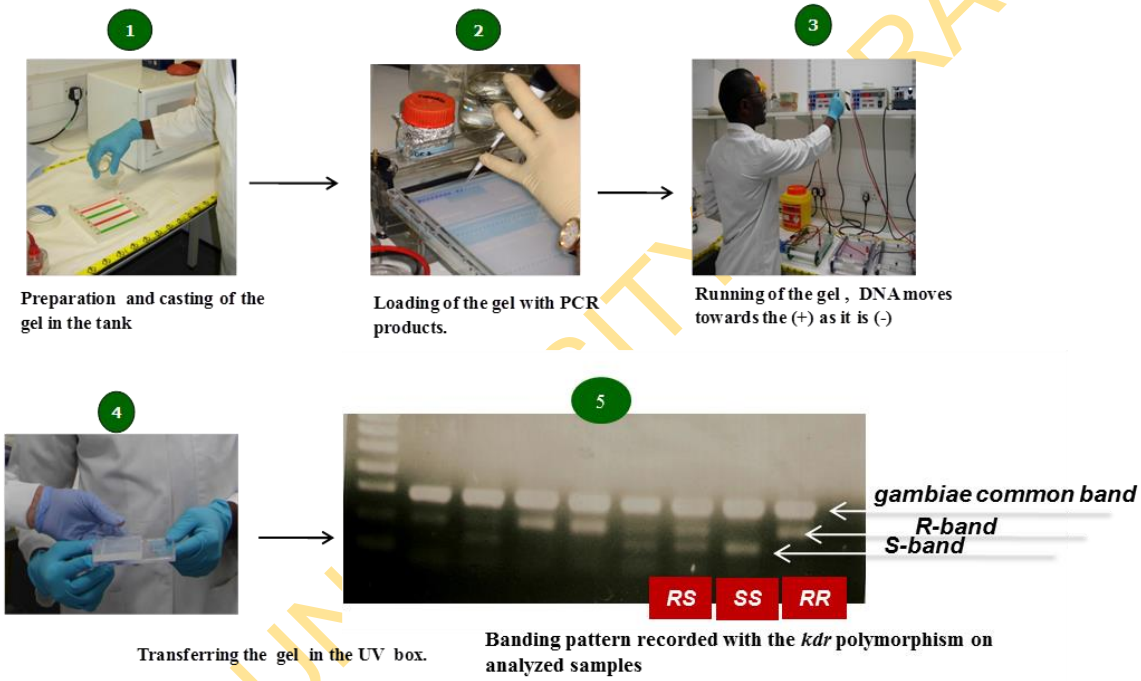

Fig. 3.7 Agarose gel migration of PCR products of amplified *Anopheles* DNA

3.2.2 Polymerase chain reaction for *A. gambiae* speciation

Each DNA extracts from mosquito samples was used as template for PCR synthesis. A thermo-cycler made GenePro model B-480 was set for amplifications of DNA fragments. For species identification (Koekemoer *et al.*,1999). A master mix solution containing 1.25µl of PCR buffer, 1.25µl of dNTPs, 1µl of each primer (Universal, *A. gambiae ss, A. arabiensis, A. melas, A. quadrianulatus*.), 0.5µl of $MgCl_2$ and 4.9µl of sterile distilled water was prepared for each PCR tube and 2µl of extracted DNA sample was added to the master mix. The thermocycler was loaded with DNA samples to be amplified and programmed at 94^{o}C for 10 minutes (denaturation phase), then 30 cycles of 94^{o}C for 30 seconds, 50^{o}C for 30 seconds and 72^{o}C for 30 seconds (hybridization and extension phase). After the amplification, the PCR product was migrated on 1% agarose gel (Figs. 3.6 and 3.7).

3.2.3 Polymerase chain reaction for detection of the knock down (kdr) mutation in *A. gambiae* populations

For the identification of *kdr* mutations in collected *Anopheles* samples, the protocol described by Martinez-Torres *et al.* (1998) was used. A master mix solution containing 2.5µl of PCR buffer, 0.5µl of dNTPs, 0.3µl of $MgCl_2$, 1.25µl of Agd_1 (primer), 1.25 µl of Agd_2 (primer), 2.5µl of the Agd_3 (primer), 2.5 µl of Agd_4 (primer), 0.125 of Taq and 13µl of sterile distilled water was prepared for each PCR tube and 1µl of extracted DNA sample was added to the master mix. The Agd_1 and Agd_2 primers correspond to the standard *A. gambiae* banding whereas the Agd_1 and Agd_3 correspond to the resistant genotype and the Agd_2 and Agd_4 to the susceptible genotype. The thermocycler was loaded with DNA samples to be amplified and programmed at 95^{o}C for 15 minutes, 94^{o}C for 30 seconds, 35 cycles of 48^{o}C for 30 seconds, 72^{o}C for 1 minute and 72^{o}C for 10 minutes. After the amplification, the PCR product was migrated on 1% agarose gel (Appendix. 4).

Specific primers used for PCR-species and PCR-kdr

The members of *A. gambiae* complex were discriminated as *gambiae ss, arabiensis, and melas*. Using respectively the following primers sequences: CTGGTTTGGTCGGCACGTTT, AAGTGTCCTTCTCCATCCTA, GTGACCAACCCACTCCCTTGA described by Koekemoer et *al.* (1999). The *kdr* gene

mutation was localized on the DNA template using 4 primers described by Martinez-Torres *et al.* (1998): ATAGATTCCCCGACCATG, AGACAAGGATGATGAACC, AATTTGCATTACTTACGACA, and CTGTAGTGATAGGAAATTTA.

3.2.3 Electrophoresis of PCR products

For the migration of PCR products, an agarose gel (1%) is prepared. 1% agarose powered is dissolved in TBE buffer, heated to boiling temperature for 10 minutes, cooled to the room temperature and 10µl of ethydium bromide added and gently swirled. The ethydium bromide is a radioactive compound which fixes the nucleotides making them to fluoresce in the presence of UV light. The gel is caste in the electrophoresis tank and wells made on it with electrophoresis comb and allowed to solidify at room temperature. The size of the comb and therefore the number of wells was selected based on the number of PCR products (samples) to migrate at a time. The voltage was set at 100 and the migration on the gel was fixed for approximately 1hour. Molecular weight markers ranging from 1000 to 100 bp were simultaneously loaded on the gel and used as the ladder for interpreting the size of each amplified DNA (Fig. 3.7).

3.3 Evaluation of potential contributions of agricultural pesticide residues and spilled petroleum products in the selection of pyrethroid resistance in *Anopheles* populations

Soil and water samples were collected from agricultural sites under synthetic pesticides application and from areas of spilled petroleum products.

3.3.1 Screening of pesticide residues in water and soil samples from vegetable sites

The screening of pesticide residues was conducted on samples from the agricultural sites of Houeyiho in Cotonou and Ajibode in Ibadan.

3.3.1.1 Screening technique

A bioassay focused on factors capable of inhibiting the normal growth of *Anopheles* larvae in breeding sites was conducted (MIM-WHO, 2003). The protocol used in this evaluation is mainly based on comparison of the growth of larvae in test breeding sites (water and soil samples from agricultural settings under pesticide pressure) and in control breeding sites (water and soil samples from similar areas with no pesticides).

3.3.1.2 Collection of water and soil samples used for the bio-assay

Surface water and soil samples (1 cm of the upper layer) were collected from the vegetable farms of Houeyiho in Benin and Ajibode in Ibadan, Nigeria (Fig. 3.8). These

samples were considered as test samples. For control samples, soil and water were collected from the premises of the insectary of the Centre for Research in Entomology of Cotonou–Akpakpa (CREC-Akpakpa) and the premises of the insectary of the Department of Zoology, of the University of Ibadan (UI) (Fig. 3.8). Both control sites have no previous history of pesticide contamination. Soil and water samples collected in the field were taken to the insectary where they were used for reconstituting *Anopheles* breeding sites, 5 replicates of each type of breeding sites were reconstituted with samples from each target locality. Breeding sites reconstitution was based on (i) a mixture of 10g of top (surface) soil from vegetable farms and 1000 ml of water from the control sites and (ii) 1000ml of water collected in the vegetable farm (water used for watering vegetables). Water samples from CREC-Akpakpa were designated as CREC-water similarly; samples from the premises of the UI-insectary were recorded as UI-water.

3.3.1.3 Monitoring of larval development in simulated breeding sites

An average of 200 eggs of the susceptible *A. gambiae* strain from kisumu was inoculated in each artificial breeding site (soil and water samples from Houeyiho and Ajibode, CREC-Akpakpa and UI-insectary). A similar inoculation was repeated with the resistant *A. gambiae* strain from ladji and for the different types of artificial breeding sites simulated. More than 4,000 eggs were inoculated and monitored during this biological evaluation. The variations in hatching rates of eggs, the larvae developmental rate and yields of rearing larvae of resistant *A. gambiae* from Ladji and susceptible *A. gambiae* from Kisumu to adult mosquitoes in test and control artificial breeding site were determined compared and plotted. During this follow-up experiment, larvae in all artificial breeding sites were fed with similar quantities and types of food (a powder from cat biscuits mixed with yeast).

3.3.1.4 History of synthetic pesticides utilization by farmers in target agricultural sites

The level of synthetic pesticide use by communities in the vegetable farms where samples were collected was investigated through quantitative and qualitative surveys. After getting farmers consents on the study, they were subjected to quantitative and qualitative questionnaires to generate data on: the families of insecticide used in vegetable farming, the various doses applied for pests control, the frequency of treatments, the origin

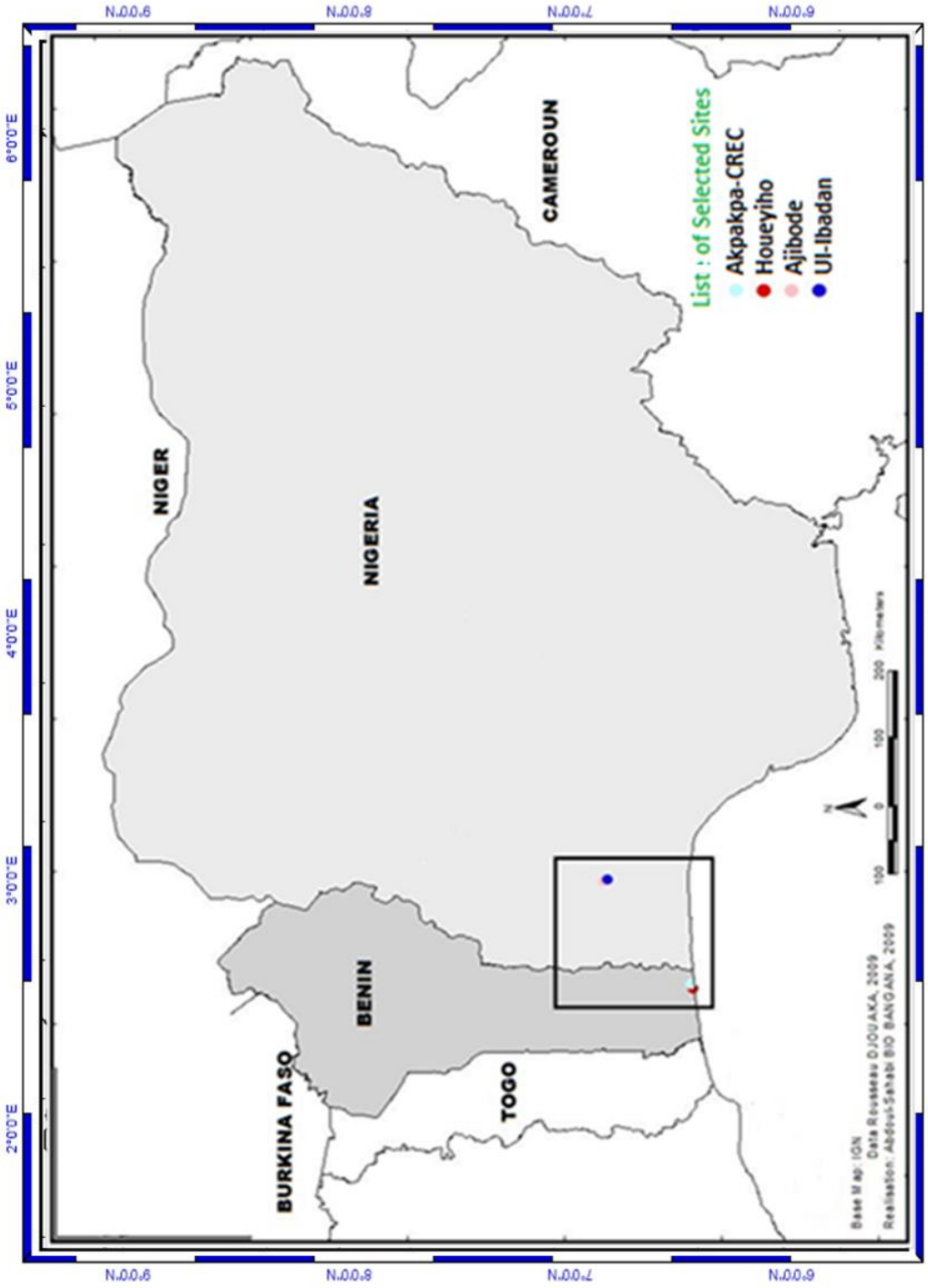

Fig. 3.8 Map of sites selected for screening of pesticides residues in vegetable farms (Akpakpa-CREC, Houeyiho, Ajibode and UI- Ibadan).

of insecticides and the place of purchase (Appendix.1). Both the discussion guide (qualitative) and the questionnaire (quantitative) were tested and validated at the International Institute of Tropical Agriculture (IITA, Benin-Cotonou) Cotonou, Station. At Houeyiho, a total of 20 farmers approved the consent forms and underwent both the quantitative and the qualitative interviews, while at Ajibode, 10 farmers were interviewed and probed on their agricultural practices. This investigation also included direct observations of farm treatments by farmers. More emphasis was placed on the qualitative aspect of this survey.

3.3.2 Evaluation of the contribution of petroleum products in the selection for insecticide resistance in *A. gambiae*

3.3.2.1 KAP studies on the empirical utilisation of petroleum products (PP) in rural communities

Prior to assessing the larvicidal activities of PP, questionnaires, focus group discussions (FGD), and in-depth questionnaires and interviews (Appendix.2) were conducted in 3 rural communities namely Gbodjo, Ladji, and Ketonou in the Southern Benin were PP are still used for larval control. The interview focused on: i) the empirical use of PP, ii) the mode of application, iii) the period when PP are mostly used for breeding site treatments and iv) the frequency of utilization of PP (Appendix.2). A total of 65 key respondents were interviewed in the 3 communities.

3.3.2.2 *Anopheles* populations used for analysing the lethal activities of PP

Two local resistant strains of *Anopheles* were used for this experiment: *A. gambiae* from Ladji *i* and *A. gambiae* from Ojoo. Both strains were used as reference resistant mosquitoes for assessing the lethal activities of PP in areas of pyrethroid resistance. *A. gambiae* populations from ladji were collected from the rural locality of Ladji, at 7 km from Cotonou, the economic capital of the Republic of Benin and *A. gambiae* populations from Ojoo were collected from the locality of Ojoo in Ibadan, Oyo state. Both strains were maintained in the insectary throughout the duration of this experiment.

3.3.2.3 Determination of lethal concentrations of 4 PP on larvae of *A. gambiae*

Larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo were exposed to four petroleum products: petrol, kerosene, engine oil and used engine oil from mechanic workshops. Known volumes of PP ranging from 0.12×10^{-3}µl to 9820×10^{-3}µl were

introduced in 255 cm^2 plates half filled with well water (approximately 100ml) and each containing 25 larvae (second to third stage larvae) of *Anopheles gambiae* from Ladji and *A. gambiae* from Ojoo (Fig. 3.9). Larvae were fed throughout the experiment with biscuit and yeast. Each breeding site treatment with PP in each plate was replicated four times for each population of *Anopheles* (*A. gambiae* from Ladji and *A. gambiae* from Ojoo), making a total of 100 larvae exposed to each tested concentration of PP. Similarly, control breeding sites with no traces of petroleum products were constituted and monitored in tandem. Mortality rates as well as the number of adults emerging from each breeding site were determined. The lowest concentration (LoC_{100}) capable of inhibiting the development of larvae to the adult stage was determined; it corresponds to 100% mortality of exposed larvae.

The highest concentration (HiC) not having any observable effect on the growth of larvae also known as the NOEL (No effect level) was determined; it corresponds to 0% mortality of exposed larvae. In between both concentrations, the LC_{50} (concentration leading to a mortality of 50 % of exposed larval population) was also determined for each PP. Data generated were pooled and used for plotting the curve of activity of each PP on strains of *A. gambiae* from Ladji and *A. gambiae* from Ojoo. Thus, the HiC and the LoC_{100} corresponding to petrol, kerosene, engine oil and waste oil was determined by dose response analysis during this laboratory assessment. In between the HiC and the LoC_{100}, the LC_{50} was determined for each PP.

3.3.2.4 Identification of the mode of action of PP on *Anopheles* larvae

Two potential modes of action of petroleum products were analysed during this study: i) the killing of larvae by "suffocation" through the oil film produced by PP at the surface of breeding sites, and ii) the direct lethal activity through contact and ingestion of dissolved particles of PP in breeding sites, referred here as "contact toxicity". Water samples with visible residues of petroleum products were collected from the locality of Ojoo, an area of spilled waste engine oils by mechanics and oil retailers. These samples from the field known as “crude samples” were used in the laboratory for the simulation of two types of breeding sites: i) The first set of breeding sites known as "unsieved or crude" was directly reconstituted by putting two litres of the water from the field into laboratory bowls. ii) The second set of breeding sites known as "sieved or clean" was reconstituted

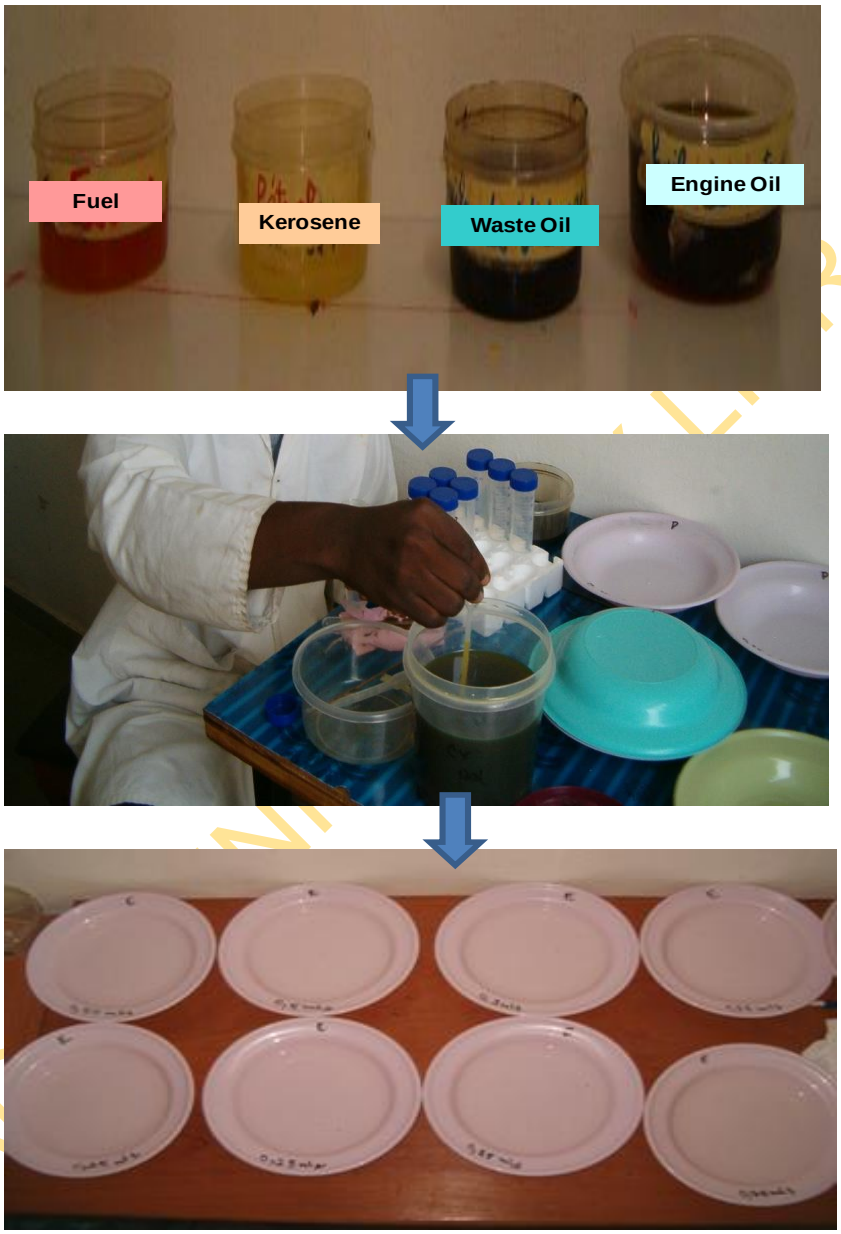

Fig. 3.9 Quantification of the lethal activity of 4 petroleum products on *Anopheles larvae* introduced in plates containing different concentrations of PP

by sieving to clean two litres of the crude sample from the field. The determination of the mode of action of petroleum products on mosquito larvae was based on statistical comparisons of larval mortalities recorded in the “unsieved or crude” simulations and the mortalities in the “sieved/clean” simulations. One hundred larvae of *A. gambiae* from Ojoo were introduced in each bowl containing sieved or unsieved petroleum and reared to adults. Four replicates were made for each type of simulation, making a total of 400 larvae monitored in "un-sieved/crude" and 400 larvae in "sieved/clean" breeding sites. Control bowls were constituted alongside using well water with no trace of petroleum products and containing the same number of *Anopheles* larvae. For each type of breeding site (sieved and un-sieved), mortality rates were determined and statistical tests of comparison used to associate larval lethality with either sieved or un-sieved simulations.

3.3.3 Analysis of associations between the presence of petroleum products in breeding sites and the emergence of pyrethroid resistant populations of *Anopheles*

The association between the nature (physico-chemical properties) of breeding sites and the susceptibility statut of emerging populations of *Anopheles* was analysed.

3.3.3.1 Physico-chemical properties of breeding sites of resistant and susceptible *Anopheles*

Fresh water samples were collected from each identified breeding site in the southwest Nigeria and the southern Benin and their physico-chemical properties determined. The physical thickness of the water and the presence of oil layer at the surface of the breeding sites were visually appreciated and recorded. The dissolved oxygen (DO) and the pH of the breeding site; 2 parameters which are likely to affect the development of *Anopheles* larvae and their susceptibility status were determined by a digital portable multi-meter (Handheld Digital-Multimeter® model M3640D). These physico-chemical parameters were either measured in the field or water samples were preserved in a cooler and later analysed in the laboratory.

3.3.3.2 Oviposition preferences of gravid females of *Anopheles* in localities where breeding sites are partially contaminated with spilled petroleum products

50 gravid females of *A. gambiae* ready to lay eggs were introduced into a cage provided with 2 different bowls of breeding sites for oviposition. The first bowl contained water with petroleum product residues collected at Ojoo and the second bowl had well

water with no trace of petroleum products and served as control sample. This experiment was carried out subsequently with *A. gambiae* from Ojoo and *A. gambiae* from UI strain introduced in cages containing the two simulated oviposition bowls.This oviposition experiment was repeated with other sets of oviposition bowls contaminated with PP residues collected at Akpakpa in Benin. Each oviposition cage was made in 4 replicates of 50 mosquitoes resulting in a total of 200 females for each strain monitored respectively on petroleum contaminated samples from Ojoo and Akpakpa. The number of eggs laid by each strain of *Anopheles* in the breeding site with or without petroleum products was counted and compared. Chi square ($\chi 2$) statistical test was used to compare the number of eggs laid in each type of simulated site and to determine the preferred oviposition habitat of pyrethroid resistant and susceptible *Anopheles.*

3.3.3.3 Monitoring of the development of *Anopheles* larvae (resistant and susceptible strains) in breeding sites with petroleum products residues

Two hundred eggs of *A. gambiae* were introduced into breeding sites containing two liters of water contaminated with petroleum residues from areas of spilled petroleum products in Ojoo and Akpakpa. The hatching rate and the number of larvae getting to pupae stage were determined for the resistant strain *A. gambiae* from Ojoo and the susceptible strain *A. gambiae* from UI. Four replicates were analysed for each experiment. Control breeding sites made of clean well water with no trace of petroleum products were also seeded with eggs of the same species and monitored in tandem with test breeding sites. The number of hatched eggs and the number of larvae progressing to the pupae stage in each type of breeding sites (oily breeding sites and the controls) and for each strain of *Anopheles* (the resistant and the susceptible strain) were determined.

3.4 Screening of candidate metabolic genes overexpressed in pyrethroid resistant populations of *A. gambiae* from Benin and Nigeria

This experiment focused on the identification of differential expressions of metabolic genes in pyrethroid resistant *Anopheles* populations which are exposed to different types of xenobiotics such as spilled petroleum and agricultural pesticides. *Anopheles* larvae exposed to petroleum products were collected from Ojoo in Nigeria whereas larvae exposed to pesticide residues were collected from the vegetable farm of

Akron. The susceptible *Anopheles* populations from the locality of Orogun in Nigeria (Fig. 3.10) served as control samples in this experiment.

3.4.1 Selection of permethrin resistant *A. gambiae* for micro-array analysis

Thousands of fourth instar larvae and pupae were collected from the breeding pools identified in the 3 target localities: Ojoo, Orogun and Akron (Fig. 3.10). Larvae and pupae were maintained in the laboratory and reared to adults (F0). Batches of 20 females of *A. gambiae* (one day old) which had emerged from larvae collected directly from the field were introduced into WHO susceptibility tubes (WHO, 1986) containing papers coated with 0.75% permethrin. After an exposure time of one hour, mosquito samples from Akron and Ojoo were transferred into insecticide free tubes and maintained for 24 hours with sugar solutions. At the end of the 24 hr, dead mosquitoes were discarded and alive or selected individuals (permethrin resistant population) were maintained in cages with sugar solutions for 3 days then after, a minimum of 30 permethrin resistant females (3 pools of 10 females also known as 3 biological replicates) from Ojoo and Akron were preserved in RNA-later solution for microarray analysis as described by David et *al*., (2005). Susceptible mosquitoes from Orogun were subjected to WHO tubes containing control papers impregnated with silicon oil (insecticide carrier) instead of permethrin. They were also maintained for 3 days with sugar solutions and a minimum of 60 females preserved in RNA-later solution.

3.4.2 Target preparation and microarray hybridizations

Total RNA (tRNA) was extracted in pools of 10 mosquitoes from the preserved samples (30 *Anopheles* from Ojoo; 30 *Anopheles* from Akron and 60 *Anopheles* from Orogun). The extraction was done with the PicoPure™ RNA isolation kit (Arcturus) according to the manufacturer's instructions. After extraction, the quantity of extracted tRNA was measured by a spectrophotometer (Nanodrop) (Fig. 3.11) and its quality was based on electrophoresis migrations of 1µL of extracted tRNA on 1% agarose gel at a voltage of 75 for 60 minutes (Fig. 3.12)

3.4.3 cDNA synthesis, labelling and hybridization

Extracted RNA was amplified using a RiboAmp™ RNA amplification kit (Arcturus). Amplified RNAs were checked for quantity by spectrophotometry, then reverse transcribed into labelled cDNA and competitively hybridized to the array. Cy3 and

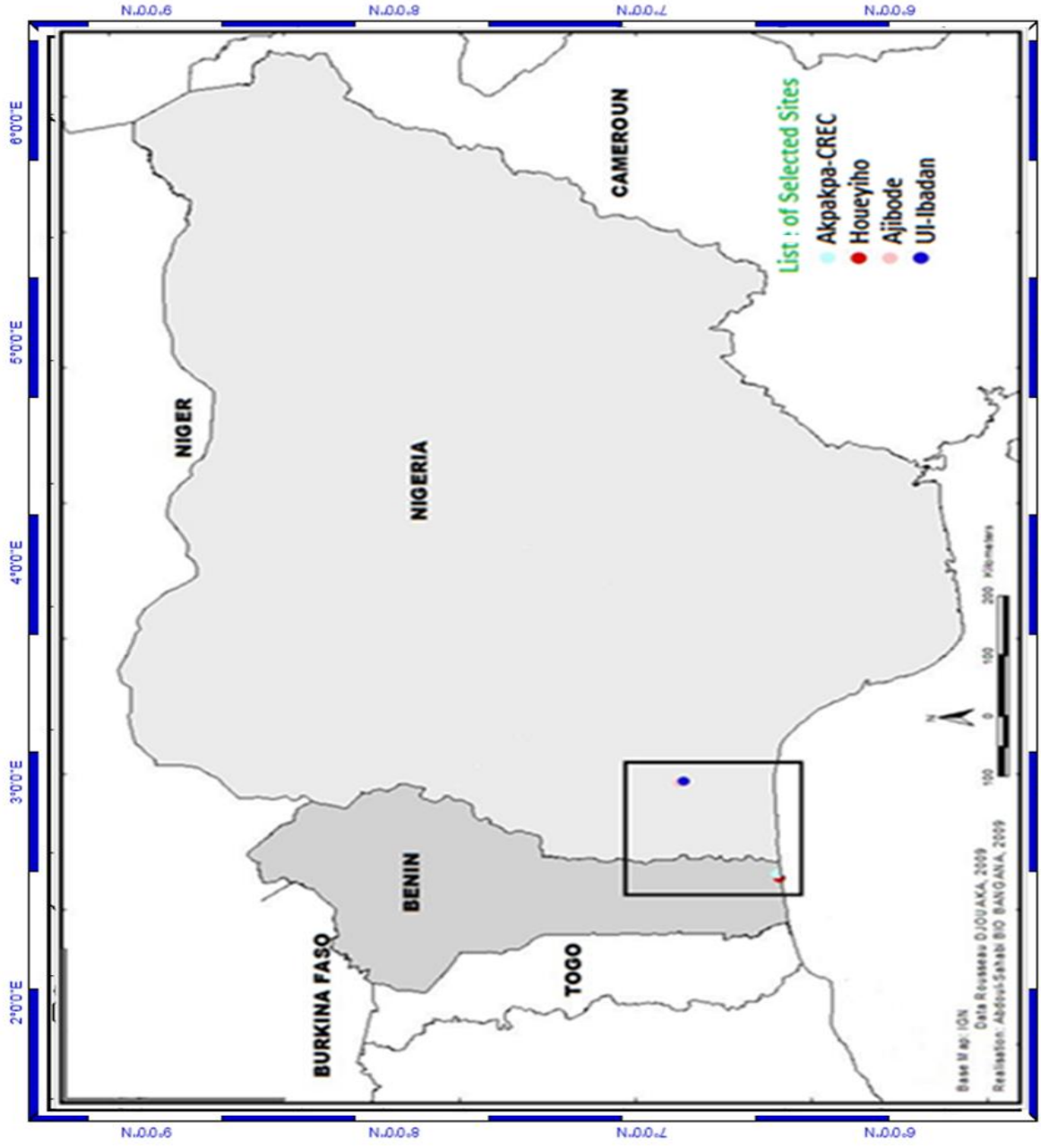

Fig. 3.10 Map showing the 3 study sites selected for candidate metabolic gene search and their locations in relation to the control site (Orogun)

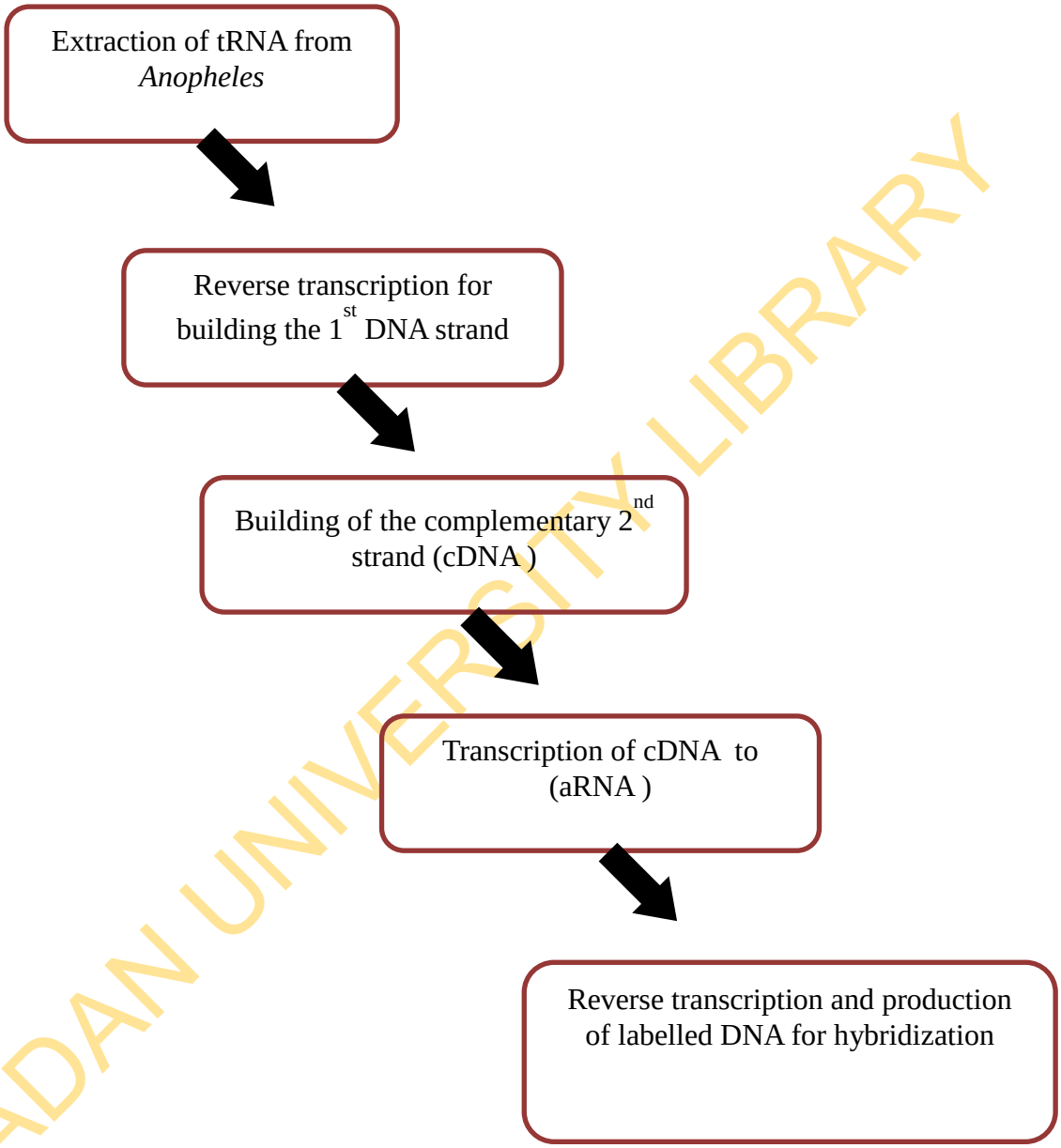

Fig. 3.11 Steps for producing and labeling cDNA of *Anopheles* samples from target sites (Ojoo, Akron and Orogun) for micro-array hybridization

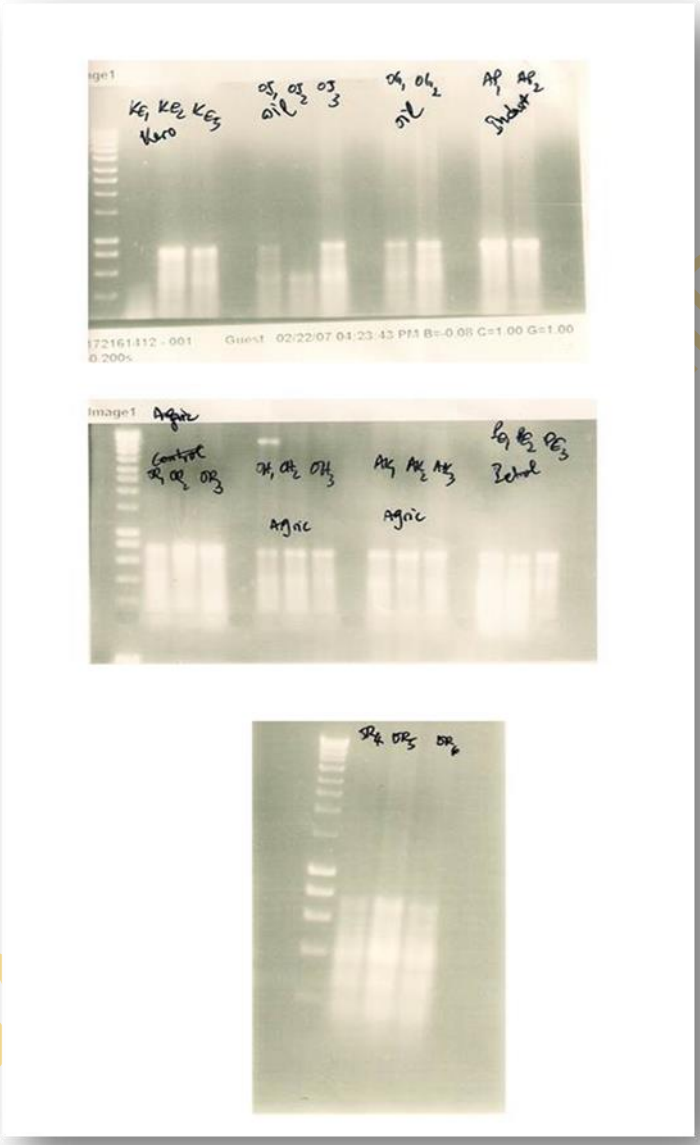

Fig. 3.12 Gel analysis of migrated total RNA prior to cDNA production

Cy5 dyes were used for labelling of test and control samples. Labels were swapped between samples, making 2 technical replicates from each biological replicate and hence a

total of six labelled targets for each comparison (test and control samples) (Fig. 3.13). Labelled cDNA targets from the agricultural site of Akron and the oil spillage locality of Ojoo sites were competitively hybridised with the susceptible population of *Anopheles* samples from Orogun to the *A. gambiae* detox chip array which was printed with a physical rearrangement of the detoxifying probes (David *et al.*, 2005; Muller *et al.*, 2008).

3.4.4 Array scanning and visualization

After visual inspection of each array, spot and background intensities were calculated from the scanned array images using GenePix Pro 5.1 software (Axon Instruments). Raw intensities were then analysed with Limma software package version 2.4 (Lima 2.4) For the comparisons between the two groups of samples, selected (resistant) vs. unselected (susceptible), estimates for technical replicates (dye-swaps) were first averaged and then compared between the two groups as described by Muller et *al*. (2008). The probes printed on the microarray include 103 cytochrome P450s, 31 esterases, 35 glutathione S-transferases and 85 additional genes such as peroxidases, reductases, superoxide dismutases, ATP-binding cassette transporters, tissue specific genes and housekeeping genes (Fig. 3.13).

3.5. Data analysis

Series of statistical analysis were conducted on the various laboratory and field data collected during this research study.

3.5.1 Analysis of data on the susceptibility level of *Anopheles* to permethrin

Mortality rates of *Anopheles* post exposure to permethrin were computerized on Excel software and the corresponding susceptibility status of *Anopheles* populations were graded as resistant (R), susceptible (S) or reduced susceptibility (RS) (WHO, 1986). Data on the latitudes and longitudes of surveyed localities were analysed with Arc-view software and projected on maps of Benin and Nigeria.

3.5.2 Questionnaires and group discussions and in-depth interviews with farmers and petroleum products users

Qualitative data from focus group discussions and in-depth interviews were tape reordered, transcribed and analysed with the software Text base beta.

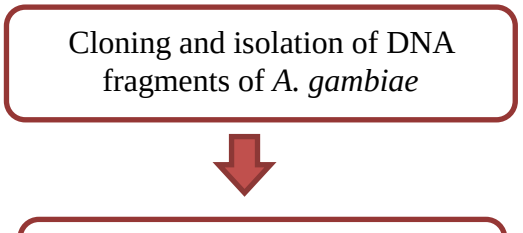

Fig. 3.13 Steps for synthesis and printing of detoxifying gene probes of *A. gambiae* on the "Detox" chip array

Quantitative data were analysed with Epi Info sofware. Data were mainly descriptive and presented as percentages.

3.5.3 Lethal activities of petroleum products on *Anopheles* larvae

Mortality rates corresponding to given concentrations of petroleum products were determined in the laboratory. Data generated from the laboratory assays were used to plot dose response curves of mortality rates and petroleum concentrations in breeding sites with Excel and SPSS software package. The HiC and LoC of each petroleum product were determined through laboratory bio-assays. The HiC and LoC of the 4 types of petroleum products tested were compared for assessing the relative efficacy of each petroleum products of *Anopheles* larvae. The mode of action of petroleum products on *Anopheles* larvae was identified by analysing the percentage mortalities of larvae in sieved and un-sieved breeding sites.

3.5.4 Cross analysis of the physico-chemical properties of breeding sites and the susceptibility status of emerging *Anopheles* populations

Data on the physico-chemical properties (DO, pH, and the presence of oil particles) of breeding sites were cross-analysed with data on the permethrin susceptibility of *Anopheles.* The mean DO and the mean pH of breeding sites producing resistant *Anopheles* was determined and compared with the mean DO and the mean pH of breeding sites producing permethrin susceptible *Anopheles.* The number of breeding sites with petroleum residues producing permethrin resistant *Anopheles* was also compared with the number of breeding sites with petroleum residues producing permethrin susceptible *Anopheles*. The statistical analysis of variance was conducted on variables for associating the presence of petroleum products in breeding sites and the emergence of pyrethroid resistant *Anopheles* in studied localities. With data collected in vegetables farms, the mean DO and the mean pH of breeding sites producing permethrin resistant *Anopheles* was also determined and compared with the mean DO and mean pH of breeding sites producing susceptible populations of *Anopheles.*

3.5.5 Analysis of data on the oviposition preference of gravid *Anopheles*, eggs hatching rates and larval developments in simulated breeding sites

The mean number of eggs laid by resistant *Anopheles* (*A. gambiae* from Ojoo) in breeding sites with petroleum residues was determined and compared with the mean number of eggs laid in control breeding sites with no petroleum residues. Similar calculations were made with the mean number of eggs laid by the susceptible strain (*A.*

gambiae from UI). The comparison of means was based on the analysis of variance, for identifying whether or not permethrin resistant and susceptible strains of *Anopheles* prefer laying their eggs in breeding sites containing petroleum products. The number of eggs laid by *A. gambiae* from Ojoo and *A. gambiae* from UI in the various simulated breeding sites was plotted (histograms) with Excel 2000 Software.

The hatching rate was calculated as the percentage of the number of eggs hatched out of the 200 eggs inoculated in each simulated breeding site. The percentage of hatched eggs of resistant *Anopheles* (*A. gambiae* from Ojoo) in simulated breeding sites containing petroleum residues was determined and compared with the percentage of hatched eggs inoculated in control breeding sites. Similar calculations were made with the percentages of hatched eggs of susceptible *Anopheles (A. gambiae* from UI*)* in petroleum contaminated and in non-contaminated (controls) breeding sites. The recorded hatching rates of eggs of resistant and susceptible *Anopheles* inoculated in simulated breeding sites was plotted (histograms) with Excel 2000 Software.

With data recorded in vegetable farms, a similar calculation was made. The percentage of hatched eggs from resistant *Anopheles* (*A. gambiae* from Ladji*)* and susceptible *Anopheles* (*A. gambiae* from Kisumu) in the various simulated breeding sites was determined and compared with the percentage of hatched eggs in control breeding sites. The larval development rate was recorded as the percentage number of pupae recorded out of the number of first instar larvae that hatched from the 200 eggs inoculated in each simulated breeding site. The number of pupae from resistant *A. gambiae* from Ojoo and susceptible *A. gambiae* from UI in simulated breeding sites containing petroleum residues was determined and compared (χ^2 analysis) with the number of pupae recorded in control breeding sites. Similar calculations and comparisons were made with data from vegetable farms. The number of pupae of resistant *A. gambiae* from Ladji and of susceptible *A. gambiae* from Kisumu recorded in the various simulated breeding sites was determined and compared with the percentage of pupae in control breeding sites. The recorded hatching rates of eggs of resistant and susceptible *Anopheles* inoculated in simulated breeding sites was plotted (histograms) with Excel 2000 Software. For all comparisons made, the level of significance for statistical tests conducted was fixed at a probability value of 5%.

3.5.6 Allelic frequencies of the kdr mutation in permethrin resistant and susceptible phenotypes of *Anopheles* mosquitoes analyzed

In each surveyed locality, the allelic frequency of the *kdr* mutation (R) was calculated based on the formulae (Wigginton, et *al.*, 2005) :

$$f(R)=\frac{2RR+RS}{2(RR+RS+SS)}$$

with RR being the homozygous *kdr* resistant mosquitoes, RS the heterozygous and SS the susceptible mosquitoes. This allelic frequency was determined for the sub populations of dead and lived females of *Anopheles* exposed to permethrin. In each studied locality, the statistical comparison of the allelic frequency in dead and live mosquito was conducted and the probability value determined for associating (or not) the *kdr* mutation to recorded resistant phenotypes. The map for the distribution of *kdr* mutations in analysed mosquito populations was made using Arc view software.

3.5.7 Analysis of micro-array spots of expressed metabolic genes identified in studied *Anopheles* populations

The colour significance on micro array analysis program was based on fold changes (more than 2 folds). The Gene Pix pro 5.1 software and the Limma 2.4 software packages were used for analysing the color intensities of expressed genes in screened populations of *Anopheles* (Fig.3.14). The lists of metabolic genes up-regulated in permethrin resistant *Anopheles* collected from the vegetable site of Akron and the spilled petroleum products site of Ojoo were determined.

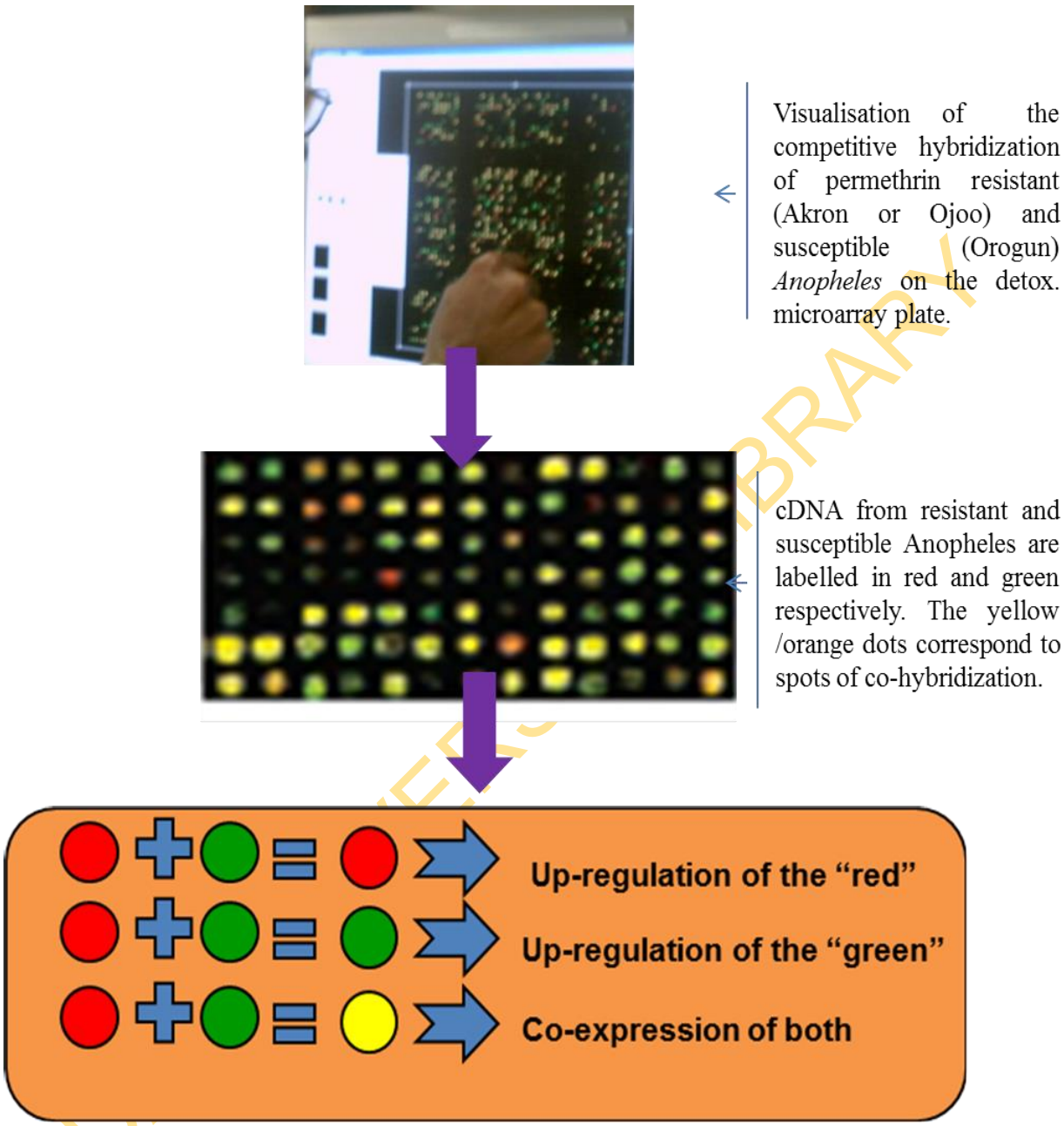

Fig. 3.14 Visualisation and analysis of scanned microarray slide.

CHAPTER 4

RESULTS

4.1 Screening of *Anopheles* populations for the susceptibility to pyrethroid in southwestern Nigeria and southern Benin

Anopheles larvae were collected in the various sites and reared to the adult stage for bioassays.

4.1.1 Susceptibility to permethrin of *A. gambiae* populations in southwestern Nigeria

Following the exposure of females of *A. gambiae* to permethrin impregnated papers, a good spread of resistance was recorded in the 6 surveyed states of Oyo, Osun, Ondo, Ekiti, Ogun and Lagos in southwestern Nigeria. Out of the 19 populations of *A. gambiae s.l.* collected from these states, 14 populations were clearly resistant to permethrin, whereas 5 were within the range of susceptibility (Table 4.1).

In Oyo state, 8 sites with *Anopheles* breeding spots were identified during this study: IITA, Ojoo, Ajibode, Basorun, UI, orogun, Challenge and Oja-tuntun. Permethrin resistance was clearly recorded in *Anopheles* populations from Basorun (mortality rate of *Anopheles* = 70%), Ojoo (mortality rate of *Anopheles* = 80%), Ajibode (mortality rate of *Anopheles* = 90%), Challenge (mortality rate of *Anopheles* = 81%) and Oja-tuntun (mortality rate of *Anopheles* = 81%). *A. gambiae* populations collected from UI, IITA and Orogun were all susceptible: UI (mortality rate of *Anopheles* =100%), IITA (mortality rate of *Anopheles* =100%) and Orogun (mortality rate of *Anopheles* =100%).

In Osun state, 2 sites with *Anopheles* breeding spots were identified during this study: Modakeke and Lagere. Permethrin susceptibility was recorded in *Anopheles* populations from Modakeke (mortality rate of *Anopheles* = 97%) while permethrin resistance was found in *Anopheles* populations from Lagere (mortality rate of = 94%).

In Ondo state, 2 sites with *Anopheles* breeding spots were identified: Illesha garage (Akure) and Owena. *Anopheles* populations from the two localities were all resistant to permethrin: Ilesha garage (mortality rate of *Anopheles* = 89%) and Owena (mortality rate of *Anopheles* =75%).

In Ekiti state, 2 sites with *Anopheles* breeding spots were identified: Ati-kankan 1 and Ati-kankan 2 both at Ado-Ekiti. Permethrin resistance was recorded with *Anopheles*

TABLE 4.1 Susceptibility to permethrin of *A. gambiae* in southwestern Nigeria

States	Localities (main city)	Latitude	Longitude	*Anopheles* tested	Mortality rates	Susceptibility
Oyo	IITA	7°22'12.0"	3°53'24.0"	95	100%	Susceptible

	(Ibadan)					
	Ojoo (Ibadan)	7°27'52.9"	3°54'58.7"	80	80%	Resistant
	Ajibode (Ibadan)	7°19'48.0"	3°54'00.0"	85	90%	Resistant
	Bashorun (Ibadan)	7°25'18.4"	3°56'08.3"	75	70%	Resistant
	UI (Ibadan)	7°26'21.8"	3°53'21.8"	80	100%	Susceptible
	Challenge (Ibadan)	7°26'17.9"	3°56'14.1"	80	81%	Resistant
	Orogun (Ibadan)	7°18'36.0"	3°54'36.0"	80	100%	Susceptible
	Oja-tuntun (Ogbomoso)	8°08'28.2"	4°14'3.5"	83	81%	Resistant
Osun	Lagere (Ife)	7°28'37.1"	4°33'17.9"	78	94%	Resistant
	Modakeke (Ife)	7°28'37.1"	4°32'19.9"	80	97%	Reduced Sus.
Ondo	Ilesha garage (Akure)	7°16'13.3"	5°09'57.7"	77	89%	Resistant
	Owena (Owena)	7°24'03.2"	5°00'45.0"	78	75%	Resistant
Ekiti	Ati-kankan 1 (Ado-ekiti)	7°37'06.0"	5°13'14.4"	83	85%	Resistant
	Ati-kankan 2 (Ado-ekiti)	7°37'06.5"	5°13'14.4"	80	88%	Resistant
Ogun	Imowo (Ijebu-ode)	6°50'57.5"	3°55'51.4"	76	96%	Reduced Sus.
	Mobafulo (Ijebu-ode)	6°47'59.1"	3°53'52.0"	75	80%	Resistant
	Ogere 1 (Lagos-Ibadan road)	07°26'12.3"	4°37'16.2"	4	75%	Resistant
	Ogere 2 (Lagos-Ibadan road)	07°26'12.3"	4°37'16.2"	40	88%	Resistant
Lagos	Badagry	6°24'36.0"	2°53'24.0"	50	98%	Susceptible

Susceptibility levels: From 97% to 100% mortalities (97% excluded) correspond to susceptible insect populations; 95% to 97% mortalities (95% excluded) correspond to reduced susceptibility in insect populations; below 95 % mortalities correspond to resistant insect populations (WHO/ANVR/MIM, 2003).

populations from the two sites. 85% mortality rate of *Anopheles* was recorded at Ati-kankan 1 and 88% at Atikankan 2.

In Ogun state, 4 sites with *Anopheles* breeding spots were identified during this study: Imowo (Ijebu-Ode), Mobalufo (Ijebu-Ode), Ogere 1 (Lagos-Ibadan road), and Ogere 2 (Lagos Ibadan road). Permethrin resistance was recorded with *Anopheles* populations from Ogere 1 (mortality rate of *Anopheles* = 75%); Ogere 2 (mortality rate of *Anopheles* = 88%) and Mobalufon (mortality rate of *Anopheles* = 80%). A reduced susceptibility pattern was noticed with *Anopheles* populations from Imowo (mortality rate of *Anopheles* = 96%).

In Lagos state, *Anopheles* breeding sites were found at Badagry where permethrin susceptibility was recorded with the *Anopheles* populations (mortality rate of *Anopheles* = 98%). Samples from Lagos city could not reach the insectary for bio-assays as they all died during the trip back to the laboratory. In most screened breeding sites, cadavers of larvae were found on water surface.

The latitudes and the longitudes of the 19 surveyed sites were used for the mapping of susceptibility status of *Anopheles* populations to permethrin in southwestern Nigeria. Data generated showed that 13 bioassayed populations were found to be resistant to permethrin. (Fig. 4.1).

4.1.2 Susceptibility to permethrin *of A. gambiae* collected in southern Benin

Following the exposure of females *A. gambiae* to permethrin impregnated papers and 24h monitoring of mortality rates of exposed females, resistance was recorded in the 6 surveyed divisions (Zou, Plateau, Mono, Couffo, Oueme, Atlantique) of the Southern Benin. Out of the 18 populations of *A.gambiae sl.* collected from these divisions, 17 populations were clearly resistant to permethrin, whereas only one population was susceptible (Table 4.2).

In the Zou division, 2 sites with *Anopheles* breeding spots were identified: Seto and Abomey. Permethrin resistance was clearly recorded in *Anopheles* populations from Seto (mortality rate of *Anopheles* = 80%) and Abomey (mortality rate of *Anopheles* = 83%).

In Plateau division, 3 sites with *Anopheles* breeding spots were identified during the surveyed period: the site of Pobe, Sakete and Ifangni. Resistance was recorded in

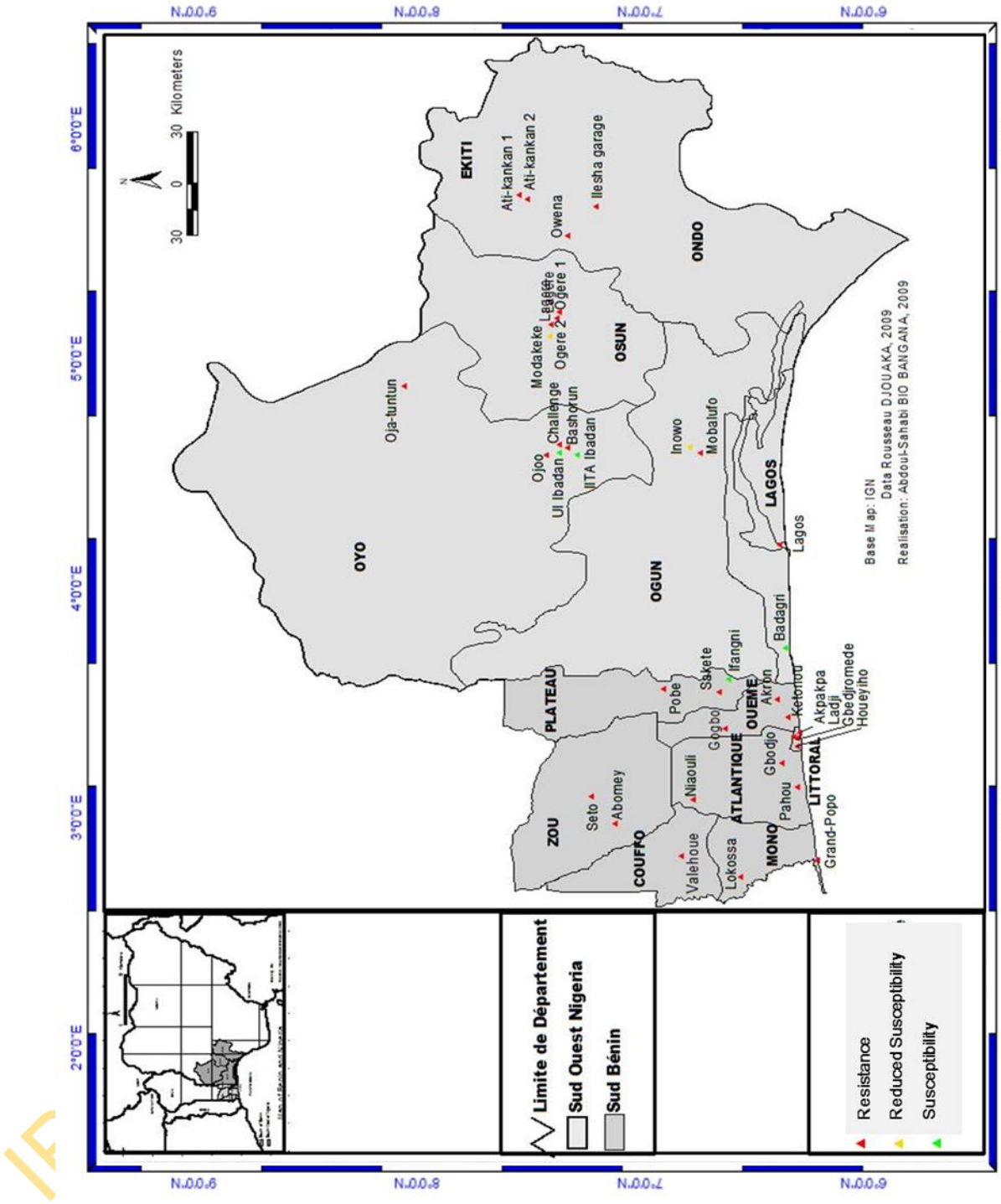

Fig. 4.1 Map of permethrin susceptibility status of *Anopheles* populations in the surveyed localities of southwestern Nigeria and southern Benin

TABLE 4.2 Susceptibility of *A. gambiae* populations to permethrin in southern Benin

Divisions	Localities	Latitude	Longitude	*Anopheles* Tested	Mortality Rates	Susceptibility
Zou	Seto	2°59'39.4"	2°04'43.2"	50	80%	Resistant
	Abomey	7°10'48.0"	1°58'48.0"	60	83%	Resistant
Plateau	Pobe	6°57'36.0"	2°40'48.0"	50	80%	Resistant
	Sakete	6°42'36.0"	2°36'00.0"	55	91%	Resistant
	Ifangni	06°40'12.0"	2°43'48.0"	100	98%	Susceptible
Mono	Lokossa	6°36'36.0"	1°42'36.0"	80	93%	Resistant
	Grand- Popo	6°16'12.0"	1°48'00.0"	60	90%	Resistant
Couffo	Valehoue	8°01'34.6"	2°29'49.3"	75	92%	Resistant
Oueme	Gogbo	6°41'05.2"	2°28'37.1"	80	90%	Resistant
	Akron	6°25'48.0"	2°38'24.0"	103	23%	Resistant
	Ketonou	6°24'00.0"	2°32'24.0"	70	81%	Resistant
Atlantique	Niaouli	6°49'48.0"	2°32'24.0"	75	67%	Resistant
	Gbodjo	6°25'48.0"	2°18'00.0"	116	83%	Resistant
	Pahou	6°21'36.0"	2°10'48.0"	106	31%	Resistant
	Akpakpa	6°21'00.0"	2°27'36.0"	110	69%	Resistant
	Ladji	6°23'40.1"	2°26'32.5"	100	65%	Resistant
	Gbedjromede	6°21'36.0"	2°25'48.0"	112	36%	Resistant
	Houeyiho	6°21'36.0"	2°23'24.0"	76	70%	Resistant

Susceptibility levels: From 97% to 100% mortalities (97% excluded) correspond to susceptible insect populations; 95% to 97% mortalities (95% excluded) correspond to reduced susceptibility in insect populations; below 95 % mortalities correspond to resistant insect populations (WHO/ANVR/MIM, 2003).

Pobe (mortality rate of *Anopheles* = 80%) and Sakete (mortality rate of *Anopheles* = 91%). *A. gambiae* populations from the site of Ifangni were susceptible (mortality rate of *Anopheles* = 98%).

In Mono division, two sites with *Anopheles* breeding spots were identified: Lokossa and Grand-Popo. Permethrin resistance was recorded in *Anopheles* populations from Lokossa (mortality rate of *Anopheles* = 93%) and Grand-Popo (mortality rate of *Anopheles* = 90%).

In Couffo division, only one site, Valehoue, with *Anopheles* breeding spots was identified. Permethrin resistance was recorded in *Anopheles* populations with a mortality rate of 92 %.

In Oueme division, 3 sites with *Anopheles* breeding spots were identified: Gogbo, Akron and Ketonou. Permethrin resistance was recorded in *Anopheles* populations from Gobo (mortality rate of *Anopheles* = 90%), Akron (mortality rate of *Anopheles* =23%) and Ketonou (mortality rate of *Anopheles* = 81%).

In Atlantique division, 7 sites with *Anopheles* breeding spots were identified during this study: the sites of Niaouli, Gbodjo, Pahou, Akpakpa, Ladji, Gbedjromede, Houeyiho. Permethrin resistance was clearly recorded in *Anopheles* populations from Niaouli (mortality rate of *Anopheles* = 67%), Gbodjo (mortality rate of *Anopheles* =83%), Pahou (mortality rate of *Anopheles* = 31%), Akpakpa (mortality rate of *Anopheles* = 69%), Ladji (mortality rate of *Anopheles* = 65%), Gbedjromede (mortality rate of *Anopheles* = 36%) and Houeyiho (mortality rate of *Anopheles* = 70%) (Table 4.2).

The latitudes and the longitudes of the 18 surveyed sites were used for the mapping of permethrin susceptibility in the southern Benin (Fig. 4.1). Data generated revealed wide presence of resistance in most surveyed sites (Fig. 4.1).

4.2. Molecular characterization of *Anopheles* populations from surveyed sites in Nigeria and Benin (PCR-*species*, PCR-*forms* and PCR-*kdr*)

PCR analyses were conducted on a total of 288 female *Anopheles*. Out of the total of 288 *Anopheles* analysed, 141 of these individuals survived lethal doses of permethrin while 147 individuals died after exposure to permethrin.

4.2.1 Molecular characterisation of *Anopheles* from the southwestern Nigeria

The polymerase chain reaction for species differentiation identified 76 *A. gambiae ss.* (26%) and 212 *A. arabiensis* (74%) (Fig.4.2a). At Ojoo and Ajibode all samples analysed were *A. gambiae ss.* whereas at the locality of Challenge both *A. gambiae ss* and *A. arabiensis* were found living in sympatry with 73% and 27% for *A. gambiae ss.* and *A. arabiensis* respectively. Except at Challenge where, *Anopheles* species collected were a mix of *A. gambiae* ss and *A. arabiensis* (Fig.4.2b), populations of *Anopheles* found in other surveyed localities of this region were mainly *A. arabiensis* (Table 4.3). When identified samples of *A. gambiae ss.* were subjected to PCR-form differentiation, they were all identified as "M" forms. Molecularly characterized samples of *Anopheles gambiae ss* and *arabiensis* from the surveyed localities were subjected to PCR-*kdr* for investigations on the sodium channel structure. Only 1 mosquito found in the locality of Challenge in Ibadan had a modified sodium channel at heterozygous form (RS). Analysis conducted on *A. arabiensis* samples did not show any trace of *kdr* mutations (Table 4.4). The map of molecular mechanisms of permethrin resistance developed by *Anopheles* populations in this region of Nigeria showed a high presence of non-*kdr* mutants among females that had survived permethrin exposure (Fig. 4.3)

4.2.2 Molecular characterisation of mosquito populations from the southern Benin

PCR analyses were conducted on a total of 438 female *Anopheles.* 196 of these individuals survived lethal doses of permethrin and 242 individuals died after exposure to permethrin (Table 4.5). The polymerase chain reaction for species differentiation identified 407 *A. gambiae ss*. (93%) and 31 *A. melas* (7%). No *A. arabiensis* was identified in this region of Benin (Fig. 4.4). Two members of the *A. gambiae* complex namely *A. melas* and *A. gambiae ss*. were found in sympatry in the sites of Houeyiho, Ketonou, Pahou and Grand popo. The proportions of *A. melas* at Houeyiho, Ketonou, Pahou and Grand- popo were 1%, 2%, 5% and 3% respectively whereas those of *A. gambiae ss* were respectively 99%, 98%, 95%, and 97 % (Table 4.5). When samples of *A. gambiae ss*. were subjected to PCR-form differentiation, they were all identified as "M" forms. Elevated frequencies of *kdr* alleles ranging from 0.9 to 0.5 were found in the 196 females *Anopheles* that had survived permethrin exposure (Table 4.6). The map of molecular mechanisms of permethrin resistance developed by *Anopheles* populations

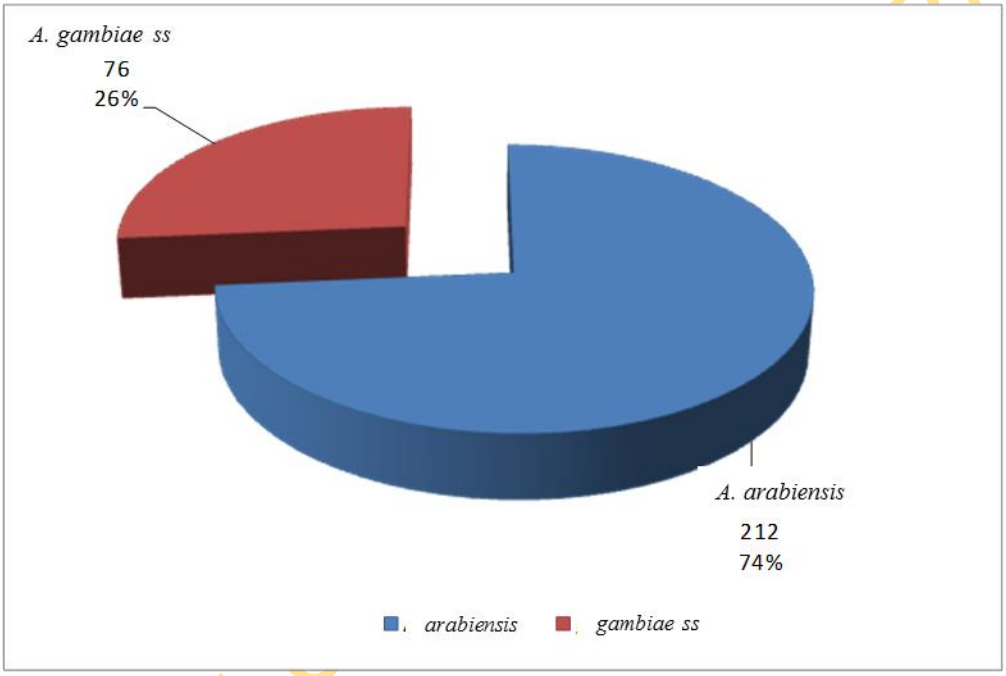

Fig. 4.2a Members of the *A. gambiae* complex identified in southwestern Nigeria

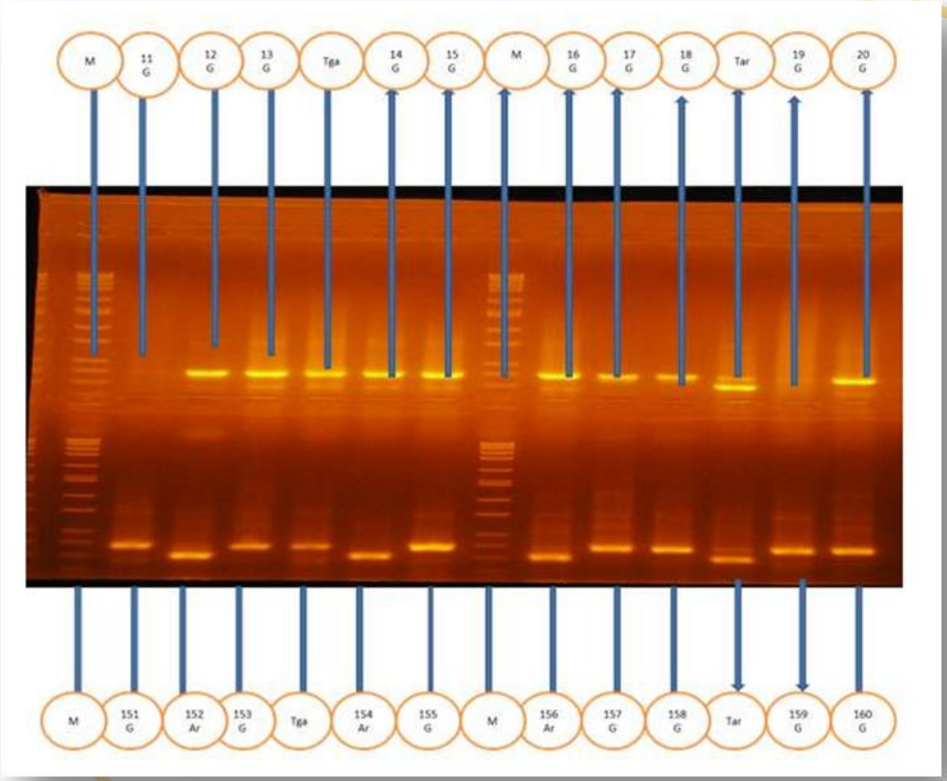

Fig. 4.2b The banding pattern of samples from Challenge, Ibadan showing *A. gambiae ss* (coded "g") and *A. arabiensis* (coded "Ar") living together in similar site.

TABLE 4.3 Distribution of members of *A. gambiae* complex in studied localities in Nigeria

Divisions	Localities	No. Tested	PCR-Species % *gambiae ss*	PCR-Species % *arabiensis*	PCR-Species % *melas*	PCR- Forms % "S"	PCR- Forms % "M"
Oyo	IITA	20	100%	-	-	-	100%
	Ojoo	30	100%	-	-	-	100%
	Ajibode	24	100%	-	-	-	100%
	Bashorun	20	100%	-	-	-	100%
	UI	18	100%	-	-	-	100%
	Challenge	30	73%	27%	-	-	100%
	Orogun	26	100%	-	-	-	100%
	Oja-tuntun	20	-	100%	-	-	-
Osun	Lagere	10	-	100%	-	-	-
	Modakeke	6	-	100%	-	-	-
Ondo	Ilesha garage	10	-	100%	-	-	-
	Owena	20	-	100%	-	-	-
Ekiti	Ati-kankan 1	10	-	100%	-	-	-
	Ati-kankan 2	10	-	100%	-	-	-
Ogun	Imowo	8	-	100%	-	-	-
	Mobafulo	10	-	100%	-	-	-
	Ogere1	2	-	100%	-	-	-
	Ogere2	8	-	100%	-	-	-
Lagos	Badagry	6	-	100%	-	-	-

TABLE 4.4 Allelic frequencies of the *kdr* mutation in *Anopheles* species from the studied localities in Nigeria

Divisions	Localities	Survivors					Dead				
		Anopheles tested	RR	RS	SS	*f-kdr*	*Anopheles* tested	RR	RS	SS	*f-kdr*
Oyo	IITA	0	-	-	-	-	0	-	-	-	-
	Ojoo	15	0	0	0	0	15	0	0	0	0
	Ajibode	8	0	0	0	0	8	0	0	0	0
	Bashorun	15	0	0	0	0	15	0	0	0	0
	UI	0	-	-	-	-	0	-	-	-	-
	Challenge	15	0	1	14	0.03	15	0	0	0	0
	Orogun	0	-	-	-	-	0	-	-	-	-
	Oja-tuntun	15	0	0	0	0	15	0	0	0	0
Osun	Lagere	4	0	0	0	0	4	0	0	0	0
	Modakeke	2	0	0	0	0	2	0	0	0	0
Ondo	Ilesha garage	8	0	0	0	0	8	0	0	0	0
	Owena	15	0	0	0	0	15	0	0	0	0
Ekiti	Ati-kankan 1	12	0	0	0	0	12	0	0	0	0
	Ati-kankan 2	9	0	0	0	0	9	0	0	0	0
Ogun	Inowo	3	0	0	0	0	3	0	0	0	0
	Mobafulo	5	0	0	0	0	5	0	0	0	0
	Ogere 1	1	0	0	0	0	1	0	0	0	0
	Ogere 2	4	0	0	0	0	4	0	0	0	0
Lagos	Badagry	1	0	0	0	0	1	0	0	0	0

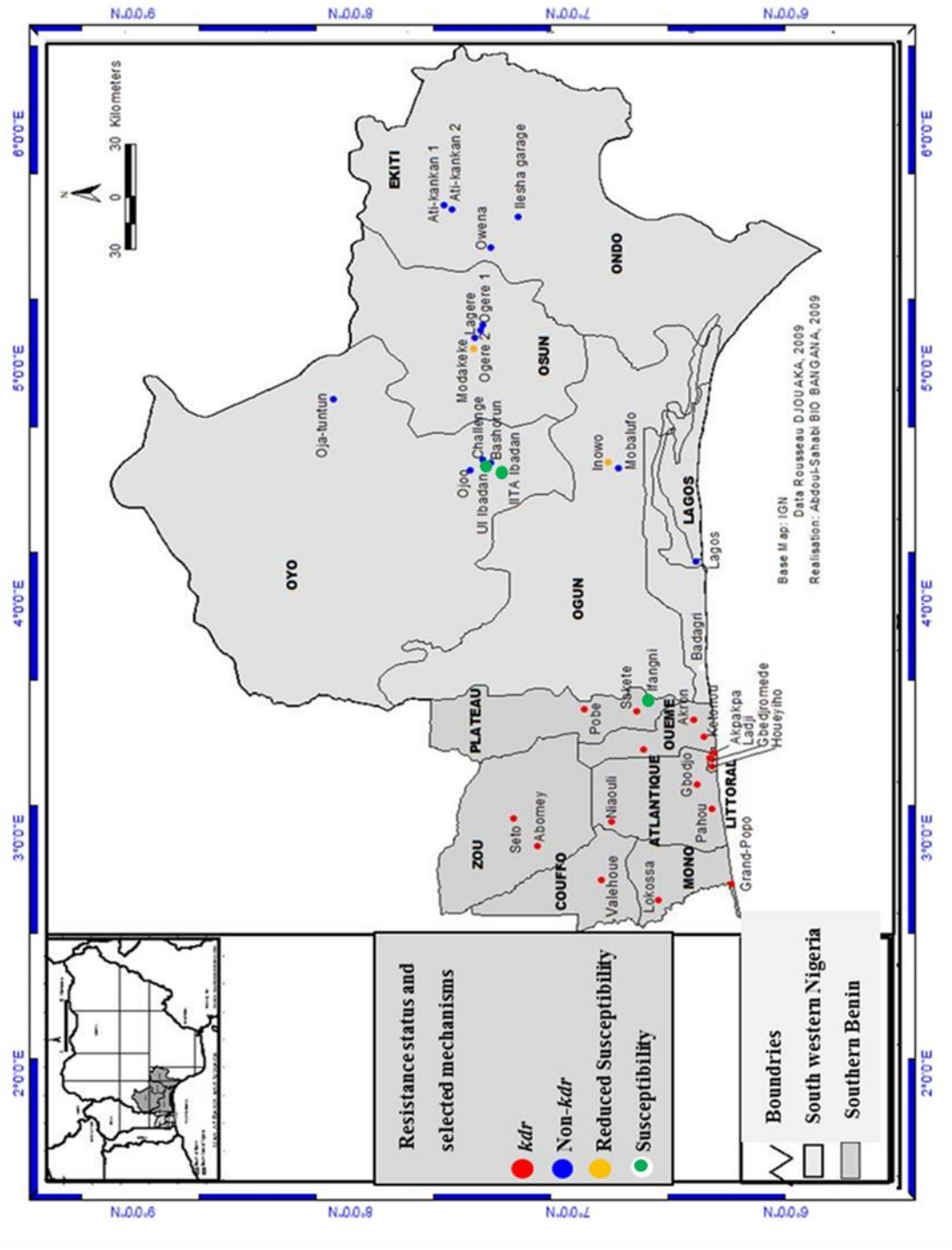

Fig. 4.3a Mechanisms of permethrin resistance identified in southwestern Nigeria and southern Benin

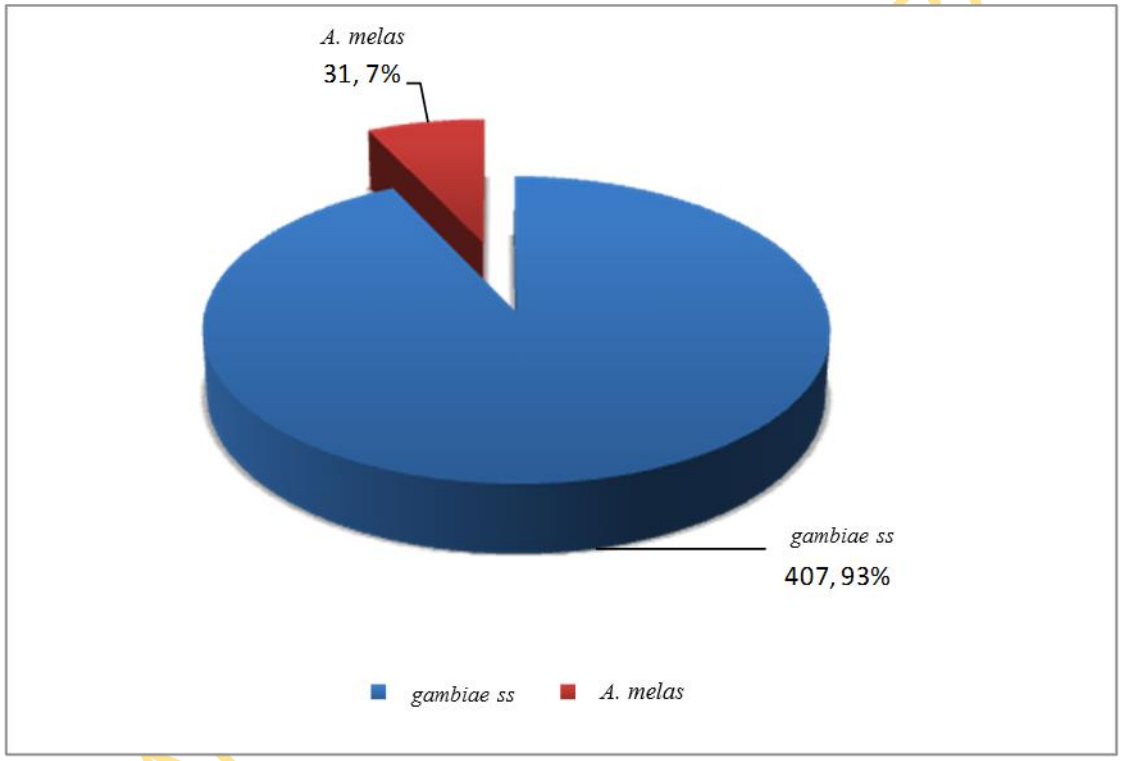

Fig. 4.4 Members of the *A. gambiae* complex identified in the southern Benin

TABLE 4.5 Distribution of members of *A. gambiae* complex in studied localities in Benin

Divisions	Localities	*Anopheles* tested	PCR- Species % *gambiae* ss	% *arabiensis*	% *melas*	PCR-Forms % S	% M
Zou	Seto	20	100%	-	-	-	100%
	Abomey	24	100%	-	-	-	100%
Plateau	Pobe	15	100%	-	-	-	100%
	Sakete	16	100%	-	-	-	100%
	Ifangni	10	100%	-	-	-	100%
Mono	Lokossa	18	100%	-	-	-	100%
	Grand- Popo	21	97%	-	3%	-	100%
Couffo	Valehoue	21	100%	-	-	-	100%
Oueme	Gogbo	23	100%	-	-	-	100%
	Akron	30	100%	-	-	-	100%
	Ketonou	30	98%	-	2%	-	100%
Atlantique	Niaouli	30	100%	-	-	-	100%
	Gbodjo	30	100%	-	-	-	100%
	Pahou	30	97%	-	5%	-	100%
	Akpakpa	30	100%	-	-	-	100%
	Ladji	30	100%	-	-	-	100%
	Gbedjromede	30	100%	-	-	-	100%
	Houeyiho	30	99%	-	1%	-	100%

TABLE 4.6 Distribution of the *kdr* alleles in *Anopheles* species from the studied localities in Benin

Divisions	Localities	Survivors					Dead				
		Anopheles tested	RR	RS	SS	*f-kdr*	*Anopheles* tested	RR	RS	SS	*f-kdr*
Zou	Seto	10	5	2	3	0.6	10	0	2	8	0.1
	Abomey	12	9	2	1	0.83	12	0	1	11	0.04
Plateau	Pobe	10	5	3	2	0.65	10	0	2	8	0.1
	Sakete	6	3	2	1	0.66	15	0	3	12	0.25
	Ifangni	0	0	0	0	0	15	0	0	15	0
Mono	Lokossa	3	1	2	0	0.66	15	0	2	13	0.33
	Grand- Popo	6	4	1	1	0.75	15	0	1	14	0.08
Couffo	Valehoue	6	2	3	1	0.58	15	0	4	11	0.33
Oueme	Gogbo	8	5	2	1	0.75	15	0	1	14	0.06
	Akron	15	5	8	2	0.6	15	0	6	9	0.2
	Ketonou	15	8	5	2	0.7	15	1	3	11	0.16
Atlantique	Niaouli	15	5	8	2	0.6	15	0	2	13	0.06
	Gbodjo	15	10	3	2	0.76	15	0	3	12	0.1
	Pahou	15	12	2	1	0.86	15	0	1	14	0.03
	Akpakpa	15	8	4	3	0.66	15	0	5	10	0.16
	Ladji	15	13	2	0	0.93	15	0	6	9	0.2
	Gbedjromede	15	10	5	0	0.83	15	1	4	10	0.2
	Houeyiho	15	5	9	1	0.63	15	1	3	11	0.16

in this southern region of Benin showed a high presence of *kdr* mutants among females that had survived permethrin exposure (Figs. 4.3a and 4.3b)

4.3 Evaluation of potential contributions of agricultural pesticides to selection of pyrethroid resistance in *Anopheles* populations breeding around vegetable farms

Several analyses of soil and water samples were conducted in the laboratory to assess the presence of pesticide residues in water bodies surrounding farms under synthetic pesticides treatments.

4.3.1 The use of synthetic pesticides in the vegetable farm of Houeyiho in Benin

In the vegetable farm of Houeyiho, all the 20 farmers interviewed agreed that it is impossible to grow vegetables without using pesticides. Some vegetables like cucumber, lettuce and green cabbages are very sensitive to pests and have to be treated very often to avoid pests attack. Farmers also agreed that without pesticide treatments, the yield is very low and most vegetables are destroyed before getting to maturity. Among the pesticides used, 2 were the most mentioned: the Decis® (deltamethrin EC 10.75 g/l), and Kinikini® (mixture of cyfluthrin and malathion) (Table 4.7). Other local products made from neem (*Azadirachta indica*) seeds and papaya leaves were also mentioned by farmers as being effective in controlling cabbage nematodes. The supply chain for pesticides was also probed but very few farmers could provide information on where and how they got the pesticides; more than 50% of interviewed farmers got the pesticides from informal or non-registered suppliers. In most cases, vegetable farmers received a basic training from extension workers of the ministry of agriculture in Benin. The training is focussed on pest management strategies such as the recognition of pests, periods of treatment, the type of pesticides to use and, the basic health safety measures. More than 30% of interviewed farmers do not respect manufacturer's instructions on the use of pesticides. In the vegetable farm at Houeyiho, cabbages were treated 2 times each week and during the 65 days for the maturity of cabbage; this corresponds to an average of 18 treatments for each complete round of vegetables development. Investigations in vegetable farms also revealed that 72 treatments of pesticides are done annually at Houeyiho.

4.3.2 The use of synthetic pesticides in the vegetable farm of Ajibode in Nigeria

At Ajibode, farmers also agreed that it was impossible to grow vegetables without using pesticides. The pesticides mainly used by farmers are lambda cyhalothrin

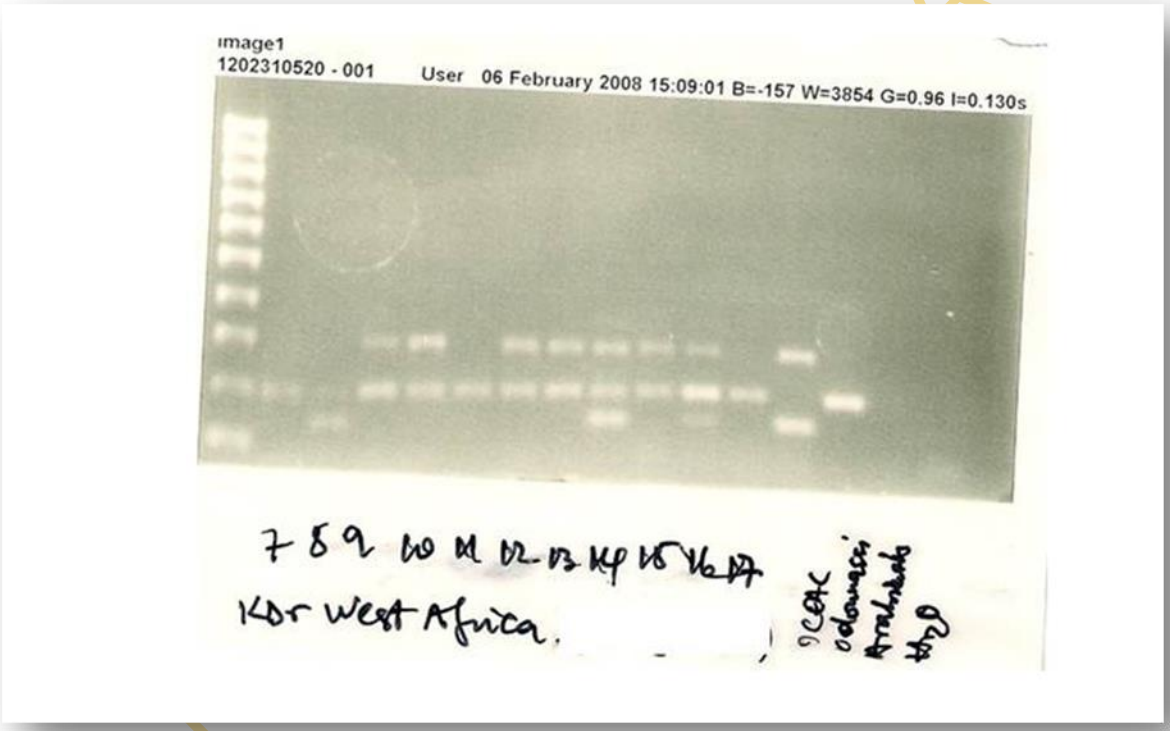

Fig. 4.3b The *kdr* Banding pattern recorded with mosquito samples from Benin

commercially known as Karate® and cypermethrin known as Cyper-Di-force®. No traditional mixture of plant leaves and seeds is used for vegetable pests control at Ajibode (Table 4.7). Empty containers of pesticides were found at the Ajibode farm. Trainings on good practices for pests management in vegetable farms are not properly organized at Ajibode. Farmers use pesticides irrespectively of the indicated doses. The number of pest treatments in vegetable farming increases with the intensity of pests attack recorded in the farms.

4.3.3 Susceptibility to permethrin of *A. gambiae* collected around vegetable farms of Houeyiho and Ajibode

76 and 85 females *Anopheles* collected around vegetable farms of Houeyiho and Ajibode were exposed to permethrin impregnated papers. Mortality rates of 70% and 90% were recorded at Houeyiho and Ajibode respectively. *Anopheles* populations collected around the vegetable farms were recorded to be resistant to permethrin (Table 4.2)

4.3.3.1 Assessment of the presence of pesticide residues in *Anopheles* breeding sites found in surveyed vegetable farms through monitoring of eggs hatching rates

The control breeding sites used in this experiment (the control breeding site made of well water collected at the CREC and referred here as CREC-water) offered favourable conditions for the hatching of eggs of both *A. gambiae* from Kisumu and Ladji (more than 70% hatching rates for both strains). When 10 g of surface soil from agricultural areas under insecticide treatments were added into 1000 ml of water of the control breeding, a decrease in hatching rates was observed with both strains but more significantly with the susceptible strain *A. gambiae* from Kisumu. The hatching rates of *A. gambiae* from Kisumu and *A. gambiae* from Ladji dropped from 75% and 86% in control breeding sites to 7% and 37% respectively in the test breeding sites (Fig. 4.5). The statistical comparison of both results revealed a relatively high toxic impact of the simulations on *A. gambiae* from Kisumu eggs (P=0.000). Data from this experiment revealed the inhibition of the hatching rate of *Anopheles* eggs in breeding sites reconstituted with soil samples collected in vegetable farms under synthetic pesticide treatments. With breeding sites reconstituted with soil from irrigation pool mixed with the irrigation water, a relatively low hatching inhibition was recorded. Both strains *A. gambiae* from Kisumu and *A. gambiae* from Ladji gave hatching rates of 58%

TABLE 4.7 The use of pesticides in the vegetable farm of Houeyiho in Benin and Ajibode in Nigeria

	Locations	
Vegetable farming practices	**Houeyiho (Benin)**	**Ajibode (Nigeria)**
Main synthetic insecticide used	Decis® (Deltamethrin EC 10,75 g/l) Kinikini® (cyfluthrin + malathion)	Karate® (lambda cyhalothrin) Cyper-Di force® (Cypermethrin)
Locally made insecticides	Neem extracts+ papaya leaves and grains	No extract identified
Treatment doses for pests control in vegetable farms	40 g of (Neem + papaya mixture) is diluted in 15L of water	About 50ml of the product (Karate®) is diluted in 15 L of water
Frequency of vegetable farms treatment with synthetic and local pesticides	The number of treatment is high, and depends on the level of pest attacks	The number of treatment is high, and depends on the level of pest attacks
Types of vegetables grown by farmers	*Brassica oleracea, Amaranthus blitoides, Daucus carota, Cucumis sativus, Lactuca sativa*	*Brassica oleracea, Amaranthus blitoides,*
Origin of pesticides used by farmers	Formal and informal chain supply system	Pesticides used by farmers are obtained commercially

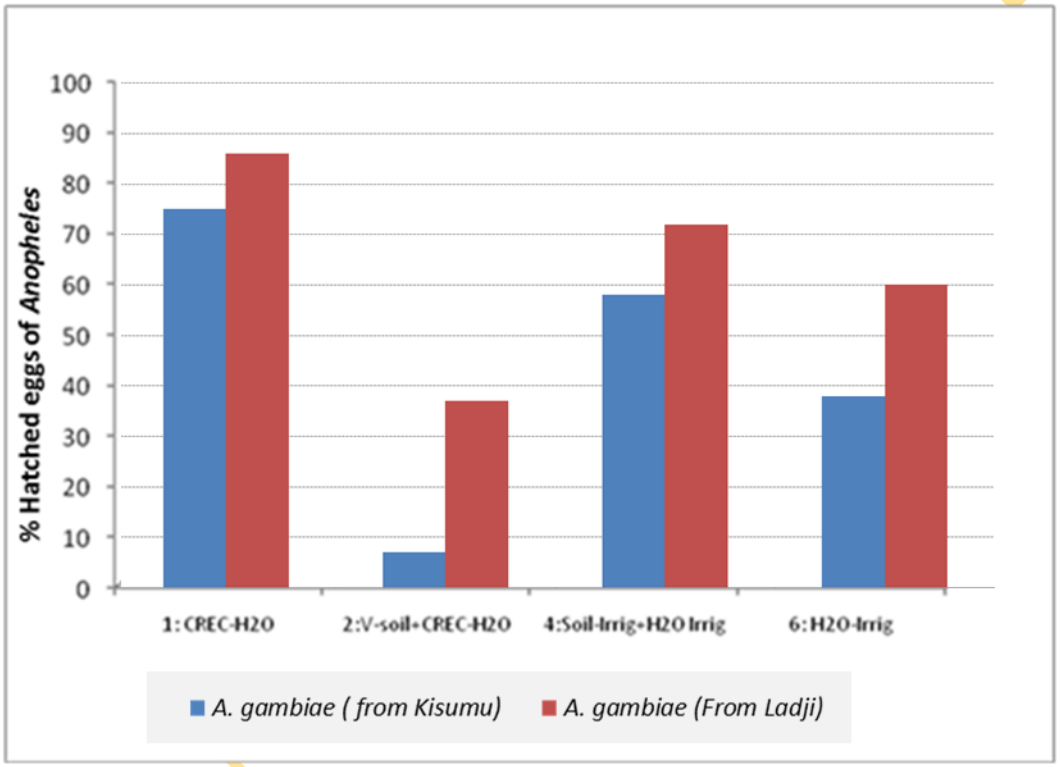

Fig. 4.5 Hatching rate of resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Houeyiho

and 72% respectively (Fig. 4.5). In the last simulation made with irrigation water alone, the recorded hatching rate for eggs of *A. gambiae* from Kisumu and *A. gambiae* from Ladji was 38% and 60% respectively (Fig. 4.5).

For samples from the vegetable site at Ajibode, Nigeria, the control breeding sites used was UI-water collected from a spring identified in the Zoological garden. This control offered favourable conditions for the hatching of eggs of both *A. gambiae* from Kisumu and *A. gambiae* from Ladji (70% and 72% hatching rates respectively). When 10 g of the surface soil from the agricultural area of Ajibode was added into 1000 ml of water from the control site (UI-water), a remarkable inhibition of the hatching rates of *A. gambiae* from Kisumu and *A. gambiae* from Ladji was recorded. The hatching rates dropped from 70% and 72% as earlier recorded in control breeding sites to 5% and 20% respectively in the test breeding sites (Fig. 4.6).

In the second set of simulated breeding sites reconstituted with soil from the irrigation pool mixed with the irrigation water, a relatively low hatching inhibition was recorded. Hatching rates of 55% and 65% were recorded with eggs of *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively (Fig. 4.6).

In the third simulation made exclusively with irrigation water from the Ajibode farm, the hatching rate for eggs of *A. gambiae* from Kisumu and *A. gambiae* from Ladji was 30% and 55% respectively.

4.3.3.2 Assessment of the presence of pesticides residues in *Anopheles* breeding sites found in surveyed vegetable farms through monitoring of larval development rates

The monitoring of larval development rates in the control samples made of well water from the CREC (CREC-water), showed that 98% and 92% of larvae of *A. gambiae* from Ladji and *A. gambiae* from Kisumu respectively got to the pupae stage (Fig. 4.7). In the test breeding sites constituted of samples from the agricultural settings of Houeyiho, a decrease in the development rate was recorded in the two simulations made in the laboratory. In the first test simulation made of soil from the vegetable farm diluted with CREC-water, 42% and 61% of *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively got to the pupae stage. In the second test simulation made of wet soil and irrigation water from the vegetable farm, 85% and 96% of *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively got to the pupae stage (Fig. 4.7).

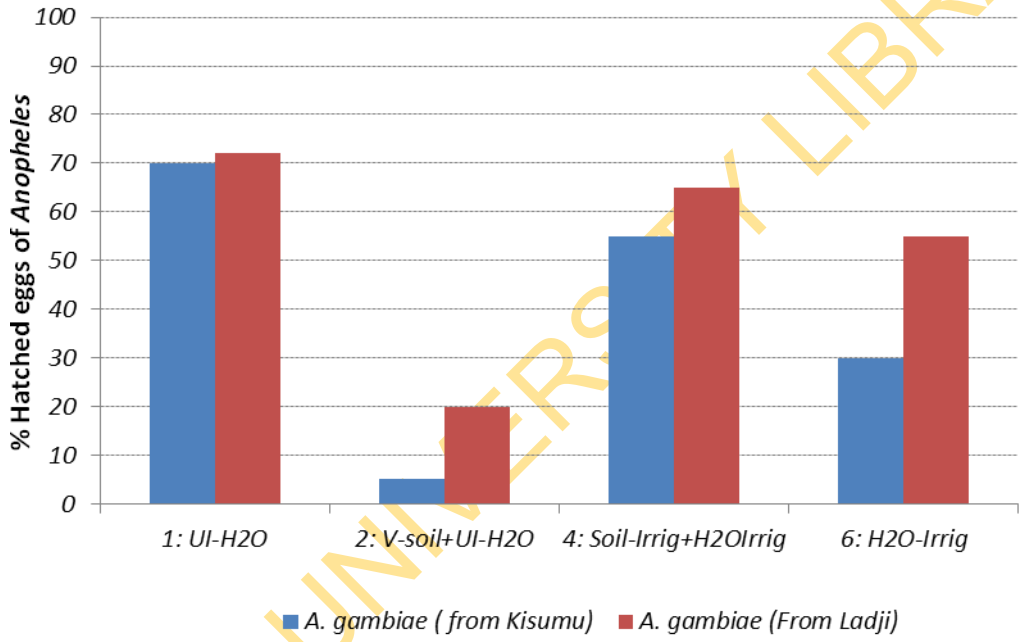

Fig. 4.6 Hatching rate of resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Ajibode

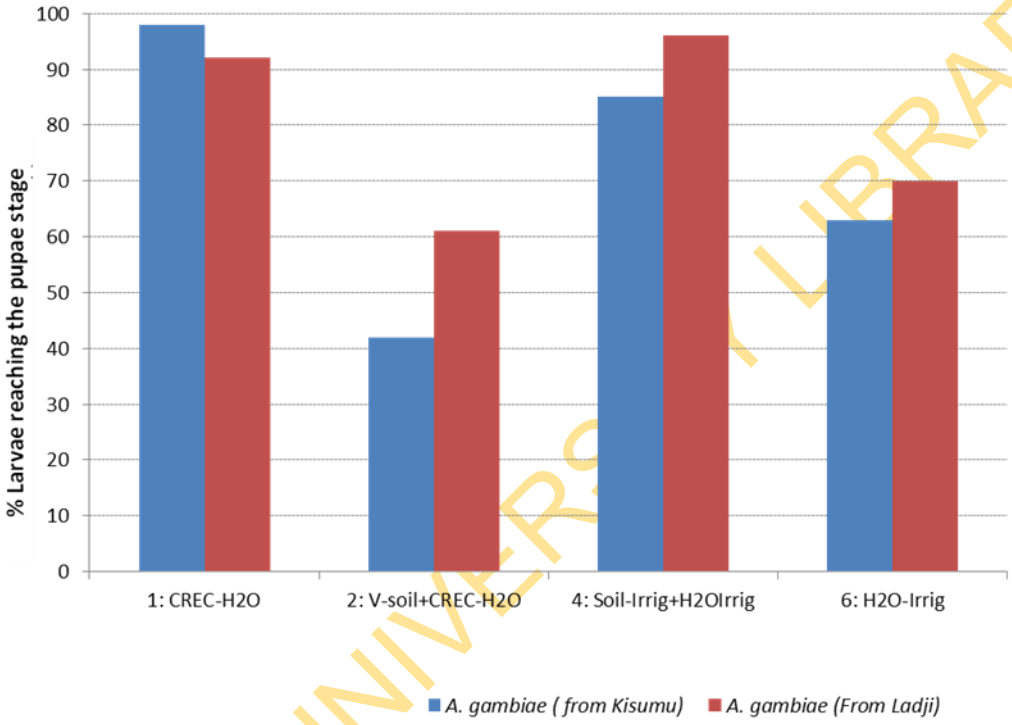

Fig. 4.7 Larval development of resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Houeyiho

In the third set of test breeding sites made of irrigation water alone, 63% and 70% larval development rates were recorded for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively (Fig. 4.7).

In the vegetable site of Ajibode, the control breeding sites made with 1000 ml water collected from a spring flowing in the Zoological garden (UI-water) gave a mean percentage of 90% and 89% larvae of *A. gambiae* from Ladji and *A. gambiae* from Kisumu respectively getting to the pupae stage (Fig. 4.8). When 10 g of the surface soil from the agricultural area of Ajibode was added to 1000 ml of water of the control breeding site, the development of larvae showed a relatively low rate of first instars larvae reaching the pupae stage: 40% and 50% for larvae of *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively (Fig. 4.8).

In breeding sites reconstituted with soil from irrigation pool mixed with the irrigation water from Ajibode, 75% and 80% of larvae of *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively reached the pupae stage (Fig. 4.8).

In the last set of breeding sites made exclusively with the irrigation water from Ajibode farm, 55% and 60% of larvae of *A. gambiae* from Ladji and *A. gambiae* from Kisumu respectively got to pupae stage (Fig. 4.8).

4.3.3.3 Assessment of the presence of pesticides residues in *Anopheles* breeding sites found in surveyed vegetable farms through monitoring mosquito productivity

The proportions of *A. gambiae* eggs reaching adult stage were low in simulated breeding sites. In the control breeding site made with CREC-water, 46% and 48% of the 200 eggs of *A. gambiae* from Kisumu and the 200 eggs of *A. gambiae* from Ladji respectively reached the adult stage. In breeding sites containing 100 g of surface soil from the vegetable site of Houeyiho, the yield of adult production dropped significantly to 2% and 21% for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively compared to the yield recorded in the control breeding sites (46% and 48% for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively) (Fig. 4.9). When eggs were inoculated into the artificial breeding sites made with soil and watering water from the agricultural site of Houeyiho, an adult production of 49% and 68% was recorded for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively. In the last set of breeding sites reconstituted with water of irrigation from the vegetable farm of Houeyiho, 20% of

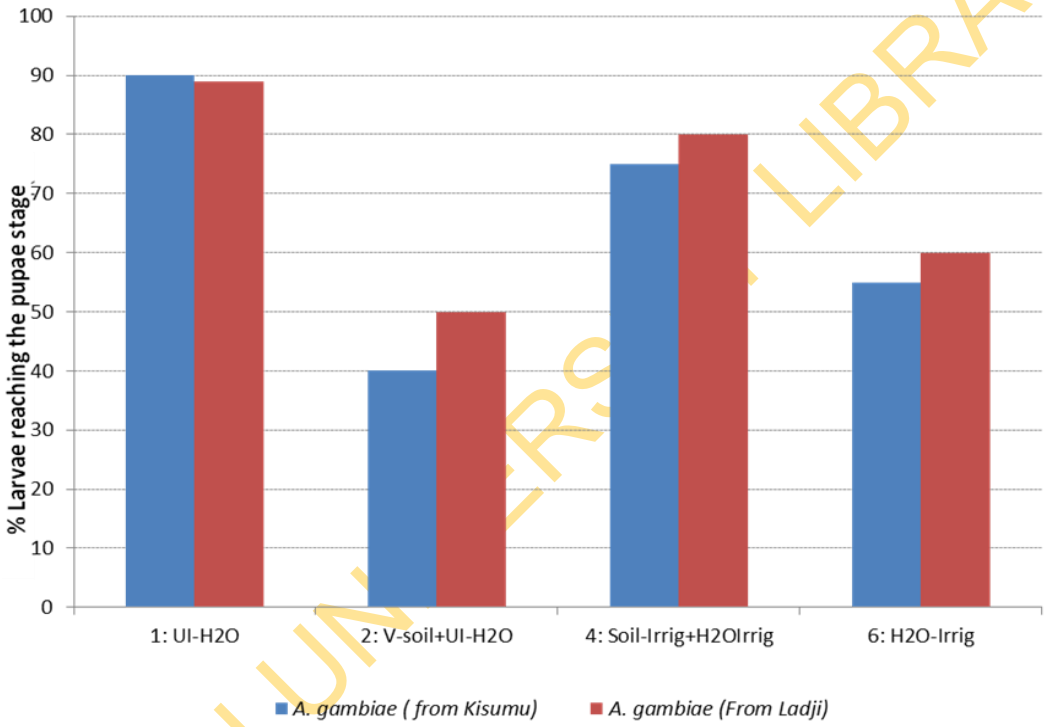

Fig. 4.8 Larval development of resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Ajibode

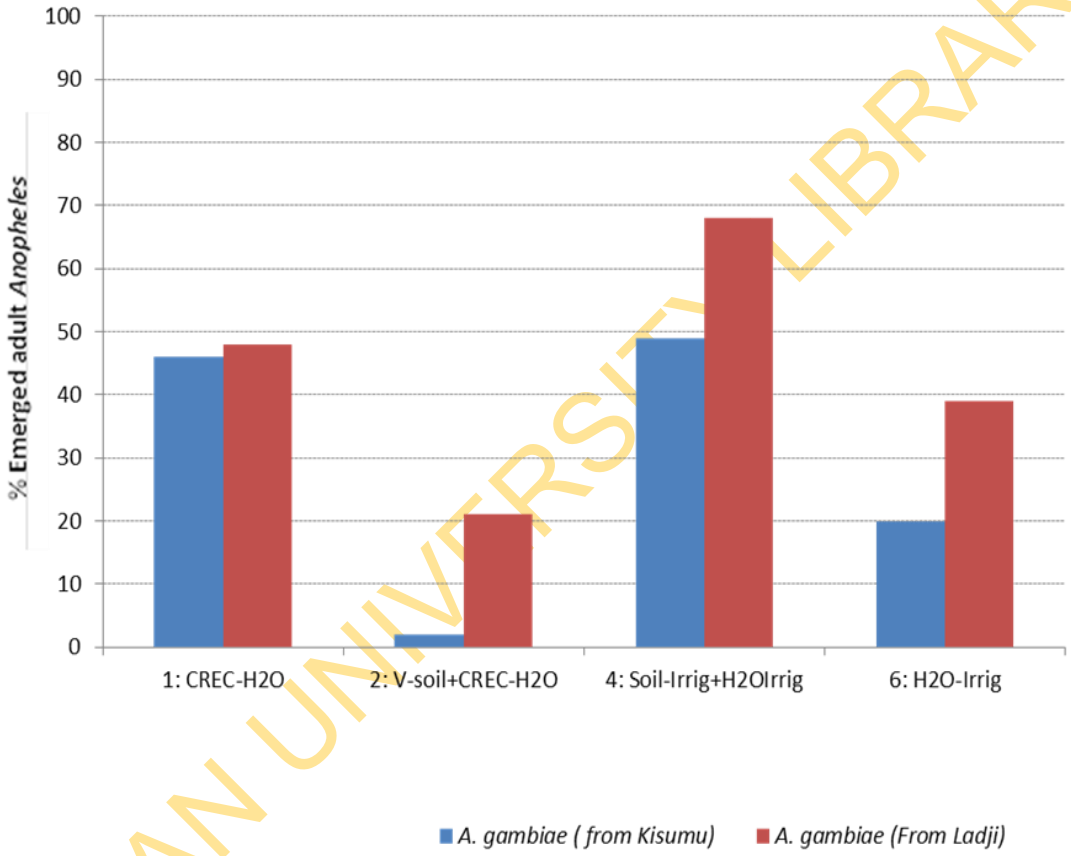

Fig. 4.9 The yield of rearing resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Houeyiho

adults of *A. gambiae* from Kisumu and 39% of *A. gambiae* from Ladji were produced from the 200 eggs inoculated. The growth of the resistant strain *A. gambiae* from Ladji was less affected by simulations than that of the susceptible strain *A. gambiae* from Kisumu. The yield of resistant strains was always high compared to susceptible strains: 2%, 49% and 20% for *A. gambiae* from Kisumu versus 21%, 68% and 39% for *A. gambiae* from Ladji in the various simulated breeding sites (Fig. 4.9).

With samples from Ajibode, the proportions of adults *Anopheles* recorded in the control breeding site (UI-water) were 50% and 53% of the 200 eggs of *A. gambiae* from Kisumu and the 200 eggs of *A. gambiae* from Ladji respectively. In breeding sites containing 100 grams of surface soil from the vegetable site at Ajibode, the yield of adult production was very low with both strains: 5% and 10% for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively *A. gambiae* from Ladji (Fig. 4.10). When eggs were inoculated into breeding sites reconstituted with soil and watering water from the agricultural site of Ajibode, an adult production of 35% and 60% was recorded for *A. gambiae* from Kisumu and *A. gambiae* from Ladji respectively (Fig. 4.10). In breeding sites made of irrigation water from the vegetable farm of Ajibode, 15% of adults of *A. gambiae* from Kisumu and 30% of *A. gambiae* from Ladji were produced from the 200 eggs inoculated (Fig. 4.10).

4.4 Evaluation of the potential contributions of spilled petroleum products in the selection of pyrethroid resistance in *A. gambiae* populations

Several experiements were conducted in the field and the laboratory to assess the lethal effect of petroleum products on mosquito larvae, their modes of action on mosquito larvae and their contributions in the selection of pyrethroid resistance in malaria vectors.

4.4.1 The empirical utilisation of petroleum products for mosquito control in rural communities of the southern Benin

The use of petroleum products for mosquito control in rural communities of the southern Benin was confirmed through interviews conducted in 4 communities Gbodjo, Ladji, and Ketonou. Petroleum products are used against several insects of great nuisance like: mosquito larvae, flies and cockroaches. The petroleum products mainly used in these communities are kerosene and waste engine oil from mechanics. Others, such as petrol and engine oil, are said by the interviwed individuals to be used occasionally.

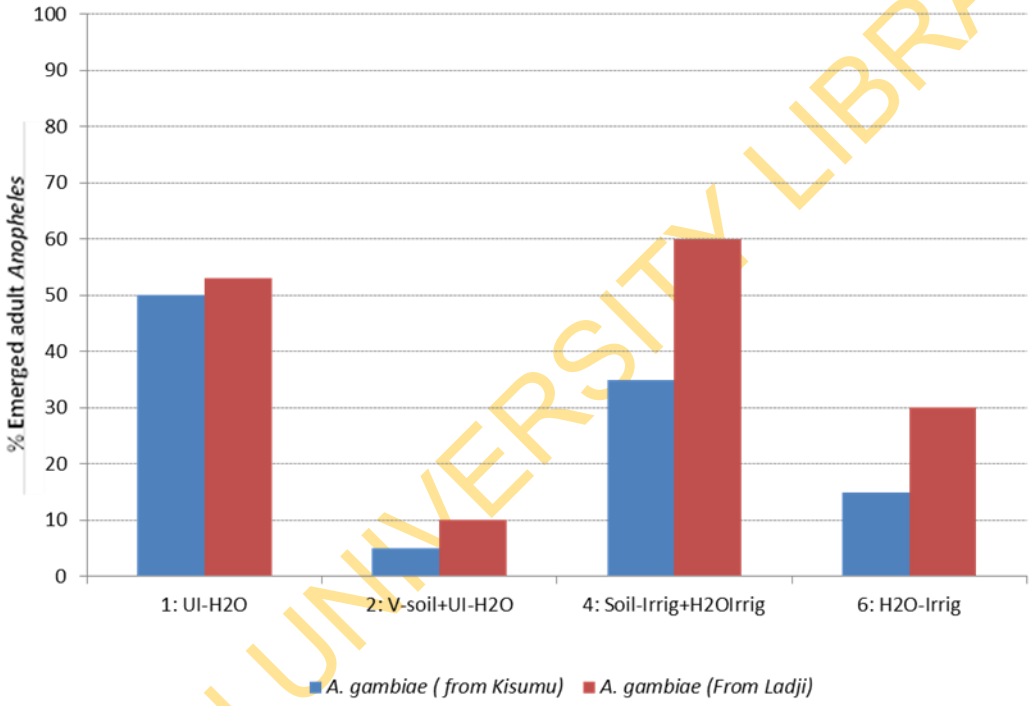

Fig. 4.10 The yield of rearing resistant and susceptible strains of *Anopheles* in simulated water and soil samples from vegetable farms of Ajibode, Ibadan

These products are either sprayed on the ground, on table surfaces, on standing water points and in latrines. Out of a total number of 65 key respondents interviewed in the three communities, 73% spray kerosene in standing water points and latrines to control mosquito growth and nuisance, whereas 9% use waste engine oil, 5% engine oil and 2% petrol (Fig. 4.11). Information extracted from the interviewed populations also showed that 11% do not know much about using petroleum products for pests control. This investigation also revealed that this traditional technique of controlling vectors using petroleum products is transferred in the community from parents to offspring without any scientific explanation to support the efficacy of this strategy.

4.4.2 Analysis of the lethal effect of petrol on permethrin resistant larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo

Experiments conducted in the laboratory on the larvicidal activities of petrol on larvae of *A. gambiae* from Ladji showed 100% mortality when breeding sites contained in laboratory bowls were treated with petrol at concentrations of $7.856 \times 10^{-3}\mu l/cm^2$. The lowest concentration (LoC_{100}) of petrol capable of producing 100 % mortality was $7.856 \times 10^{-3}\mu l$ for each cm^2 of treated breeding sites (Fig. 4.12.). The highest concentration (HiC) of petrol yielding no larvicidal activity on the resistant strain *A. gambiae* from Ladji was $11.8 \times 10^{-3}\mu l$ for each cm^2 of sprayed surface. The LC_{50} of petrol on larvae of *A. gambiae* from Ladji was $2.946 \times 10^{-3}\mu l/cm^2$. Treatments of breeding sites with concentrations of petrol below $7.856 \times 10^{-3}\mu l/cm^2$ did not kill 100% of larvae of *A. gambiae* from Ladji but rather selects some survivals (Fig. 4.12.). When this experiment was conducted with the permethrin resistant strain *A. gambiae* from Ojoo, the larvicidal activities of petrol on this strain showed 100% mortality when breeding sites were sprayed with petrol at concentration of $10.120 \times 10^{-3}\mu l/cm^2$. The lowest concentration (LoC_{100}) of petrol capable of producing 100% mortality was $10.120 \times 10^{-3}\mu l$ for each cm^2 of treated breeding site.

The highest concentration (HiC) of petrol yielding no larvicidal activity on the resistant strain *A. gambiae* from Ojoo was recorded at concentration of $393 \times 10^{-3}\mu l$ for each cm^2 of sprayed surface. The LC_{50} of petrol on larvae of *A. gambiae* from Ojoo was $7856 \times 10^{-3}\mu l/cm^2$. Treatments of breeding sites with concentrations of petrol below $10.120 \times 100^{-3}\mu l/cm^2$ kill less than 100% of larvae of *A. gambiae* from Ojoo and therefore

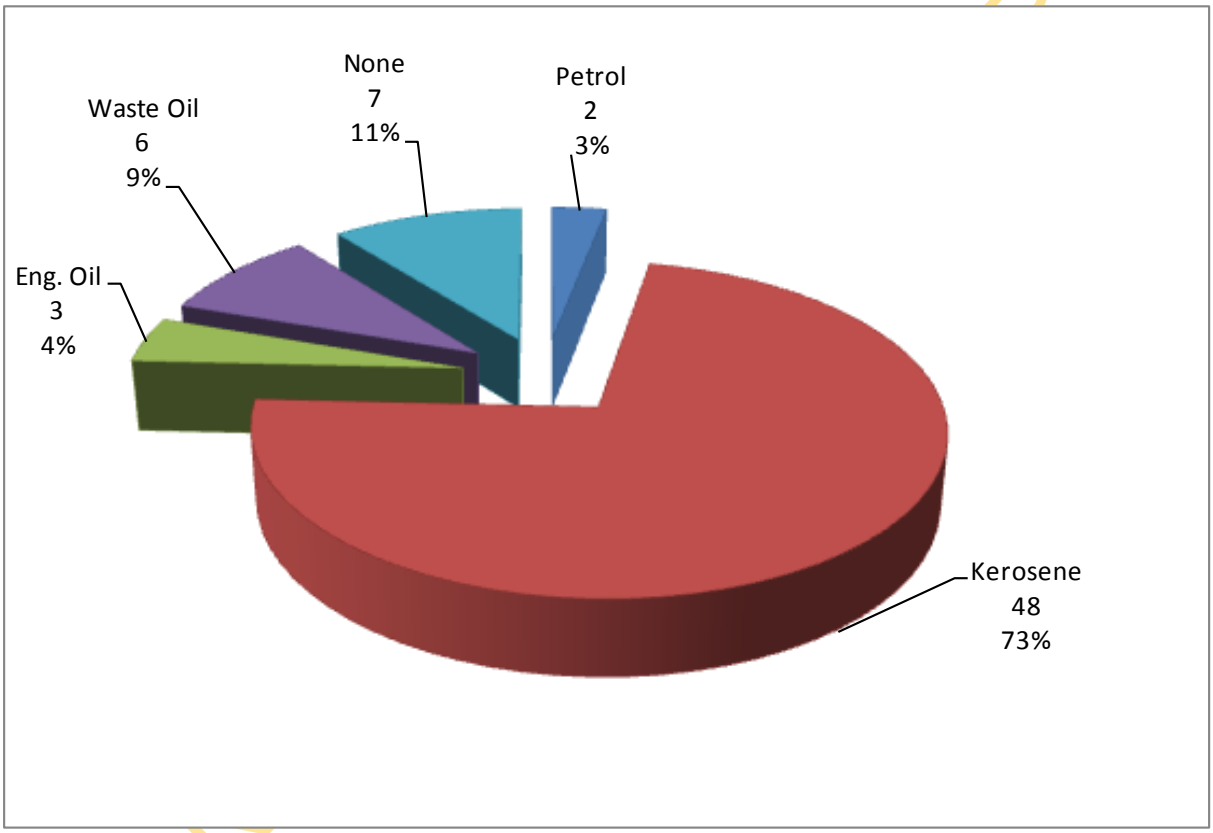

Fig. 4.11 Utilisation of petroleum products for mosquito control in rural communities in southern Benin

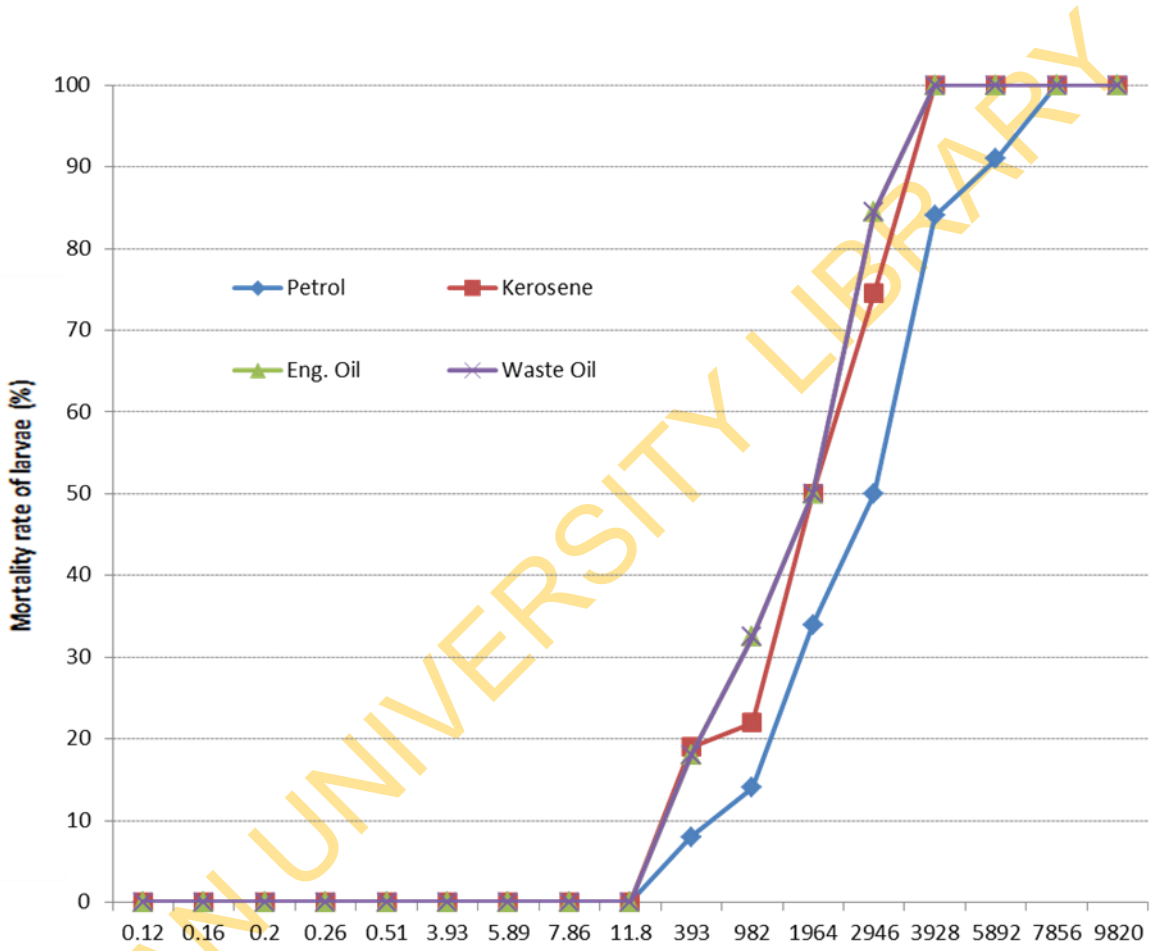

Fig. 4.12 Larvicidal effect of petroleum products on larvae of *A. gambiae* from Ladji.

select some survivals (Fig. 4.13).

4.4.3 Analysis of the lethal activity of kerosene on permethrin resistant larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo

The treatment of breeding sites with kerosene was very effective on larvae of *A. gambiae* from Ladji at concentrations ranging from 11.8 × 10-3µl to 3.930 × 10^{-3}µl per cm^2. At these concentrations, larval mortalities increased from 0% to 100% (Fig. 4.12). Larvicidal activities of kerosene on larvae of *A. gambiae* from Ladji showed 100% mortality when breeding sites contained in laboratory bowls were mixed with petrol at concentration of 3.930 × 10^{-3}µl/cm^2. The lowest concentration (LoC_{100}) of kerosene capable of producing 100% mortality was, therefore, recorded at 3.930 × 10^{-3}µl for each cm^2 of treated breeding site (Fig. 4.12). The highest concentration (HiC) of kerosene yielding no larvicidal activity on the resistant strain *A. gambiae* from Ladji was recorded at concentrations of 11.8 × 10^{-3}µl for each cm^2 of sprayed surface.

The LC_{50} of kerosene on larvae of *A. gambiae* from Ladji was 1.964 × 10^{-3}µl/cm^2. Treatments of breeding sites with concentrations of kerosene below 3.930 × 10^{-3}µl/cm^2 (the LoC_{100}) did not kill 100% of larvae of *A. gambiae* from Ladji but rather selected some survivals (Fig. 4.12).

When this experiment was repeated on the permethrin resistant strain *A. gambiae* from Ojoo, the larvicidal activity of kerosene on this strain showed 100% mortality when breeding sites were mixed with kerosene at concentrations of 10.020 × 10^{-3}µl/cm^2. The lowest concentration (LoC_{100}) of kerosene capable of producing 100% mortality was 10.020 × 10^{-3}µl for each cm^2 of treated breeding sites (Fig. 4.13). The highest concentration (HiC) of kerosene yielding no larvicidal activity on the resistant strain *A. gambiae* from Ojoo was 393 × 10^{-3}µl for each cm^2 of treated surface. The LC_{50} of kerosene on larvae of *A. gambiae* from Ojoo was 5892 × 10^{-3}µl/cm^2. Treatments of breeding sites with concentrations of kerosene below 10.020 × 100^{-3}µl/cm^2 killed less than 100% of larvae *A. gambiae* from Ojoo and resulted in the selection of few survivals (Fig. 4.13).

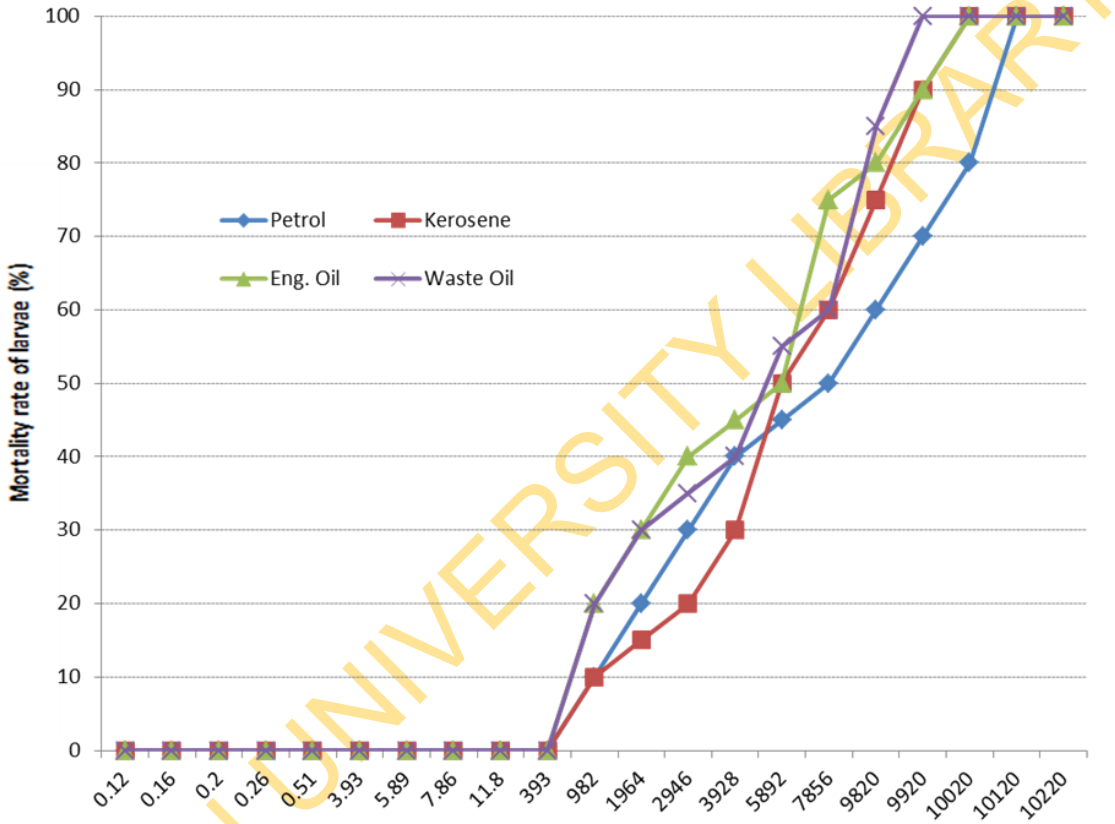

Fig. 4.13 Larvicidal effect of petroleum products on larvae of *A. gambiae* from Ojoo

4.4.4 Analysis of the lethal effect of new engine oil on permethrin resistant larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo

Engine oil was effective on the resistant strain *A. gambiae* from Ladji after treatments of simulated breeding sites with concentrations between $11.8 \times 10^{-3}\mu l/cm^2$ and $3.930 \times 10^{-3}\mu l/cm^2$. At these concentrations, larval mortalities increased from 0% to 100% (Fig. 4.12). Larvicidal activities of engine oil on larvae of *A. gambiae* from Ladji showed 100% mortality when breeding sites contained in laboratory bowls were treated with engine oil at concentration of $3.930 \times 10^{-3}\mu l/cm^2$. The lowest concentration (LoC_{100}) of engine oil capable of producing 100% mortality was $3.930 \times 10^{-3}\mu l$ for each cm^2 of treated breeding site (Fig. 4.12). The highest concentration (HiC) of engine oil yielding no larvicidal activity on the resistant strain *A. gambiae* from Ladji was $11.8 \times 10^{-3}\mu l$ for each cm^2 of treated surface. The LC_{50} of engine oil on larvae of *A. gambiae* from Ladji was $1.964 \times 10^{-3}\mu l/cm^2$. Treatments of breeding sites with concentrations of engine oil below $3.930 \times 10^{-3}\mu l/cm^2$ (the LoC_{100}) killed less than 100% of larvae of *A. gambiae* from Ladji and resulted in the selection of few individuals (Fig. 4.12).

When this experiment was replicated with the permethrin resistant strain *A. gambiae* from Ojoo, the larvicidal activity of engine oil on this strain showed 100% mortality when breeding sites were treated with engine oil at concentration of $10.020 \times 10^{-3}\mu l/cm^2$. The lowest concentration (LoC_{100}) of engine oil capable of producing 100% mortality was $10.020 \times 10^{-3}\mu l$ for each cm^2 of treated breeding sites (Fig. 4.13). The highest concentration (HiC) of engine oil yielding no larvicidal activity on the resistant strain *A. gambiae* from Ojoo was recorded at concentration of $393 \times 10^{-3}\mu l$ for each cm^2 of sprayed surface (Fig. 4.13). The LC_{50} of engine oil on larvae of *A. gambiae* from Ojoo was $5.892 \times 10^{-3}\mu l/cm^2$. Treatments of breeding sites with concentrations of engine oil below $10.020 \times 110^{-3}\mu l/cm^2$ killed less than 100% of larvae of *A. gambiae* from Ojoo and resulted in the selection of few individuals survivals (Fig. 4.13).

4.4.5 Analysis of the lethal activity of used engine oil on permethrin resistant larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo

Treatments with waste engine oil exhibited mortalities of 100% at concentrations equal or higher than $3.930 \times 10^{-3}\mu l/cm^2$. This concentration represents the lowest concentration of used engine oil (LoC100) producing 100% mortalities on larvae of *A.*

gambiae from Ladji (Fig. 4.12). The highest concentration (HiC) of used engine oil yielding no larvicidal activity on the resistant strain *A. gambiae* from Ladji was 11.8×10^{-3}µl for each cm^2 of treated surface. The LC_{50} of used engine oil on larvae of *A. gambiae* from Ladji was 1.964×10^{-3}µl/cm^2. Treatments of breeding sites with concentrations of used engine oil below 3.930×10^{-3}µl/cm^2 (the LoC_{100}) killed less than 100% of larvae of *A. gambiae* from Ladji and resulted in the selection of few individuals (survivals post treatments) (Fig. 4.12).

When this experiment was replicated with the permethrin resistant strain *A. gambiae* from Ojoo, the larvicidal activity on this strain showed 100% mortality when breeding sites were treated with used engine oil at concentration of 10.020×10^{-3}µl/cm^2. The lowest concentration (LoC_{100}) of waste engine oil capable of producing 100% mortality was recorded at 10.020×10^{-3}µl for each cm^2 of treated breeding sites (Fig. 4.13). The highest concentration (HiC) of used engine oil yielding no larvicidal activity on the resistant strain *A. gambiae* from Ojoo was 393×10^{-3}µl for each cm^2 of treated surface (Fig. 4.13). The LC_{50} of used engine oil on larvae of *A. gambiae* from Ojoo was 5892×10^{-3}µl/cm^2. Treatments of breeding sites with concentrations of engine oil below 10.020×100^{-3}µl/cm^2 killed less than 100% of larvae of *A. gambiae* from Ojoo and resulted in the selection of few individuals (Fig. 4.13).

The comparison of activities of the 4 petroleum products on permethrin resistant larvae of *A. gambiae* from Ladji and *A. gambiae* from Ojoo showed a relatively low lethal activity of petrol on *Anopheles* larvae (Figs. 4.12 and 4.13) as recorded with the determined HiC and LoC values (Figs. 4.14 and 4.15).

4.4.6 Identification of the mode of action of PP on *A. gambiae* larvae

A. gambiae from Ladji was chosen in this experiment because of its relatively consistent susceptibility to petroleum products compare to *A. gambiae* from Ojoo. The analysis of the mode of action of petroleum products on larvae of *A. gambiae* from Ladji showed that 100% (n = 400 exposed larvae) of larvae were killed in laboratory bowls containing water with mixtures of petroleum products collected in the field with oil pellicle (oil film) covering the surface of the water. However, when this pellicle was completely removed through series of sieving, the mortality decreased to 96% therefore, associating 4% of larval mortalities to suffocations from oil pellicle developed as a result of PP present in

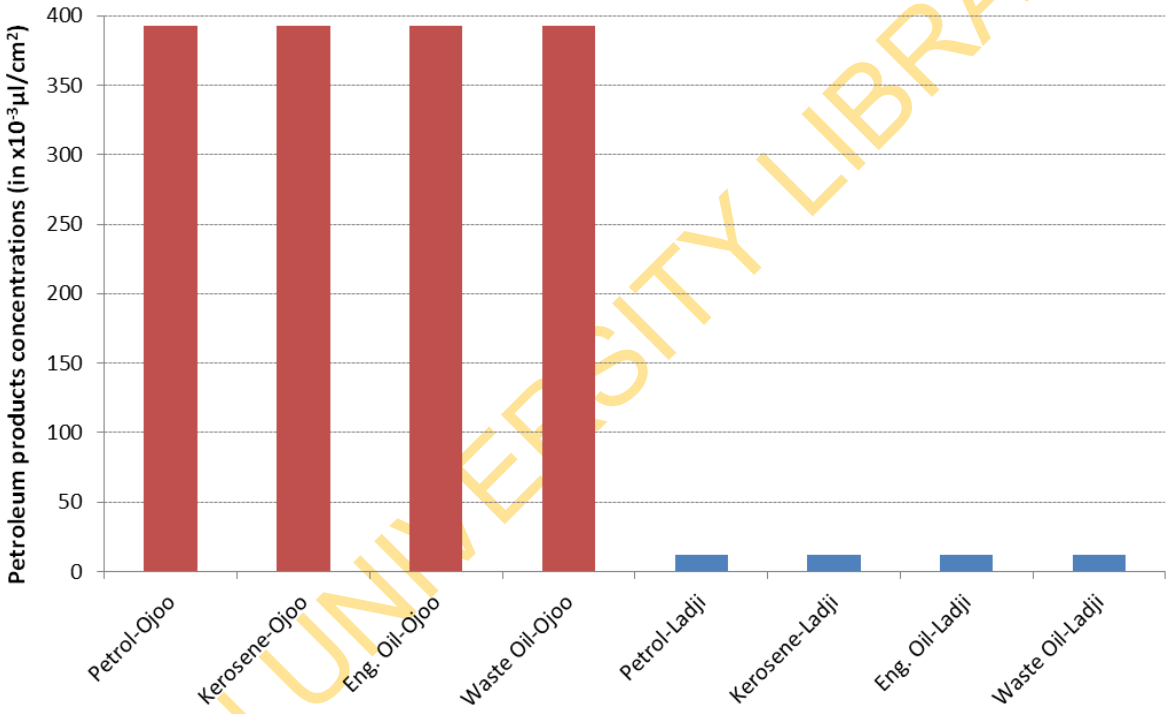

Fig. 4.14 Comparative analysis of recorded HIC of petroleum products on *A. gambiae* from Ladji and *A. gambiae* from Ojoo larvae

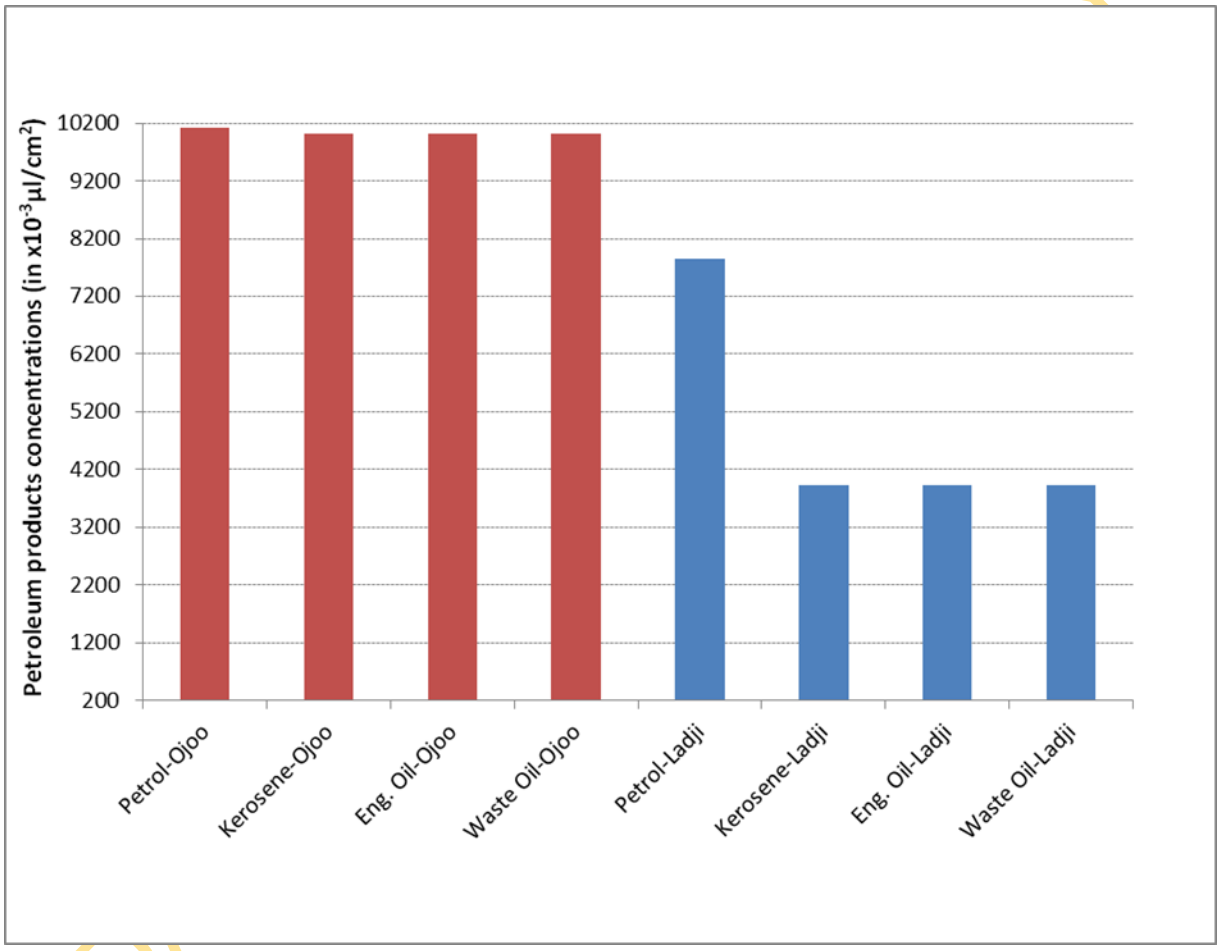

Fig. 4.15 Comparative analysis of recorded LoC_{100} of petroleum products on *A. gambiae* from Ladji and *A. gambiae* from Ojoo larvae

water surface (Fig. 4.16).

4.4.7 Analysis of physico-chemical properties of breeding sites producing pyrethroids resistant and susceptible populations of *A. gambiae* in southwestern Nigeria and southern Benin

The nature of breeding sites was analysed in target localities. Two types of breeding sites were identified in the 19 localities surveyed in southwestern Nigeria. The first contained petroleum products particles and were nominated as "oily breeding sites", while the second had clean water with no petroleum residues and were nominated as "non-oily breeding sites". Out of the 19 sites surveyed in Nigeria, 13 were "oily breeding sites" and 6 were "non-oily breeding sites". The mortality rates to permethrin of *Anopheles* populations emerging from oily breeding sites and non-oily were 82.1% and 97.2% respectively. Breeding sites identified in the field with petroleum particles produced more permethrin resistant populations of *Anopheles* than breeding sites not showing traces of petroleum products (P=0.001) (Table 4.8).

The mean pH of breeding sites producing permethrin resistant populations of *A. gambiae* was 7.9 whereas that of breeding sites producing permethrin susceptible populations of *A. gambiae* was 7.5. The mean DO of breeding sites producing permethrin resistant *Anopheles* was 14.34mg/l, while that of breeding sites producing permethrin susceptible *Anopheles* was 34.5mg/l (Table 4.9). In the surveyed sites of southern Benin, two types of breeding sites were identified in the 18 localities surveyed: 9 turbid breeding sites containing food detritus and vegetation (nominated as "turbid breeding sites") and 9 clean breeding sites with neither food particles nor vegetation; these were recorded as "non-turbid" breeding sites.

The mortality rates to permethrin of *Anopheles* populations emerging from turbid breeding sites and non-turbid breeding sites were 65.5% and 75.62% respectively. Although both types of breeding sites produced permethrin resistant *Anopheles*, it was found that the turbid breeding sites identified in the southern Benin could produce more mosquitoes with permethrin resistant status than the clean "non-turbid" breeding sites (Table 4.10). The mean pH of turbid breeding sites was found at 7.8 compared to the mean pH of clean non-turbid breeding sites which was 7.3. The mean DO recorded with turbid breeding sites was 12.2mg/l compared to the mean DO recorded with clean

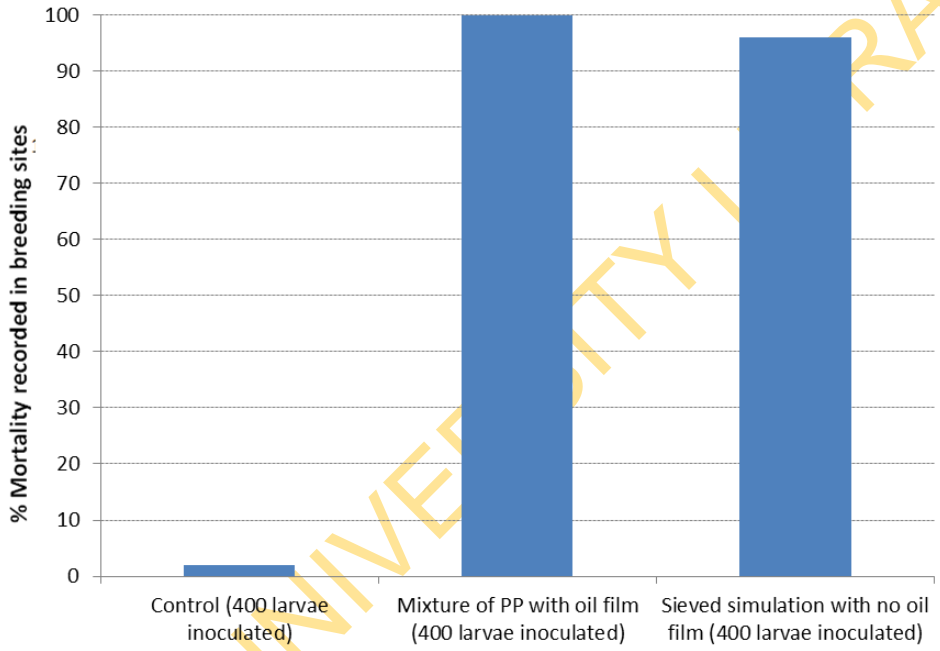

Fig. 4.16 Mode of action of petroleum products on *Anopheles* larvae (Mortality rates recorded with sieved and crude or raw petroleum products from field contaminated breeding sites)

TABLE 4.8 Comparison of the mean mortality rates to permethrin of *A. gambiae* populations produced by oily and non-oily sites in Nigeria

	Oily breeding sites	Non Oily breeding sites
Number of breeding sites surveyed during the study	13	6
Number of tested females of *A. gambiae* from identified breeding sites	849	546
Mortality rates (mean) recorded with *Anopheles* from each type of breeding sites following exposure to permethrin	82.1%	97.2%
Variance (mortality rates in surveyed localities)	4.33	47.7
Pv. Comparing the mean mortalities (following exposure to permethrin) recorded with mosquito emerging from oily and non-oily breeding sites	Pv=0.00151	

TABLE 4.9 Physico-chemical parameters (pH and DO) of breeding sites producing susceptible and resistant populations of *Anopheles* in southwestern Nigeria

Localities in Nigeria	Breeding site type	Oxygen level in the breeding site (mg/l)	pH level	*Anopheles* tested	Mortality rates (%)
IITA	No oil	40	7.2	95	100
Ajibode	No oil	25	9	85	90
Ojoo	Oily	12	7.9	80	80
Challenge	Oily	12	75	80	81
Lagere	Oily	20	7.7	94	94
Ati-kankan 1	Oily	15	7.6	83	85
Ati-kankan 2	Oily	15	7.7	80	88
Mobafulo	Oily	13	7.9	75	80
Ogere 1	Oily	10	7.8	4	75
UI	No oil	52.5	7.9	80	100
Orogun	No oil	30	7.1	80	100
Modakeke	No oil	35	7	80	97
Inowo	No oil	19.5	9	76	96
Badagry	No oil	30	7	50	98
Bashorun	Oily	12	8	75	70
Oja-tuntun	Oily	13	7.8	83	81
Ilesha garage	Oily	16.5	7.6	77	89
Owena	Oily	10	9	78	75
Ogere 2	Oily	13	7.6	40	88

TABLE 4.10 Comparison of the mean mortality rates to permethrin of *A. gambiae* populations produced by turbid and non-turbid sites in southern Benin

Surveyed breeding sites	Turbid breeding sites (with few traces of oil)	Clean breeding sites (no oil traces)
Number of breeding sites	9	9
Mean dissolved oxygen	12.2	27.4
Mean pH level of breeding sites	7.8	7.3
Mean mortality rates recorded with permethrin following exposure of emerging *Anopheles*	65.7%	75.6%
Permethrin resistance level of emerging *Anopheles*	High	Less high

breeding sites 27.4mg/l (Table. 4.11).

4.4.8 Identification of the preferred types of breeding sites selected by pyrethroids susceptible and resistant strains of *Anopheles* for ovipositions

When gravid females of the permethrin susceptible strain *A. gambiae* from UI were released in cages containing several breeding sites reconstituted with water samples collected in the locality of spilled petroleum products of Ojoo in Nigeria, out of a mean number of 1,900 eggs laid in each cage, 4.5% (86 eggs) of these eggs were oviposited in oily breeding sites and 95.5% (1814 eggs) in the clean breeding site (control site). With gravid females of the permethrin resistant strains *A. gambiae* from Ojoo released in similar cages, out of a mean number of 2,425 eggs laid in cages, 77% (1875 eggs) of these eggs were oviposited in breeding sites with clean water (control site) whereas 23% (550 eggs) of these eggs were laid in breeding site with petroleum debris (Fig.4.17). Neither the resistant strain *A. gambiae* from Ojoo nor the susceptible strain *A. gambiae* from UI preferred laying eggs in oily breeding sites. However, it was observed that the resistant strain *A. gambiae* from Ojoo could lay a relatively high number of eggs in oily breeding sites compared to the susceptible strain
A. gambiae from UI (23% of eggs laid by *A. gambiae* from Ojoo versus 4.5% of eggs laid by *A. gambiae* from UI*)*. When this experiment was replicated using water samples collected in the locality of spilled petroleum products of Akpakpa in southern Benin, the permethrin resistant strain *A. gambiae* from Ojoo laid 28% (725 out of 2525) of eggs in the oily breeding sites and 72% (1800 out of 2525) in clean water (Fig. 4.17).

With the susceptible strain *A. gambiae* from UI released in similar cages, 5% (105 out of 1880) of oviposited eggs were deposited in oily breeding sites and 75% (1775 out of 1880) were laid in clean breeding sites (Fig. 4.17). With samples from Benin, neither the resistant strain *A. gambiae* from Ojoo nor the susceptible strain *A. gambiae* from UI preferred laying eggs in oily breeding sites. However, it was observed that the resistant strain *A. gambiae* from Ojoo could lay a relatively high number of eggs in oily breeding sites compared to the susceptible strain from UI (28% of eggs laid by *A. gambiae* from Ojoo versus 5 % of eggs laid by *A. gambiae* from UI).

TABLE 4.11 Physico- chemical parameters (pH and DO) of breeding sites producing susceptible and resistant populations of *Anopheles* in southern Benin

Localities	Breeding site type	O_2 level in mg/l	pH level	individuals	Mortality in %
Seto	Turbid	14	7	50	80
Grand- Popo	Turbid	12	7	60	90
Valehoue	Turbid	13	8	75	92
Akron	Turbid	10	8.5	103	23
Niaouli	Turbid	12	8.1	75	67
Akpakpa	Turbid	12	9	110	69
Ladji	Turbid	12	7	100	65
Gbedjromede	Turbid	12	7	112	36
Houeyiho	Turbid	13	9	76	70
Abomey	Clean	24	7.1	60	83
Pobe	Clean	20	7.6	50	80
Sakete	Clean	25	7.2	55	90
Ifangni	Clean	30	7	100	98
Lokossa	Clean	25	8.5	80	93
Gogbo	Clean	35	7	80	90
Ketonou	Clean	33	7.5	70	81
Gbodjo	Clean	30	7	116	83
Pahou	Clean	25	7.2	106	31

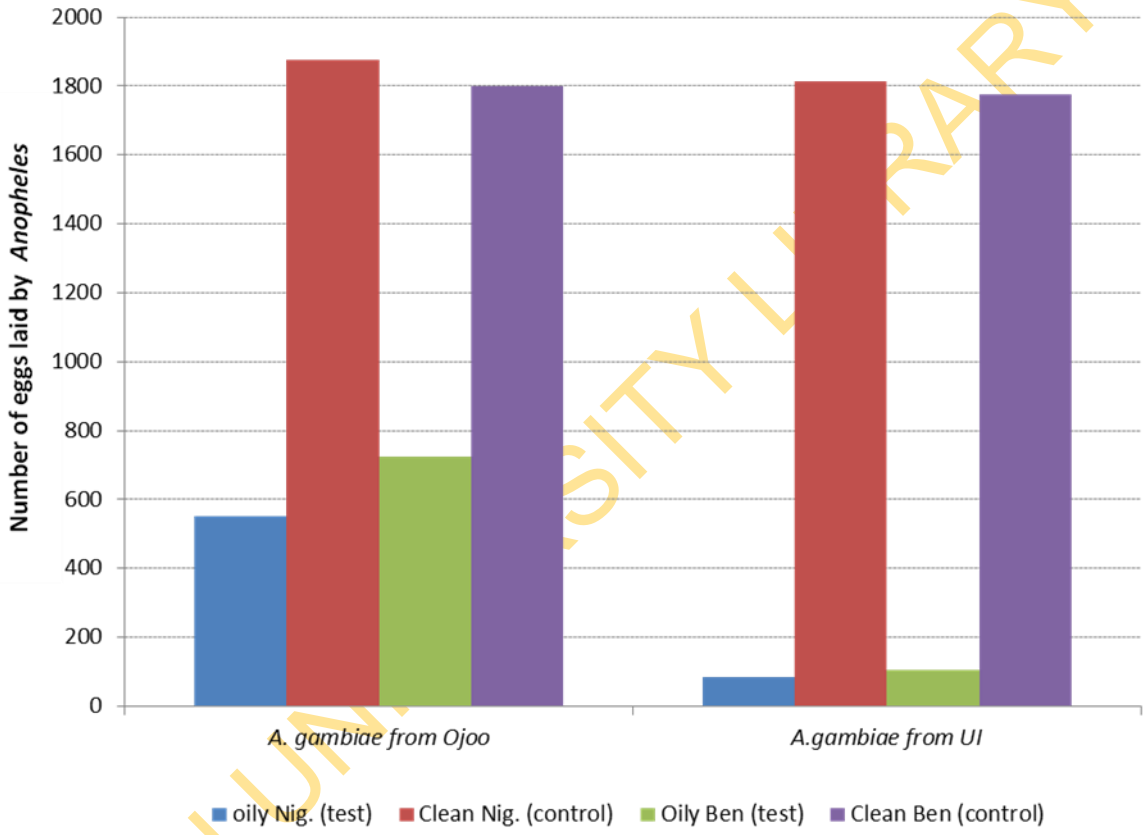

Fig. 4.17 Number of eggs laid by resistant (*A. gambiae* from Ojoo) and susceptible (*A. gambiae* from UI) strains of *Anopheles* in oily and non-oily breeding sites from southwestern Nigeria and southern Benin

4.4.9 Hatching rate of eggs laid by pyrethroid susceptible and resistant strains in oily breeding sites

The control breeding sites used in this experiment: The "clean breeding site from Ojoo in Nigeria" offered favourable conditions for the hatching of eggs of both *A.gambiae* from Ojoo and *A. gambiae* from UI. 84% (168 hatched eggs out of 200 eggs laid) and 87% (174 eggs out of 200 eggs laid) hatching rates were recorded in the control breeding sites with eggs of *A. gambiae* from Ojoo and *A. gambiae* from UI respectively (Fig. 4.18). When eggs of the resistant strain *A. gambiae* from Ojoo and of the susceptible strain *A. gambiae* from UI were laid in oily breeding sites simulated with samples from Ojoo, a decrease in hatching rates was observed with both strains. The hatching rates of *A. gambiae* from Ojoo and *A. gambiae* from UI dropped from 84% and 87% in control breeding sites to 63% and 61% in the test breeding sites (Fig. 4.18).

In the site of Akpakpa, the locality of spilled petroleum of Benin, the control breeding site (the clean breeding site from Akpakpa in Benin) offered favourable conditions for the hatching of eggs of both *A. gambiae* from Ojoo and *A. gambiae* from UI. 80% (160 hatched eggs out of 200 eggs laid) and 75% (150 hatched eggs out of 200 eggs laid) hatching rates were recorded in the control breeding sites with eggs of *A. gambiae* from Ojoo and *A. gambiae* from UI respectively (Fig. 4.18). When eggs of the resistant strains *A. gambiae* from Ojoo and of the susceptible strains *A. gambiae* from UI were laid in oily breeding sites reconstituted with samples from Akpakpa, a decrease in hatching rates was observed with both strains. The hatching rates of *A. gambiae* from Ojoo and *A. gambiae* from UI dropped from 80% and 75% in control breeding sites to 70% and 63% in test breeding sites (Fig. 4.18).

4.4.10 Development of larvae of pyrethroid susceptible and resistant strains in oily breeding sites (rate of larvae getting to pupae stage)

The rates of first instar larvae getting to the pupae stage were low in all breeding sites containing traces of petroleum products. Very few larvae (1%) of the permethrin susceptible strain *A. gambiae* from UI could reach the pupae stage in breeding sites containing petroleum products residues from south western Nigeria (only 1 pupae was recorded out of the 122 L_1 larvae that had emerged from hatched eggs) compared to the control breeding sites which yielded 64% of first instar larvae getting to pupae

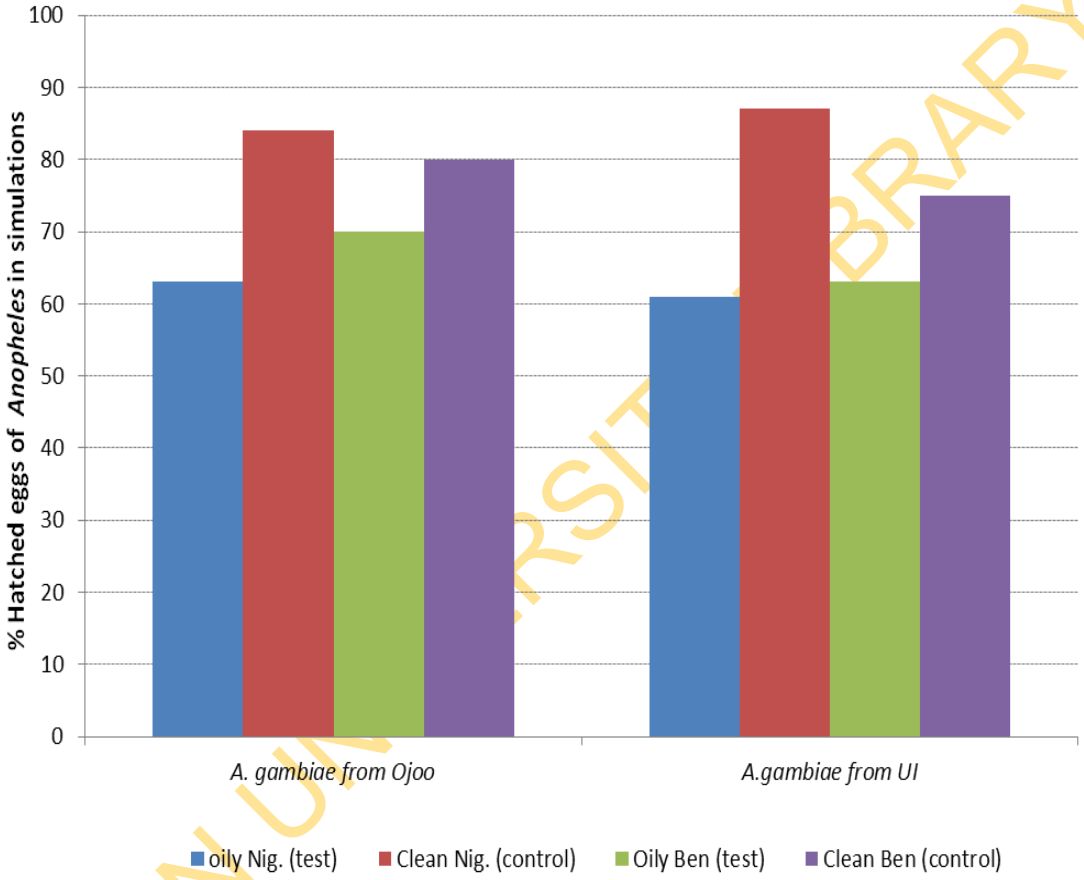

Fig. 4.18 Hatching rate of eggs laid by resistant *(A. gambiae* from Ojoo) and susceptible (*A. gambiae* from UI) strains of *Anopheles* in oily and non-oily breeding sites from Nigeria and Benin

stage (Fig. 4.19).With the permethrin resistant strains *A. gambiae* from Ojoo, 27% (34 out of 126 hatched individuals) of larvae were able to reach the pupae stage when reared in water with oily residues from Ojoo compared to the development rate recorded with samples in the control breeding sites where 66% of first instar larvae got to the pupae stage (Fig. 4.19).

In the site of Akpakpa, the locality of spilled petroleum of Benin, the simulated control breeding sites (clean breeding Benin) offered favourable conditions for the development to pupae stage of first instar larvae of both *A. gambiae* from Ojoo and *A. gambiae* from UI: 63% and 60% respectively. In breeding sites with petroleum residues, the rates of first instar larvae getting to the pupae stage were low. Very few larvae (5%) of the permethrin susceptible strain *A. gambiae* from UI could reach the pupae stage in breeding sites containing mixed petroleum products residues collected in petroleum polluted localities from southern Benin compared to the development rate of 60% recorded in the control (Fig.4.19).

With the permethrin resistant strain *A. gambiae* from Ojoo, 35% of larvae were able to reach the pupae stage when reared in water with oil residues from Akpakpa in Benin compared to the development rate of 63% recorded in the control breeding sites (Fig. 4.19).

4.5 Identification of detoxifying genes up-regulated in pyrethroids resistant *Anopheles* from sites under synthetic agricultural pesticides utilisation and sites of spilled petroleum products

A competitive hybridization was conducted on a microarray detox chip using permethrin resistant *A. gambiae* populations from the agricultural site of Akron and the site of spilled petroleum products of Ojoo and, the permethrin susceptible *A. gambiae* population from Oregun.

4.5.1 Genotyping and bioassay of *Anopheles* populations prior to micro-array analysis

The genotypic analysis of *Anopheles* populations from the 3 localities (Akron, Ojoo and Orogun) selected for competitive hybridisation on micro-array chips showed that all mosquitoes samples were A. gambiae ss. When subjected to molecular forms analysis, results revealed that all sanples selected were A. gambiae ss of the "M"

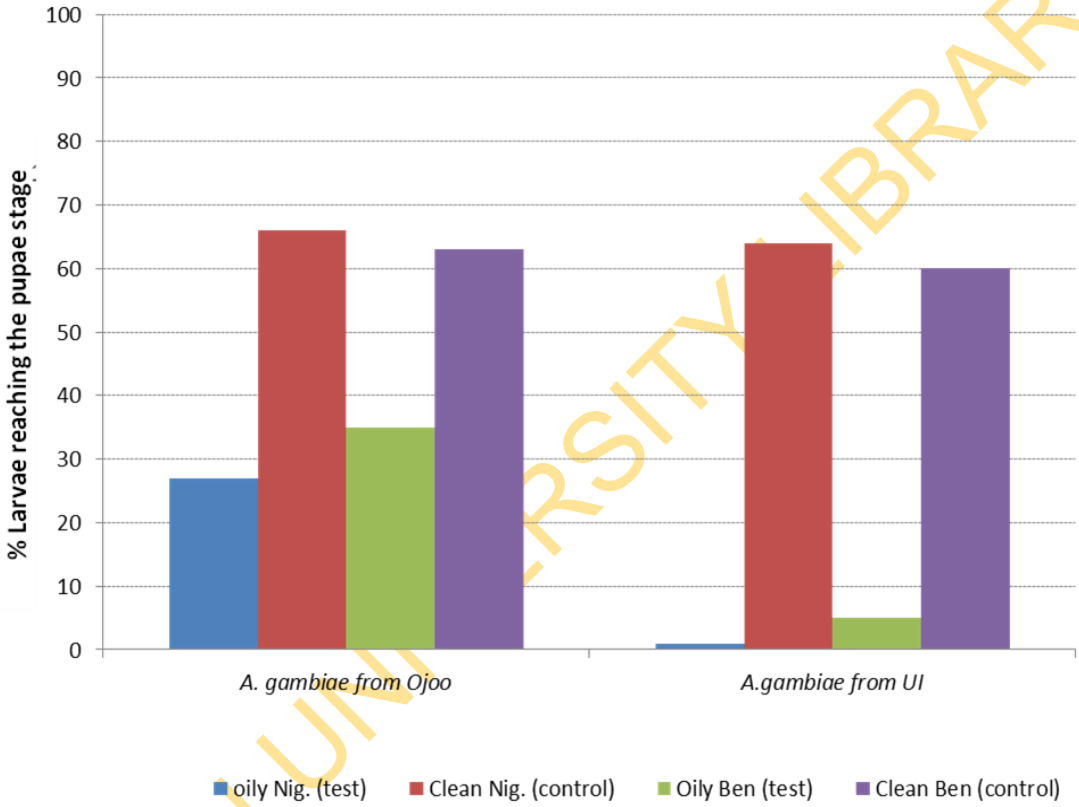

Fig. 4.19 Rate of larvae (larvae from hatched eggs) of *A. gambiae* from Ojoo and *A. gambiae* from UI developing to pupae stage in oily and non-oily breeding sites from southwestern Nigeria and southern Benin

molecular form (Table 4.12). No individual was found with the East-*kdr* mutation. The West-*kdr* was found at high frequencies in *A. gambiae* from Akron populations, and neither mutation occurred in either *A. gambiae* from Orogun or *A. gambiae* from Ojoo.

4.5.2 Identification of metabolic genes over transcribed on *Anopheles* samples from agricultural setting

The competitive hybridization of resistant *A. gambiae* samples from Akron and the susceptible *Anopheles* from Orogun on a "detox-chip" array exhibited different gene expression profiles (Fig. 4.20). The agricultural site of Akron revealed a remarkable expression of 5 genes from 2 groups of detoxification genes: the CYP group and the CPLC group (not well documented yet). During samples analysis, over-expressions of the CYP6P3, CYP325D2 and CYP6M2 genes all belonging to the CYP sub class and the CPLC8 and CPLC3 belonging to the group of genes involved in the cuticle synthesis were recorded. The expression levels obtained with these genes (CYP6P3, CYP325D2, CYP6M2, CPLC8 and CPLC3) in the resistant mosquitoes from Akron were respectively 12.37; 5.05; 2.48; 8.03 and 5.24 fold changes as compared with the susceptible field population (*A. gambaie* from Orogun). The most striking result recorded with samples from the agriculture site of Akron was the 12.4-fold over expression of the cytochrome P450, CYP6P3 (Table 4.13 and Fig. 4.20).

4.5.3 Identification of metabolic genes over transcribed on *Anopheles* samples from oil spillage site

A total of 9 detoxification genes were significantly expressed on samples from Ojoo, the locality under oil spillages. 5 of the 9 genes (CYP6P3, CYP6N1, CYP6AG2, CYP6M2 and CYP6AK1) belong to the CYP sub-class whereas 2 (GSTD1-6 and GSTD11) were from the GST sub-class and the last 2 (CPLC8 and TPX2) are involved in the cuticle synthesis. The expression levels obtained with these genes (CYP6P3, CYP6N1,

4.5.4 Comparative expression profiles of metabolic genes in permethrin resistant *A. gambiae* from Akron in southern Benin and Ojoo in the southwestern Nigeria

In both resistant populations of mosquitoes (*A. gambiae* from Akron and *A. gambiae* from Ojoo) studied, the expression profiles of transcripts revealed the elevated expression of CYP6AG2, CYP6M2, CYP6AK1, GSTD1-6, GSTD11, CPLC8 and TPX2 in the resistant *A. gambiae* strain from Ojoo with respectively 7.37; 4.84; 2.71; 2.58; 2.19;

TABLE 4.12 Molecular form, percentage mortality and *kdr* frequency of *A. gambiae* from southern Benin and southwestern Nigeria

Collection site	Cytotype (N=30)	% Mortality with Permethrin (N=80)	West-*kdr* Frequency (N=30)	East-*kdr* Frequency (N=30)
Orogun (control site)	M	100	0	0
Akron (Agricultural)	M	23	0.86	0
Ojoo (Oil contamination)	M	80	0	0

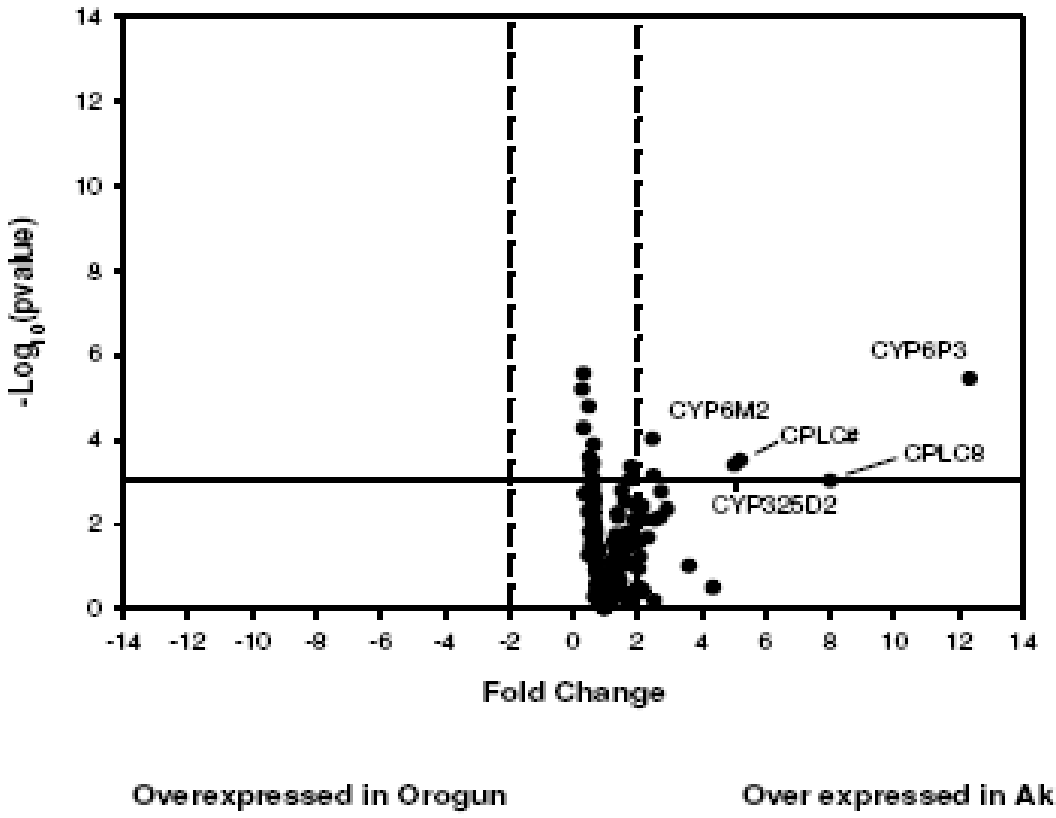

Fig. 4.20 Candidate metabolic genes over transcribed on resistant *Anopheles* mosquitoes from the agricultural site of Akron when co-hybridized with the susceptible strain *A. gambiae* from Orogun

TABLE 4.13 Candidate metabolic genes from micro-array analysis of resistant and susceptible field samples from Akron and Orogun

Site	Identified Genes	Expressions level (fold changes)	Pvalues (log)
Akron (Agricultural site)	CYP6P3	12.37	5.42
	CYP325D2	5.05	3.58
	CYP6M2	2.48	4.01
	CPLC8	8.03	4.01
	CPLC	5.24	3.49

4.07; 2.54; 2.62; and 5.02 fold changes as compared with the susceptible field population (Orogun) (Table 4.14 and Fig. 4.21). Two P450s namely CYP6P3 and CYP6M2 earlier identified on resistant populations of *A. gambiae* from Akron were also overexpressed on pyrethroid resistant samples from Ojoo.

elevated expression of the *CYP6P3* gene: more than 12 folds expression with sample from Akron and 7.37 folds with samples from Ojoo (Fig. 4.22). The CPLC8 belonging to the family of cuticular genes implicated in the synthesis and the thickness of the cuticle in mosquitoes was also over transcribed in both samples. The expression levels recorded with CPLC8 was more than 8 folds with *Anopheles* populations from the agricultural site of Akron and 2.62 folds on samples from the oil spillage locality of Ojoo (Fig. 4.22).

The last similarity recorded among the 2 resistant populations of *A. gambiae* analysed was the up-regulation of the CYP6M2 gene. The CYP6M2 gene which belongs to the subclass of CYP was expressed more than 2 times higher in pyrethroid resistant populations of *A. gambiae* from Benin and Nigeria. Fold changes of 2.48 and 2.58 were recorded for *A. gambiae* from Akron and *A. gambiae* from Ojoo respectively when competitively hybridized with *A. gambiae* from Orogun (Fig. 4.22).

TABLE 4.14 Candidate metabolic genes up-regulated in resistant populations of *Anopheles* from Ojoo

Site	Identified Genes	Expressions level (fold changes)	P-values (log)
Ojoo (Oil contamination site)	CYP6P3	7.37	9.27
	CYP6N1	4.84	12.94
	CYP6AG2	2.71	9.25
	CYP6M2	2.58	8.39
	CYP6AK1	2.19	9.58
	GSTD1-6	4.07	8.49
	GSTD11	2.54	6.11
	CPLC8	2.62	8.46
	TPX2	5.02	11.06

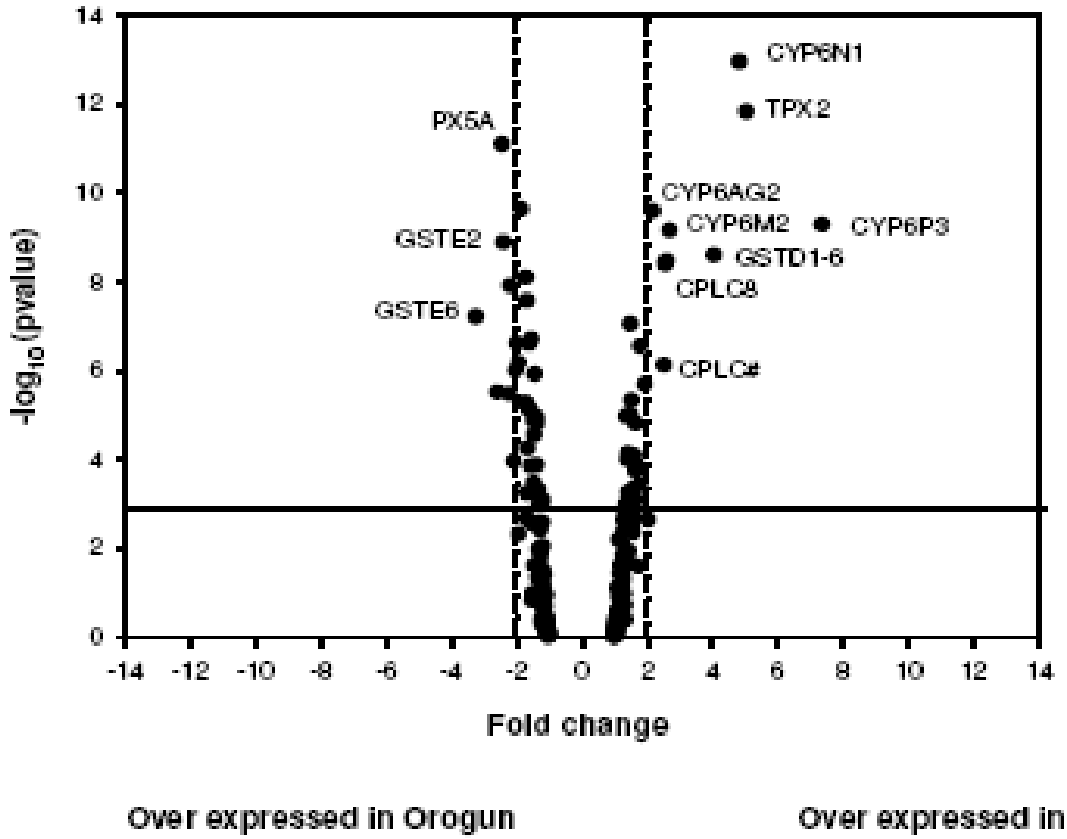

Fig. 4.21 Candidate metabolic genes over transcribed on resistant *Anopheles* mosquitoes from the oil spillage locality of Ojoo when co-hybridized with the susceptible strain *A. gambiae* from Orogun

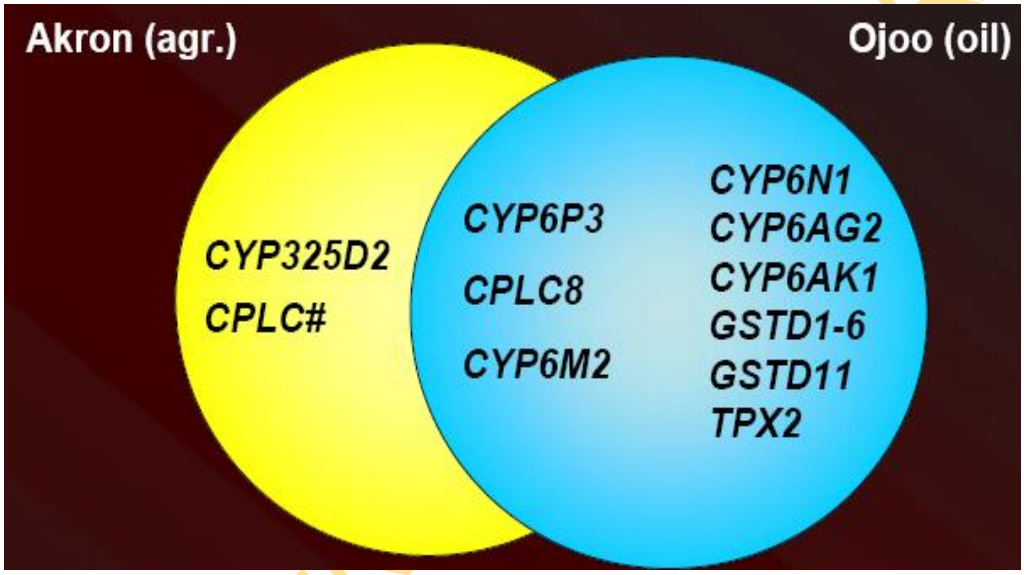

Fig. 4.22 Cohort of expressed genes in resistant population of *A. gambiae* from the Agricultural site of Akron in Benin and the site of spilled oil of Ojoo in Nigeria

Chapter 5

Discussion

5.1 The susceptibility pattern of *Anopheles* populations to pyrethroid in southwestern Nigeria and southern Benin

Series of insecticide bio-assays conducted in this study revealed a wide spread of insecticide resistance in southwestern Nigeria. The presence of *Anopheles* populations capable of withstanding diagnostic doses of permethrin was initially reported in southwestern Nigeria by Awolola *et al.* (2003) and Mojca *et al.* (2003). Although these initial studies were confined to Lagos and Ogun states respectively, the spread of *Anopheles* resistance seems to go beyond those 2 states. *Anopheles* populations collected from 13 localities out of the 19 surveyed in the 6 states covered during this study were resistant to permethrin. In West African countries such as Benin, Burkina Faso and Cote d'Ivoire, several studies have shown the presence of permethrin resistant populations of *Anopheles* (Akogbeto *et al.,* 1999; Chandre *et al.,* 1999; Diabate *et al.,* 2003; N'guessan *et al.,* 2003). The Republic of Benin shares a highly populated and very business active geographic border with the southwestern region of Nigeria therefore, the permethrin resistance observed in southwestern Nigeria could originate from migrations of resistant strains of *Anopheles* from Benin Republic where high levels of resistance were documented as early as 1999 (Akogbeto *et al.,* 1999) or, could be locally selected by specific environmental factors and human activities.

The use of agricultural pesticides was less recorded in surveyed localities of southwestern Nigeria. Agricultural pesticides utilisation was recorded only in the locality of Ajibode where vegetable farming is practiced at low scale. *A. gambiae* populations from Ajibode were found resistant to permethrin (mortality rate of 90%). Many reviews have implicated synthetic pesticide utilisation in agriculture as a potential source of insecticide resistance selection in malaria vectors (Yadouleton et *al.*, 2009). The limited utilisation of agricultural pesticides in surveyed sites, suggests that other environmental factors or human practices account for the selection of pyrethroid resistance in malaria vectors in southwestern Nigeria. However, the relatively low rates of permethrin resistance recorded in all surveyed sites probably explain the progressive flow of pyrethroid resistant genes in *Anopheles* populations of the southwestern Nigeria.

In the southern Benin, pyrethroid susceptible populations of *Anopheles* were almost inexistent. Out of the 18 populations of mosquitoes analysed, only one population

exhibited considerable level of susceptibility (mosquito populations from the locality of Ifangni). Ifangni is a rural area located in the south eastern Benin. Here, communities have low incomes and agriculture is mainly for household consumption and not market oriented. The local agriculture practiced at Ifangni is less demanding and farmers use little or no pesticides; this probably explains the absence of pyrethroid resistance in this locality. High levels of resistance were recorded in the southern Benin, with mortality rates to permethrin being as low as 23%. Very low mortalities were recorded in *Anopheles* populations collected in or around the vegetable farms of Akron (Mortality of 23%) and Pahou (31%) which are constantly under pesticide treatments. This result confirms the contribution of agricultural pesticides utilisation in the selection for resistance in malaria vectors (Diabate *et al.,* 2002; N'guessan *et al.,* 2003; Chouaibou *et al.,* 2009). Vegetable cropping is consistently becoming an important source for incomes to many households in urban and periurban settings (Yadouleton et *al.,* 2009). Consumers demand for vegetables is periodical and significantly increases during festivity periods. Vegetable farming is highly pesticide dependent with peaks of pesticide treatments corresponding to periods of high market demands or high pest attacks (Yadouleton et *al.,* 2009).

Agricultural pesticides used by farms have similar active ingredients as those used in public health for mosquito control therefore; these pesticides could easily contribute to the selection for resistance in malaria vectors. The intensity of pyrethroid resistance was relatively low in southwestern Nigeria compare to southern Benin. This difference probably results from the nature of factors selecting for resistance in both countries. In Benin the selection is mainly from agricultural pesticides utilisation whereas in Nigeria, most breeding sites producing resistant *Anopheles* populations contained traces of petroleum products, suggesting a cross resistance phenomenon between spilled petroleum products and pyrethroids.

5.2 Genotyping of permethrin resistant phenotypes

The southwestern region of Nigeria shares a border with the southern Benin where resistance has been documented as being mostly related to *kdr* mutations (Akogbeto *et al.,* 1999). The proximity of the two countries could lead to suspicion of the presence of *kdr* alleles in *Anopheles* mosquitoes from Nigeria. Contrary to this suspicion, results obtained from PCR-*kdr* analysis did not show an association between permethrin resistant

phenotypes and the presence of *kdr* alleles in analysed mosquito populations (one *kdr* mutant *Anopheles* out of 514 analysed). These results stand in favor of a low gene flow among *Anopheles* populations from Benin and Nigeria. This reproductive barrier could be justified by a remarkable presence of 2 different members of *A. gambiae* complex in both countries: *A. arabiensis* in the south western Nigeria and *A. gambiae ss.* in Benin. In a recent study conducted by Noutcha and Anumudu (2009), at Igbora a rural community in Ibadan, both members of *A. gambiae* complex (*A. gambiae ss.* and *A. arabiensis)* where found to be the main malaria vectors in the community. The low frequency of the *kdr* mutation recorded in the 19 surveyed localities of the south western Nigeria confirms earlier findings of Awolola *et al. (*2003), on *Anopheles* populations from Lagos and Mojca *et al.* (2003) who reported low presence of *kdr* mutations in *Anopheles* samples from Ogun state in southwestern Nigeria.

In the southern Benin, 2 members of *A. gambiae* complex were identified: *A. gambiae* ss and A. *melas*. The distribution of both species in Benin was initially documented by Akogbeto *et al.* (1990). This study confirms the main occupancy of the southern Benin by *A. gambiae ss*. which constitutes 93% of the total *A. gambiae sl.* populations analysed. When samples of *A. gambiae* ss were further genotyped, they were all characterized as "M" molecular forms as earlier reported by Akogbeto *et al.* (2003).

Classical molecular analysis and micro-array assays conducted on samples from the southwestern Nigeria and the southern Benin revealed that several detoxifying genes are up-regulated in permethrin resistant individuals. These findings demonstrate that permethrin resistance does not relate exclusively to changes occurring on the sodium channel (*kdr* mutation) but also to several other genes such as *Ace-1* (Weill *et al*. 2004) and series of detoxifying genes (David *et al.*, 2005) which could be externally expressed as permethrin resistant phenotypes.

5.3 The hatching rate of *Anopheles* strains in breeding sites simulated with soil and water samples from vegetable farms

The biological screening of pesticide residues from water and soil samples collected in agricultural sites in Benin and Nigeria suggest that samples from agricultural settings under pesticide pressure contain inhibitory factors responsible for the reduced growth rate of susceptible larvae of *A. gambiae* from Kisumu, with a lesser inhibitory

effect on the development of the resistant Ladji strain. Hatching of *Anopheles* eggs when introduced in simulated breeding sites made with soil and water from agricultural sites of Houeyiho and Ajibode is a rapid phenomenon and does not give room for expression of inhibitory factors on embryos. The recorded hatching rates should be considered as signal of toxicity for samples collected within from agricultural settings under synthetic pesticide treatments.

5.4 Water and soil samples from vegetable farms contain compounds that inhibit *Anopheles* larval development.

A. gambiae from Ladji is a permethrin resistant strain selected from a relatively polluted locality, the locality of Ladji in peripheral region of Cotonou. This strain had probably developed over time capacities to withstand low levels of toxicity. This assumption could explain the low inhibitory impact of breeding sites on the hatching of *A. gambiae* from Ladji eggs as recorded in this study. Similar to hatching rates, larval development also varied with respect to strains (Kisumu or Ladji) of *Anopheles* inoculated and the types of artificial breeding sites simulated.

In both vegetable farms (Houeyiho and Ajibode), breeding sites simulated with top soil collected around vegetables seemed to inhibit larval growth more than simulations with watering water and soil from watering pools. In these two simulations, inhibitory effects were less spectacular. In breeding sites generated with water mixed with soil from watering pools, 85% of *Anopheles* Kisumu eggs were able to reach the pupal stage, whereas, in simulations with water mixed with soil collected around vegetables, only 42% of larvae were able to reach the pupal stage. A similar trend was recorded with samples from Ajibode in Nigeria. This consistent difference in results (85% and 42% developmental rate) suggests an unequal distribution of pesticide residues after treatment in the vegetable farm of Houeyiho. Soil directly under vegetable plants is more contaminated with pesticide residues; it is subjected to several treatments and therefore receives a good amount of pesticide particles during treatments. During rain falls, these pesticide residues are washed and sediments in mosquito breeding sites were they exercise a selection pressure on mosquito larvae.

5.5 The implication of pesticide residues in the emergence of pyrethroid resistance in malaria vectors

Several chemicals (agricultural inputs) are used in vegetable farming at Houeyiho and Ajibode for pests control. These chemicals are mainly pyrethroids for Ajibode and, a combination of pyrethroids, organophosphates and carbamates for Houeyiho. These compounds are used as single formulations or as combinations of two to three insecticides of different families for generating a synergistic effect of insecticides and a better pest management. After pesticide treatments in agricultural settings, residues of insecticides get into mosquito breeding sites. These residues have lethal effects on larvae of some populations of mosquito whereas they exert a selective pressure on other populations, leading to a gradual tolerance of insecticide concentrations and the emergence of resistance. Insecticides used in public health against disease vectors are similar to those used for years in agriculture. In Benin Republic, pyrethroids were introduced in agriculture in the 1970s and, after 30 years of continuous use, cases of resistance are certain to be found in some populations of insects. Most authors incriminate pesticides used in agricultural farms as the main source of selection of resistance in mosquito species (Georghiou *et al.*, 1991; Chandre *et al.*, 1999, N'guessan et *al.*, 2003; Diabate *et al.*, 2002).

The intensification of exodus of young people from villages to towns, as a result of unemployment, has led to the development of agricultural spaces within urban and periurban areas for vegetable farming. These farms found in many West African countries are active throughout the year because of constant and high demands of urban populations. To keep the productivity high and avoid shortages of vegetables for urban consumers, farms are treated at relatively high frequencies. At Houeyiho an average of 72 pesticide treatments is conducted annually by farmers. This constant treatment of farms keeps high the level of insecticide residues in the soil and may explain the elevated toxicity observed with top soil. Data from this study indicate that factors inhibiting the hatching of *A. gambiae* eggs and the development of their larvae are insecticide residues resulting from agricultural treatments with pesticides. The indirect biological assay developed by WHO (MIM-WHO, 2003) and used in this study revealed the presence of pesticides residues in mosquito breeding sites found in and around vegetable farms. Results obtained established the implication of synthetic agricultural pesticides in the selection for pyrethroid resistance in mosquito populations breeding in and around vegetable farms.

5.6 The treatment of mosquito breeding sites with petroleum products and the selection of pyrethroid resistance in malaria vectors

Although abandoned long ago by National programs of malaria control in most African countries, petroleum products are still used in several rural communities considered in this study. This is probably due to the availability of these products, which are openly sold in many streets of West African countries such as Nigeria, Benin, Togo, and Niger. In Nigeria, several selling spots of engine oil were identified along the Ibadan Lagos express way. In Benin in addition to engine oil, petrol and gasoil are sold beside the roads by retailers mainly women and their children. The low income of communities in rural settings also accounts immensely in the slow adoption of synthetic insecticides and, therefore, solidifies their attachment to petroleum products as the main malaria vector control tool. The use of petroleum products for vector control in studied rural communities is well known to the communities, is transferred from generation to generation and seems to have become a cultural practice. The availability of these products and their relative cost-effectiveness suggests this method of mosquito control may have some benefit to the communities in rural areas.

A relatively low efficacy of petrol on *Anopheles* larvae was recorded in this study; this could be explained by its high volatility compared to kerosene, engine oil and used engine oil. The high volatility of petrol does not allow its persistence in the breeding sites. This low persistency results in a low residual effect of this product in treated breeding sites. The relatively high efficacy of kerosene, engine oil and waste oil is likely to be due to their elevated persistency in breeding sites post treatments. The HiC and the LoC_{100} values determined for each petroleum product are key operational values: the LoC_{100} indicates the lowest effective quantity of petroleum products that kills 100% of *A. gambiae* larvae during breeding site treatments. The LoC_{100} is a cost effective concentration for larviciding. On the other hand, the HiC also known as the NOEL corresponds to the quantity of petroleum products "wasted" in the environment during treatments of breeding sites. This value defines the threshold at which resistant populations of *Anopheles* could be gradually selected. The HiC values recorded in this study reflects the high probability of petroleum residues found in mosquito breeding sites to contribute to the emergence of pyrethroid resistance in analysed *Anopheles* populations.

Poupardin et *al.*, (2008) demonstrated that several xenobiotics found in mosquito habitats contribute in the selection of insecticide resistance. It is possible that these products might have contributed through cross-resistance to the numerous cases of pyrethroid resistance recorded in Nigeria and Republic of Benin.

The empirical use of PP by communities and the absence of knowledge on ideal concentrations to use during the treatment of breeding sites have over the years contributed to the spread in the environment of none active quantities of PP which in return would have contributed to the selection of resistant populations of mosquitoes. It is possible that the emergence of pyrethroid resistance in studied localities has partially resulted from the presence in *Anopheles* breeding sites of petroleum residues at concentrations below the LoC_{100} recorded in this study. The constant exposure of *Anopheles* larvae to "friendly" concentrations of petroleum (HiC) has probably induced over the years a cross resistance to pyrethroids.

5.7 Identification of the mode of action of PP on *A. gambiae* larvae

When *A. gambiae larvae* were reared in laboratory bowls containing water samples mixed with petroleum and oil pellicles at the surface, a percentage mortality of 100 was recorded within 24 hrs. However, when the surface pellicle (oil film) was removed through series of sieving the mortality rate of larvae remained as high as 96%. It is possible that some soluble active compounds of petroleum products such as benzene, toluene, and xylene (McAuliffe, 1987) dissolve, diffuse into the water and are ingested by *Anopheles* larvae after treatment of the breeding sites. These compounds keep elevated larval mortalities despite the absence of oil film. It is likely that the mode of action of petroleum products in mosquito larvae is mostly through "contact toxicity" followed by the ingestion of dissolved petroleum compounds rather than the "suffocation" from the oil film (Appendix.5).

5.8 The inhibitory effects of petroleum on *Anopheles* oviposition and larvae development

A low tolerance to oily breeding sites was recorded with pyrethroid susceptible *Anopheles* compare to resistant *Anopheles* during oviposition. This low tolerance translates the high selection pressure exercised on *Anopheles* species in localities where most breeding sites are covered with petroleum products. Similar to inhibiting oviposition,

petroleum products had a negative impact on the development of larvae of susceptible and resistant populations of *A. gambiae*. When a differential analysis of mortalities was conducted on the susceptible and the resistant populations, the destructive impact of petroleum appeared more prominent on the susceptible *A. gambiae* from UI compare to the resistant *A. gambiae* from Ojoo. This result suggests a high selection pressure of spilled petroleum products on *Anopheles* at larval stage. The inhibition of oviposition and larvae development by petroleum products in the laboratory justifies low presence of susceptible populations of *Anopheles* in oily breeding sites screened in the surveyed localities. In locality of spilled petroleum products, clean breeding sites which are free from petroleum particles may contain both resistant and susceptible strain of *Anopheles* whereas, it was observed that petroleum contaminated breeding sites mainly produce permethrin resistant strains of *Anopheles*. This segregational occupation of oily and non-oily breeding sites by resistant and susceptible populations of *Anopheles* probably explains the active selection played by spilled petroleum products on mosquito species in some localities of southwestern Nigeria and southern Benin.

5.9 Existence of a cross resistance between petroleum products and permethrin in sampled *Anopheles*

To establish the links between oil spillage and the selection of pyrethroid resistance several research steps were followed: At the initial step, the lethal activity of petroleum products on *Anopheles* larvae was established and the dose response curves of lethal activities of petroleum products on *Anopheles* larvae revealed the existence of "Larvae-friendly" concentrations of petroleum on mosquitoes. At the second setp of this research, the mode of action of petroleum residues on mosquito larvae was established and this revealed that, when petroleum products are spilled in *Anopheles* breeding sites, the majority of larvae are killed by contact toxicity rather than suffocation.The thirth step of this analysis was mainly based on laboratory simulations and results obtained showed that petroleum products in *Anopheles* breeding sites exercise a severe selection pressure by killing most susceptible strains of *A. gambiae* and allowing the emergence of individuals found to be resistant to permethrin. Ojoo and Orogun are two localities of the southwestern Nigeria at less than 2 km. At Ojoo, *A. gambiae* populations are pyrethroid resistant whereas at Orogun, populations of *A. gambiae* are susceptible to permethrin.

These differences of susceptibility in both localities are probably justified by the nature of breeding sites which are mostly oily at Ojoo and relatively clean at Orogun.

The reduced oxygen concentration identified in most breeding sites containing petroleum particles suggests that mosquitoes living in such breeding sites have developed elevated oxidative activities as survival strategies. This was confirmed through microarray analysis. The absence of the *kdr* mutations on samples emerging from oily breeding sites further confirms that petroleum products select for metabolic mechanisms of resistance and not target site mutations (*kdr* mutation). *A. gambiae* populations breeding in water contaminated with petroleum products develop elevated detoxifying activities for surviving petroleum residues found in their habitats. This study has established the existence of a corss resistance between petroleum products and pyrethroids.

Nigeria is the first oil producer in Africa with over 2,455.260 barrels per day (EIA-US, 2010). Oil dumping is an old and generalized practice in this country. The management of used petroleum products is a critical and very sensitive issue in this part of the world and leads to wide-spread environmental pollution and the development of various health hazards. Petroleum products are spilled on a wide-scale in the environment by mechanics, retailers, trailers and old cars. These products are washed by rains and accumulate in low-lying areas where, mixed with water, they constitute breeding spots for mosquitoes. *Anopheles* may lay their eggs in these oily breeding sites and selection pressures may be exercised during series of larval development cycles.

5.10 Identification of detoxifying genes up-regulated in pyrethroids resistant *Anopheles* from sites of spilled petroleum products and from agricultural areas under pesticides utilisation

The impact of pyrethroid resistance in *A. gambiae* from the Southern Benin, believed to be mainly driven by *kdr*, has been implicated in the failure of LLIN's in the region (Nguessan *et al.*, 2007). N'Guessan et *al.* (2007) used biochemical analysis of the mosquitoes to identify metabolic resistance and ruled out the involvement of P450s, GSTs and COEs. Analysing resistant populations of *A. gambiae* and *Culex quinquefasciatus* from the same locality, Corbel et *al.* (2007) demonstrated the presence of *P450s*, *GSTs* and *COEs*. This study confirmed the involvement of metabolic genes and moved a stage

further from biochemical analysis, by identifying series of metabolic genes up regulated in pyrethroid resistant strains.

The gene showing greatest levels of over-expression in resistant populations of *Anopheles* analysed is *CYP6P3*. *CYP6P3* is the ortholog of *CYP6P9* from *A. funestus*, a mosquito in which target site resistance has not been reported. This gene has been genetically linked to pyrethroid resistance in *A. funestus* (Wondji *et al.*, 2007) and is highly over expressed in a pyrethroid resistant colony (*FUMOZ-R*) from Mozambique compared with the susceptible line *FANG* from Angola (>38 fold) (Amenya *et al.*, 2008). Similarly in this study the *CYP6P3* fold change between resistant and susceptible populations were the highest recorded by the detox chip for any gene to date (7.4-fold, Ojoo and 12.4-fold, Akron). Interestingly, Muller (2008) employed the detox chip to investigate the effects on gene expression in *A. arabiensis* before and after cotton spraying campaign and reported *CYP6P3* to be down regulated after pesticides treatments. It is possible that *CYP6P3* is switched-on in *Anopheles* populations collected around vegetable farms during pesticides treatments and, after treatments this gene is switched off.

The second metabolic gene identified in this study is *CYP6M2* which was over-expressed in the two pyrethroid resistant populations of *Anopheles* collected around vegetable farms and from areas of spilled petroleum products. Studies conducted by Muller (2008) on *A. gambiae* from Ghana also showed that this gene is up-regulated in pyrethroid resistant strains of *Anopheles*. These combined results from different authors working in different sites strongly support a role for both *CYP6M2* and *CYP6P3* in conferring pyrethroid resistance in *A. gambiae* populations analysed during this research.

GSTD1-6 and *GSTD11* belong to the delta class of GSTs with the former being one of four alternatively spliced variants of *GSTD1* (Ranson *et al.*, 1998). Whilst the delta class *GSTs* has been associated with resistance in other insects (Wang *et al.*, 1991; Tang *et al.*, 1994; Vontas *et al.*, 2001), this is the first study to implicate delta *GSTs* as conferring resistance in mosquitoes. *GSTs* and the peroxiredoxins are not thought to be able to metabolise pyrethroids directly. However, both classes of enzymes can protect against oxidative stress and may counteract the pyrethroid induced oxidative stress encountered by the mosquitoes (Rhee *et al.*, 2005; Tang *et al.*, 1994). *GSTs* may also play a passive

role in sequestering pyrethroids, thereby reducing the circulating levels of active insecticide (Kostaropoulos *et al.*, 2005).

The over expression of two cuticular precursor genes in both Akron and Ojoo resistant populations lends support to the hypothesis that mosquitoes may also protect themselves from insecticides by cuticular thickening, which leads to reduced penetration of insecticides. Compared with target-site and metabolic resistance, cuticular resistance is a less well understood mechanism and few studies have investigated the link between the insect cuticle and resistance (Stone *et al.*, 1969; Apperson *et al.*, 1975; Noppun *et al.*, 1989; Lin *et al.*, 1993). However, over expression of *CPLC8* has very recently been demonstrated in pyrethroid resistant *A. gambiae* from Nigeria (Awolola *et al.*, 2008) and *A. stephensi* (Vontas *et al.*, 2007). The *A. gambiae* detox chip only contains three of the estimated 295 putative cuticular proteins in this species (HeN *et al.*, 2007), and all the three belong to the *CPLC* family about which very little is known. The role of cuticular changes in resistance clearly warrants further investigation, as it may prove to be just as an important defence mechanism as metabolic and target-site resistance.

5.11 Differential expression of metabolic genes by *A. gambiae* populations collected around vegetable farms and those from localities of spilled petroleum products

Microarray analysis showed that the gene expression profiles of studied populations of mosquitoes correlate with the nature of breeding sites hosting *Anopheles* larvae. A relatively high number of metabolic genes were over-expressed on samples from oil spillage area (Ojoo) than those from Agricultural settings (Akron). 9 genes were overexpressed in the oily site compare to 5 genes in the agricultural site. The *kdr* target site mutation was identified exclusively in the agricultural site of Akron. Although this mutation seems not to interfere with the expression of metabolic resistance, it remains possible that, in the absence of the *kdr* mutation as a protective mechanism, mosquitoes tend to compensate for this absence by developing many other defensive mechanisms such as the up regulation of several metabolic genes from different subclasses. This probably explains the relatively high number of metabolic genes recorded on samples from Ojoo compare to Akron. However, it is difficult at this stage to determine the relative contributions of *kdr* and metabolic resistance in mosquitoes with both mechanisms. It is nevertheless important to note that the highest level of permethrin resistance was observed

in the Akron population, which has both *kdr* and metabolic resistance and the lowest resistance observed in the Ojoo population which displays only metabolic resistance.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

This study investigated the possible mechanisms of resistance and factors contributing to the emergence of vector resistance in southwestern Nigeria and southern Benin. It was observed that massive utilization of agricultural pesticides and spillage of petroleum products are the two main factors which account for the emergence of pyrethroid resistance in *A. gambiae* populations collected in the Southern Benin and the South western Nigeria.

The empirical use of petroleum products for treatment of mosquito breeding sites was confirmed by rural communities in Benin. Corroborative results from focus group discussions organized within communities and the laboratory analysis of samples revealed the lethal activity of petroleum products on *Anopheles* larvae. While highlighting the mode of action of petroleum on *Anopheles* larvae which is contact toxicity rather than suffocation, data from this study have also identified the existence of some non-lethal or "larval-friendly" concentrations to which *Anopheles* larvae adapt to and gradually develop a cross resistance mechanism to pyrethroids.

This study highlighted the potential impact of oily breeding sites in the development of both susceptible and resistant strains of *A. gambiae* and, the contribution of spilled petroleum products in the development and spread of pyrethroid resistance in malaria vectors in southwestern Nigeria. Similar to the implication of agricultural pesticides in the emergence of pyrethroid resistance in mosquito populations, spilled petroleum products are washed by rains and accumulate in low-lying areas where, mixed with water, they constitute breeding spots for mosquitoes. *Anopheles* will lay their eggs in these oily breeding sites and larvae emerging from hatched eggs will undergo series of selection pressure which ends with the emergence from these oily breeding spots of adult populations of *A. gambiae* which are capable to withstand lethal doses of pyrethroids.

The molecular analysis of *A. gambiae* samples from both Nigeria and Benin revealed that permethrin resistance does not relate exclusively to changes occurring on the sodium channel; other mutations such as *Ace-1* and series of detox genes are also externally expressed as permethrin resistance. The gene expression profiles of resistant and susceptible populations of *A. gambiae* directly from the field revealed the evidence of

metabolic resistance in mosquito samples from the Republic of Benin and Nigeria irrespective of the presence or absence of *kdr*. Furthermore two P450 genes, *CYP6P3* and *CYP6M2* which are strongly associated with permethrin resistance were identified in the resistant *Anopheles* following micro-array analysis. In addition, preliminary evidence for a role for cuticular resistance was provided from this study but this is an area that needs further investigation.

Evidences that insecticides used outside malaria control activities (agricultural pesticides) and the contamination of breeding pools by spilled petroleum might be causing resistance to permethrin in *A. gambiae* has important implications for resistance management and the control of malaria vectors in Benin and Nigeria.

This research is multi-sectorial and encompasses agriculture, environment and health sectors. Specific recommendations arising from this study are:

(i) There is need to establish an information exchange platform between agriculture and health policy makers on the negative impact of synthetic pesticide utilization on human health.

(ii) There is need to develop cost effective alternatives (botanicals, biological control) for agriculture pests control and disseminate the developed alternatives to farmers through the proposed platform.

(iii) Farmers should be sensitized on the links between synthetic pesticides utilization and the emergence of populations of malaria vectors resistant to public health insecticides.

(iv) Policies on environmental protection should be reinforced and more emphasis should be placed on the management of waste petroleum products at community level.

v) In rural communities where petroleum products are used for mosquito control, effective lethal doses should be made known to individuals through community sensitizations.

(vi) Researchers should work towards the development of new families of insecticides and new vector control strategies (biological control strategies) which are cost effective, less toxic to humans and the environment.

(vii) Research should be conducted on agro-ecosystems restoration as this could lead to the return of mosquito natural enemies/predators and the natural destruction of resistant malaria vectors. Agro-ecosystem restoration research is likely to provide new pathways for controlling vector borne diseases.

(viii) Further understanding of the battery of resistance mechanisms (behavioral, reduced penetration, target site and detoxification genes) developed by *Anopheles* populations when exposed to pesticides or other environmental xenobiotics is usefull for a better control of malaria vectors.

(ix) Research should be conducted on synergistic compounds which can be combined to current insecticides for repressing the expression of detox genes and restoring the efficacy of pyrethroids in areas of resistance.

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APPENDIX

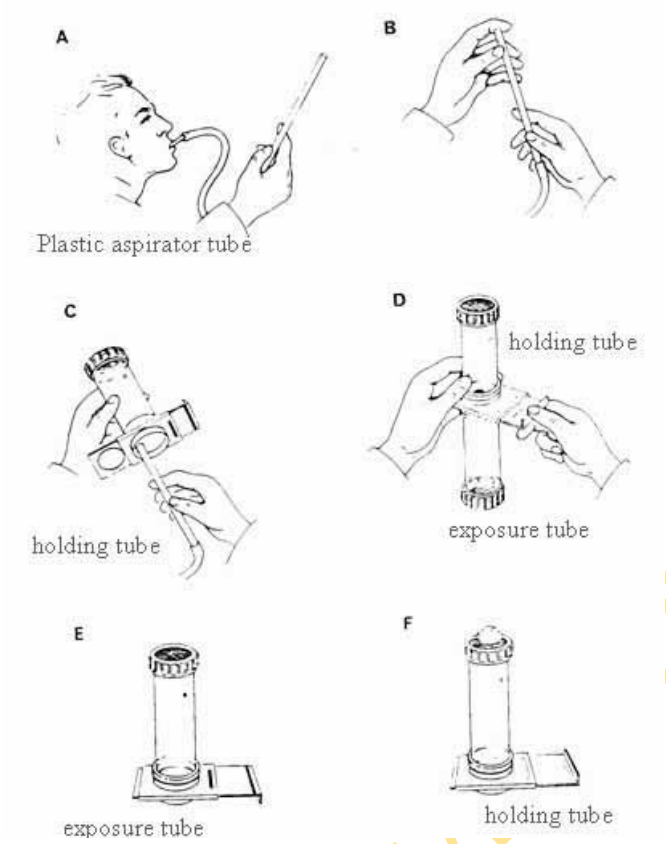
Appendix 1. Indepth interview guide on knowledge, attitudes and practices (KAP) of communities on synthetic pesticides use in Agriculture

1- Quarter/Locality
2- Status of the resource person interviewed
3- Sex and age
4- The use of pesticides in the household
5- The types of pesticides used
6- The purpose for pesticides use
7- If Agricultural use of pesticides in mentioned, discuss the types of insecticides used in accordance with the cropping system
8- Frequency of pesticide treatments in vegetable farms
9- Doses of pesticides applied for pests control in vegetable farming,
10- Origin of insecticides and the place of purchase
11- Safety precautions observed for reducing health hazard when using pesticide
12- Trainings received on the use of pesticides in agriculture.

Appendix 2. Indepth interview guide on knowledge, attitudes and practices (KAP) of communities on the use of petroleum products (PP) for mosquito control

1- Quarter/Locality
2- Status of the resource person interviewed
3- Sex and age
4- The use of PP in the household
5- The types of PP used
6- The purpose for PP use
7- If the use of PP for mosquito control in mentioned, discuss the types of PP used in accordance with the types of mosquito
8- Frequency of PP treatments in mosquito control
9- Doses of PP applied for mosquito control
10- Origin of PP and the place of purchase
11- Safety precautions observed for reducing health hazard when using PP.

Appendix3. WHO bio-assay for insecticide susceptibility using adult mosquitoes



WHO bioassay steps for analyzing the susceptibility status of Anopheles to insecticides.: (A-B-C-D) is the transfer of female Anopheles into test tubes coated with impregnated papers, (E) mosquito are kept for 1h in the exposure tube, (F) mosquitoes are taken back to holding tube for 24h observation of mortality rates.

The WHO tube test kit consists of two plastic tubes (125 mm in length, 44 mm in diameter), with each tube fitted at one end with a 16-mesh screen. One tube (exposure tube) is marked with a red dot, the other (holding tube) with a green dot. The holding tube is screwed to a slide unit with a 20 mm hole into which an aspirator will fit for introducing mosquitoes into the holding tube. The exposure tube is then screwed to the other side of the slide unit. Sliding the partition in this unit opens an aperture between the tubes so that the mosquitoes can be gently blown into the exposure tube to start the treatment and then blown back to the holding tube after the timed exposure (generally one hour). The filter-papers are held in position against the walls of the tubes by four spring wire clips: two

steel clips for attaching the plain paper to the walls of the holding tube and two copper clips for attaching the insecticidal paper inside the exposure tube.

Appendix4. Laboratory protocols and preparation of solutions

DNA extraction (Livak, 1984).

Livak grind buffer

1.6 ml 5M Nacl

5.48 g glucose

1.57 g Tris

10.16 ml 0.5 M EDTA

25 ml 20%SDS

Bring volume to 100 ml (with dH_2O), filter sterilize, store 5ml aliquots at -20oC. After thawing aliquot keep at 4oC for no more than 2 weeks.

DNA extraction

1- Heat LIVAK buffer to 65oC in heat block for 15 minutes and mix before use to re-dissolve precipitate

2- in a 1.5 ml eppendoorf grind 1 mosquito in 100 microL preheated LIVAK grind buffer. To optimize yield grind in 50microL first then rinse pestle with further 50microL and transfer immediately to 65oC

3- Incubate for 30 minutes at 65oC spin gently to collect condensations

4- add 14 microL of 8M K-acetate and mix.

5- incubate on ice for 30 minutes.

6- Centrifuge at 13000 rpm for 20 minutes at 4oC. Transfer supernatant to a new 1.5 ml eppendoorf , be careful not to transfer any debris . If desired, re-spin 20 minutes and transfer supernatant to new tube.

7- Add 200 microL EtOH, mix gently, and spin at 13,000 rpm for 15 minutes at 4oC.

8- Remove and discard supernatant, carefully rinse pellet in 100 microL ice cold 70% EtOH.

9- Dry pellet by leaving tubes on the bench for approximately 1 hour.

10- Re-suspend pellet in 10 microL TAE buffer.

Other DNA extraction solution

8M KAc

Grinding buffer

0.08M NaCl

0.16M Sucrose

0.06M EDTA

0.5% SDS

0.1M Tris-HCl

• TA (Tris EDTA) Buffer

100ml 1M Tris (pH)

20ml 0.5M EDTA

Make up volume to 1L.

• *TAE (Tris Acetic EDTA) Buffer 50X (pH 8)*

242g Tris

37.2 g Na2 EDTA.2H2O

57.1M glycial acetic acid

Make up to 1 L

• *Agarose gel*

For a 2.5% agarose gel, dissolve 10g agarose in 400ml 1X TAE

• Ethidium bromide

Dissolve 10mg EtBr crystals in 1ml distilled H2O

Appendix5. Characteristics and toxicity of petroleum products on insects

Chemical control of mosquito larvae with petroleum products (WHO, 1970)

Thickness of the product on water surface (Cm):

Diesel: 0.04

Gasoline: 0.30

Kerosene: 0.30

Boiling point:

Gasoline: 70^{o} to 150^{o}C

Kerosene: 150^{o}-300^{o}C

Gas oil, fuel oils and diesel oil: 250^{o}-350^{o}C

Effect of petroleum on insects larvae

1- The activity of petroleum oil as insect larvicide or pupicides varies with their aromatic and paraffin content and with their boiling range. Viscosity appears to be a function of boiling point.

2- The covering capacity relates to the viscosity of the oil and has a suffocating activity on insects larvae

3- Aromatic vapors are toxic and paraffin vapors are generally inert

4- The more effective larvicides are found in the fraction produced in the 232^{o}-316 ^{o}C boiling range with a 1:1 mixture of aromatic and paraffin oils.

5- The more volatile fractions have greater toxicity than the less volatile

6- Film production at water surface is long with paraffin oils (saturated fractions), intermediate with intermediate fractions and short with aromatics

7- Paraffins spread well and films persist until destroyed by evaporation; aromatics form less lenses after application

8- Loss of activity seems to be more closely related to the reduction of the area covered by the oil film than with the evaporation of the more volatile fractions.