INSECTICIDE RESISTANCE STATUS OF *ANOPHELES GAMBIAE* (DIPTERA: CULICIDAE) IN SELECTED VEGETABLE GROWING AREAS AND NON-VEGETABLE GROWING AREAS IN ACCRA METROPOLIS, SOUTHERN GHANA.

BY

EZIEFULE, CHIDINMA MIRACLE (B.Sc. (HONS))

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DECLARATION

I hereby certify that this thesis is the outcome of a research undertaken by me, Eziefule, Chidinma Miracle, towards the award of Master of Philosophy in Entomology in the African Regional Postgraduate Program in Insect Science (ARPPIS), University of Ghana, Legon. This thesis has not been submitted either in part or full, for any other degree and all references to work of other people have been duly acknowledged.

…….

EZIEFULE, CHIDINMA MIRACLE

(Student)

…..…….

DR. SAMUEL KWEKU DADZIE

(Principal Supervisor)

……

REV. DR. W.S.K. GBEWONYO

(Co-Supervisor)

……..

DR. DELPHINA A. M. ADABIE-GOMEZ

(Co-supervisor)

……..

DR. MAXWELL K. BILLAH

(ARPPIS Coordinator)

……..

ABSTRACT

The spread of insecticide resistance in *Anopheles gambiae* constitutes a major impediment to the effectiveness of malaria insecticide-based control tools and this is intensified by the use of the same insecticides in controlling both agricultural pests and public health vectors. The aim of this study was to establish the status of insecticide resistance in *Anopheles gambiae* found in vegetable growing areas (Korle-Bu and Opeibea) and non-vegetable growing areas (Mataheko and Achimota) and to determine the factors responsible for the selection of *An. coluzzii* and *An. gambiae s.s.* at the study sites. WHO susceptibility test procedures using five insecticides viz: Deltamethrin, Permethrin, Malathion, DDT and Bendiocarb was conducted in addition to a synergist (PBO) assay to assess the insecticide resistance status of *An. gambiae s.l.* population within the vegetable growing areas in comparison to the non-farming sites. Resistance was relatively high in both the farming and non-farming areas, but the exposure to PBO also enhanced the mortality of *An. gambiae s.l.* in all the sites. All the wild mosquitoes were identified to be *Anopheles gambiae s.s*. Korle-Bu had a dominance of 97.5 % *An. coluzzii*, while Opeibea had a dominance of 95 % *An. gambiae s.s.* and this was attributed to the ecological distribution and nature of the larval breeding water. The two species were found living in sympatry in the non-farming sites. High frequency of *kdr* and *ace-1* gene mutation in mosquitoes were recorded from all the sites with the highest frequencies obtained from mosquitoes collected at Opeibea. A higher frequency of *kdr* and *ace-1* gene mutation was recorded in *An. gambiae s.s.* than in *An. coluzzii*. Significantly higher enzyme activities were detected in the wild strains of *An. gambiae s.l.* compared to the susceptible Kisumu strain in all the sites except for glutathione-S-transferase (GST), where no difference was observed. Insecticide residues were determined using the gas chromatography (GC) method after fractionation of the extracts using solid phase extraction (SPE). All the samples from the study sites were found to contain detectable levels of chlorpyrifos, while

dichlorodiphenyldichloroethylene (p, p`-DDE) was only detected in vegetable growing areas of Korle-Bu and Opeibea. The detection of pesticide residues in the larval breeding water in both vegetable and non-vegetable growing areas; high frequencies of the *kdr* and *ace-1* gene mutations in all the sites; and elevated levels of enzyme activities in the mosquitoes from all the sites, revealed presence of insecticide resistance in *Anopheles gambiae* mosquitoes from the four study areas. This suggests that both agricultural activities and public health practices contribute to resistance in *Anopheles gambiae* in Accra. Enhancement of susceptibility to Deltamethrin and Permethrin in all the sites indicates the involvement of P450's in the development of resistance in the mosquitoes. Therefore, vector control products prepared with PBO can be effective even in the presence of pyrethroid resistance. Generally, resistance was only relatively higher in vegetable growing areas than non-vegetable growing areas although, was not statistically significant ($p > 0.05$). It was mostly driven by the application of insecticides by the farmers to control agricultural pest. However, more studies are recommended in other urban vegetable growing areas to understand the full scale of the impact of agricultural pesticides in the development of resistance in *An. gambiae s.l.*

DEDICATION

I hereby dedicate this research work to God Almighty who in His grace and infinite mercy saw me through to the completion of this work. May His name be praised forever.

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LIST OF ABBREVIATIONS

a.i**:** Active ingredients

Bp**:** Base pair

dNTP: Deoxyribonucleotide triphosphate

DDT: Dichlorodiphenyltrichloroethane

ECD: Electron capture detector

g: Gram

GC: Gas chromatography

KDT: Knockdown time

Kdr: Knockdown resistance

M: Molar

ml: Millilitre

µl: Microliter

mg: Milligram

µg: Microgram

pH: hydrogen potential

ppm: Parts per million

PFPD: Pulsed flame photometric detector

rpm: Revolution per minute

s.l.: *Sensu lacto*

s.s.: *Sensu stricto*

SPE: Solid phase extraction

Tm: Melting temperature

CHAPTER ONE

1.0. INTRODUCTION

Vector borne diseases comprise greater than 15 % of all infectious diseases in the world and cause more than 1 million deaths annually (WHO, 2006). Malaria has the largest disease burden worldwide, in terms of sicknesses, mortalities, financial costs and other effects, than any vector-borne disease (St. Louis and Hess, 2008). According to WHO (2012a), 200 billion cases occur every year and the number of deaths has halved in the last decades to 500,000 annually; with over 80 % of these deaths prevailing in sub-Saharan Africa; where about 70 % occur in children less than five years old and the remaining cases occurring mostly in pregnant women and the elderly.

The disease is transmitted by protozoa which belongs to the genus *Plasmodium* that infect mammals, birds and reptiles (Vezilier *et al*., 2010). There are about 120 *Plasmodium* species, of which *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* are known to infect humans. All the five species of *Plasmodium* share a common basic life cycle although there are some important differences in their pathogenicity, epidemiology, appearance, development, and host-parasite relationships. *P. falciparum,* however, is the most virulent and causes nearly most of the deaths in cases of malaria infection.

There are over 3,000 species of mosquitoes and they are classified into 41 genera according to (CDC, 2012; Person, 2014). Malaria is transmitted only by female *Anopheles* mosquitoes, and this is because they require a blood meal for the growth and development of their eggs and in addition to feeding on plant juices, to build up their energy reserves, while the males do not require a blood meal but feed only on plant juices and nectar.

The six dominant species of mosquito implicated in the transmission of malaria parasites in Africa include; *An. gambiae s.s* Giles, and *An. coluzzii* Coetzee & Wilkerson (previously called

S and M forms respectively) and *An. arabiensis* Patton (all of which are also the leading vectors of lymphatic filariasis), *An. funestus s.s* Giles, *An. pharoensis* and *An. coustanii*. All these mosquito species are prevalent throughout tropical and subtropical Africa. The *Anopheles gambiae* group consists of eight (8) sibling species that are widely distributed throughout sub-tropical Africa these include: *An. melas*, *An. merus*, *An. coluzzii*, *An. gambiae s.s*, *An. arabiensis*, *An. quadriannulatus*, *An. bwambae* and *An. amharicus*.

Anopheles funestus Giles group consists of nine (9) sibling species that are widely distributed throughout subtropical Africa (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987). All the nine members of the complex cannot be distinguished morphologically. They include: *An. funestus* Giles, *An. vaneedeni* Gillies and Coetzee, *An. leesoni* Evans, *An. parensis* Gillies, *An. rivulorum* Leeson, *An. fuscivenosus* Leeson, *An. brucei* Service, *An. aruni* Sobti and *An. confuses* Evans and Leeson (Gillies and De Meillon, 1968; Gillies and Coetzee, 1987).

Lymphatic filariasis which is referred to as a neglected tropical disease (NTD) is also a disease transmitted by mosquitoes. It is caused by the parasitic filarial nematodes which include, *Wucheraria bancrofti*, *Brugia malariae* or *Brugia timori* and is presently indigenous in approximately 72 countries (WHO, 2012b). Mosquito species which belong to the genera *Anopheles*, *Aedes*, *Culex*, *Mansonia*, *Coquilletidia* and *Ochlerotatus* genera are vectors of the lymphatic filariasis parasites. In West Africa, *Anopheles* mosquitoes are the main vectors of lymphatic filariasis (Subramanian *et al*., 1998; Appawu *et al*., 2001) and the major *Anopheles* vectors include *An. gambiae s.l.* and the *An. funestus* group (Boakye *et al*., 2004). These complexes constitute well-defined species which are identical morphologically and can occur in sympatry. In Ghana, it has been recorded that a few *Anopheles* species that occur in sympatry are vectors (Dunyo *et al*., 1996; Appawu *et al*., 2001; Boakye *et al*., 2004), and these are inclined to differ in their ability to transmit low density microfilareamia. People with the

disease can suffer deformation and permanent disabilities due to lymphoedema (swelling as a result of build-up of fluid caused by abnormal functioning of the lymph system). Elephantiasis is a debilitating condition in which parts of the body are enlarged distortedly. The psychological stigma linked with the disease is also noteworthy and can influence productivity and quality of life negatively (Anil and Tallurin, 2015).

Despite the vehement efforts by national and international bodies in its control, malaria still remains one of the most serious public health challenges in Africa (WHO, 2012a). The most effective way of controlling the transmission of malaria worldwide is to aim at the vectors; other ways are vaccination and chemotherapy. Targeting and controlling the vector is the main instrument for worldwide control of malaria. This plays a very instrumental and essential role in decreasing and, eventually in the control of malaria transmission.

The major methods used in controlling these vectors are still based on the use of insecticides treated tools, essentially Insecticide Treated Nets (ITNs) and Indoor Residual Spray (IRS) and these methods necessitates that the vectors are receptive to the insecticide being used (Overgaard, 2006).

Since 1950s, insecticides of different classes have been used successively, notwithstanding, most recent control programmes rely to a large extent on synthetic pyrethroids, which is the only class of insecticide endorsed by WHO for both insecticide-treated nets (ITNs) and indoor residual spray (IRS) (WHO, 2006; WHO, 2013). This is because of their comparably low mammalian toxicity and fast knockdown effect that induce paralysis in insects. Other classes of insecticides in addition to pyrethroids used for IRS are organophosphates, organochlorines and carbamates (WHO, 2009).

Pyrethroids are used to control pests of agricultural crops, besides, organochlorines, organophosphates and carbamates, and this extensive use has resulted in accounts of reduced effectiveness (N'Guessan *et al*., 2007; Yadouleton *et al*., 2009). The vector is exposed continuously to sub-lethal doses of these insecticides used in agriculture from the contamination of their nearby breeding sites.

The preferred habitats where these mosquito vectors that transmit malaria breed are in and around areas where agricultural activities take place. A research by Vanek *et al.* (2006), on the surveillance of the habitats of malaria vector larvae in Dar es Salaam, Tanzania, reported that habitats that were closely connected with agriculture such as marshes and furrows were more inclined to favour the breeding of *Anopheles* larvae than non-agricultural habitats. In these areas, pesticides used in agriculture apply specific weight on mosquito vectors by draining into their breeding spaces amid rainfalls or because of incidental spillage

The spread of resistance to insecticides is a major risk to the viability of the prevailing malaria control tools and is intensified by the utilization of similar insecticides in controlling both agricultural pests and vectors that influence general wellbeing (Diabate *et al*., 2002; Akogbeto *et al*., 2005; Czeher *et al.*, 2008; Djogbenou, 2009; Yadouleton *et al*., 2010). This was also reported in Ghana (Yawson *et al*., 2004; Muller *et al*., 2007; Adasi and Hemingway, 2008). Pyrethroid resistance by malaria vectors is ceaselessly being accounted for from various parts of Africa, and this has been related to the expanded utilization of ITNs and IRS (N'Guessan *et al*., 2007; Kisinza *et al*., 2011) and as of late because of the pollution of mosquito breeding habitats amid the application of agricultural pesticides (Diabate *et al*., 2002; Akogbeto *et al*., 2006; Yadouleton *et al*., 2009). Agricultural conditions can, particularly when inundated, create breeding habitats for the mosquitoes and thereby expanding vector density.

Up until now, all insecticides used in control of malaria vectors are neurotoxic. Pyrethroid and organochlorine insecticides target the voltage-gated sodium channel that stimulates impulse through the neuron membrane by the release of Na^+. Acetylcholinesterase which hydrolyses the neurotransmitter, acetylcholine in the synaptic gap is the point of action of organophosphate and carbamate insecticides.

Broadly, four types of insecticide resistance mechanisms have been depicted: target site insensitivity, metabolic, physiological (reduced penetration) and behavioural. The two main causes of resistance to insecticides in malaria vectors are modifications in the target sites and increase rate of insecticide metabolism, and these two have been intensively studied at both molecular and biochemical levels (Hemingway and Ranson, 2000). Three point mutations that make these target sites less susceptible to insecticides are the *ace-1*^{R}, the *kd*r and the *Rdl* mutations respectively (Hemingway *et al*., 2004; VectorBase, 2012).

The principal target site mutation is the knock-down resistance mutation (*kdr*) which leads to pyrethroids and DDT resistance. In *An. gambiae s.l.*, two amino acid substitutions in the voltage-gated sodium channel gene at codon 1014, a leucine to phenylalanine substitution (1014F) (Martinez-Torres *et al*., 1998) and a leucine to serine substitution (1014S) (Ranson *et al*., 2000), are implicated. Resistance to both organophosphates and carbamates in *An. gambiae* is conferred by substitution in a single amino acid of glycine to serine at position 119 in the catalytic domain of the acetylcholinesterase (AChE) gene (Weill *et al*., 2004). Gamma amino butyric acid (GABA) is a neurotransmitter of the inhibitory synapses in insect nervous system (Raymond-Delpech *et al.*, 2005). Resistance to cyclodienes like dieldrin is conferred by either a change of alanine to serine or to glycine in the GABA receptor gene at codon 296 (Du *et al.*, 2005).

Metabolic resistance involves three major categories of enzymes basically through an overproduction process. The enzymes are carboxylesterases (which acts against organophosphate and carbamate insecticides), glutathione-S-transferase (GSTs) (acting against pyrethroid, organophosphate, organochlorine insecticides) and cytochrome P450-dependent monooxygenases (metabolizes most insecticide classes, mostly together with other enzymes).

1.1. Justification/Rationale

Agricultural activities assume a vital role in transmission of malaria in both urban and peri-urban areas (Donnelly *et al*., 2005). Poverty, food insecurity and malnutrition have turned out to be problems in urban areas of Sub-Saharan Africa. In addressing these difficulties in urban communities of Sub-Saharan Africa, it also poses a challenge to public health. (Yadouleton *et al*., 2009). Urban agriculture has considerable benefits which include improving standard of living of citizens by providing food, income and employment (PADAP, 2003). Nevertheless, the socio-economic impact of urban and peri-urban agriculture is hampered by numerous factors of which the multiplication of mosquito breeding sites is one of them.

Agricultural systems which involves growing of vegetables is now more prevalent in many urban areas with Accra not being an exception. This in turn provides favourable breeding habitats for mosquitoes with possibly higher risk of malaria transmission in urban areas compared to rural areas.

A study carried out in Ghana reported higher *Anopheles* mosquitoes biting rates in urban areas with agriculture than urban areas where such practices are lacking (Klinkenberg *et al*., 2008). Previous work carried out by Achonduh *et al*. (2008) within the Accra Metropolis, revealed the presence of the *kdr* gene within *Anopheles gambiae* mosquitoes from cabbage fields and elevated levels of acetycholinesterase and oxidase activities. Residual bioactivity was detected

in runoff water and soil from the field. It was therefore speculated that the resistance detected may have been as a result of continuous exposure to lethal or sub-lethal doses of insecticides.

In as much as a number of studies on the resistance of *An. gambiae s.l.* to insecticides have been carried out in Ghana (Adasi *et al.,* 2000; Adeniran, 2002; Otieno, 2004; Yawson *et al.,* 2004; Achonduh *et al*., 2008), continuous monitoring of changing trends in resistance is essential. The information acquired will provide a premise for determining continued susceptibility to and effectiveness of insecticides used in crop growing areas and for vector resistance management.

Studies have shown a dominance of the resistant *An. coluzzii* in Korle Bu area and a dominance of the resistant *An. gambiae s.s.* in Opeibea areas all in Accra Metropolitan area (Achonduh *et al*., 2008 and Bilali *et al*., 2011). This present study aims at determining the resistance status of *Anopheles gambiae s.s.* from vegetable and non-vegetable growing areas in Accra and to investigate the factors responsible for the selection of these resistant forms in these areas (Korle Bu and Opeibea), by comparing with samples collected from two other non-crop growing areas (Mataheko and Achimota) around each of the sampling sites (about 2 km away) which served as the controls.

1.2. General objective

The general objective of this study is to determine the contribution of insecticides used in agriculture in some vegetable farms within the Accra Metropolis to the resistance status of *Anopheles gambiae* breeding in those areas in comparison with those breeding in non-crop growing areas.

1.2.1. Specific objectives

The specific objectives are to:

1. Identify members of the *Anopheles gambiae* species complex within the selected study areas of Accra Metropolis.
2. Determine the susceptibility status of *An. gambiae s.l.* to insecticides: Bendiocarb, DDT, Deltamethrin, Permethrin and Malathion in addition to a synergist, Piperonyl Butoxide (PBO) in the vegetable growing and non-vegetable growing areas.
3. Establish the frequency of knockdown resistance (*kdr*) and *ace-1* gene mutation across members of the species complex.
4. Determine the enzyme activity levels of acetylcholinesterase, mixed function oxidases (MFO), Glutathione-S-transferase (GST) and non-specific esterases in *An. gambiae s.l.*
5. Investigate the factors responsible for the dominant selection of *Anopheles coluzzii* at Korle Bu and *An. gambiae s.s.* at Opeibea.
6. Determine the presence of pesticide residues in the *An. gambiae s.l.* breeding water in both vegetable growing and non- vegetable growing areas.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. The scourge of malaria

Globally, malaria is a highly significant tropical parasitic disease, and causes greater mortality than any other communicable disease apart from tuberculosis and AIDS (WHO, 1998). The disease results from an infection with a protozoan parasite which belong to the genus *Plasmodium*. There are about six *Plasmodium* species with four out of them infecting man. The species include: *P. vivax*, *P. ovale*, *P. malariae*, *P. falciparum* infecting man and *P. knowlesi* and *P. cynomolgi* infecting primates.

Malaria is ranked as one of the most severe public health problems worldwide; with more than 3.3 billion people living in malaria endemic zones (Maigemu and Hassan, 2015). Worldwide, it has profound effect on the health and wellbeing of young children and pregnant women. It also causes undernutrition in children (National Institute of Allergy and Infectious Diseases, 2011).

Malaria is endemic in every part of Ghana, with more articulated seasonal variations in the north. The whole population of above 20 million people in Ghana are at risk of malaria infection (PMI, 2012). According to Ghana Health Service (GHS) health facility data, malaria is the main cause of sickness and deaths in children below the age of five years, causing about 33 % of hospital deaths in children below five years of age and about 40 % of all outpatient illnesses and approximately 36 % of all hospital admissions. An average of 3.3 million cases of clinical malaria are recorded in health facilities annually, of which 900,000 cases are reported to occur in children below five years of age and about 3,500 result in death in patients admitted in the hospitals (PMI, 2012).

Malaria is a major cause of poverty and has a yearly estimated cost to the economies in African countries in the range of 0.5 % to almost 9 % of gross national product (GDP) (Okorosobo *et al*., 2011). The increase in population in many malaria endemic countries has prompted strategies for local food production to enable self-sustenance and to decrease food importation which positively impacts the GDP in these countries (Pant, 1987; Service, 1991; Kaliyaperumal Karunamoorthi, 2013). Totally, malaria impact on the economy has been evaluated to cost Africa $12 billion annually. The financial effect comprises costs of medical services, working days lost due to ill health, days lost in training, lowered profitability because of mental distress from cerebral malaria and loss of speculation and tourism.

2.2. Lymphatic filariasis disease burden

Lymphatic filariasis (LF), is also a major public health challenge in tropical countries (WHO, 1992). It is a neglected tropical disease (NTD). It is estimated that over 1 billion people are at risk of contracting the disease and more than 128 million have one of the different clinical conditions related with filarial infection. The clinical symptoms of lymphatic filariasis (LF) vary with respect to endemic areas and the type of parasites implicated (Sasa, 1976). In some parts of Africa for example, Tanzania, the most clinical presentation of the disease is hydrocoele while lymphodema and elephantiasis are considerably less prevalent (Partono, 1987). Whereas, in India both hydrocoele and lymphodema are seen with practically equal prevalence (Pani *et al.*, 1991). Several studies have recorded a minimal dominance of female with chronic disease when hydrocoeles are precluded (Brabin, 1990). Other studies from Ghana recorded a male dominance of the disease (Gyapong *et al*., 1994).

The management of the disease have been impacted recently by the push for integrated control of NTD's responsive to mass drug administration (MDA) (Gyapong *et al*., 2010) and the effect of vector control on lymphatic filariasis transmission (Bokarie *et al*., 2009). Controlling the

vector is one of the five approaches approved by the WHO for prevention, control, elimination and eradication of NTD's in its 2012 road map for execution (WHO, 2012b). The WHO strategy for the eradication of LF was essentially based on chemotherapy before 2012, however, the effect of vector control on lymphatic filariasis transmission is now greatly felt (Ottesen, 2006; Bockarie and Molyneux, 2009; WHO, 2012b). Understanding the different vectorial roles in lymphatic filariasis transmission and their effects for rapid interruption of transmission in West Africa is also very important in malaria control efforts.

2.3. The mosquito vectors

The main mosquito species implicated in the transmission of malaria in Africa includes *An. gambiae s.s.*, *An. coluzzii*, *An. arabiensis* and *An. funestus s.s.*, and are all widely distributed in tropical and subtropical Africa (Chabi *et al*., 2016). *Anopheles arabiensis* are more prevalent in arid habitats and *An. coluzzii* is confined to West-Central Africa (Sinka *et al*., 2010; Coetzee *et al*., 2013). The differences in the ecology of these species, influences the effectiveness and appropriateness of control measures (Chabi *et al*., 2016). The distinction in ecology implies that *An. coluzzii* larvae preferentially utilizes sites that are more associated with anthropogenic activities, for instance, sites formed by irrigation, rice cultivation and urbanization (Lehmann and Diabate, 2008; Costantini *et al*., 2009; Simard *et al*., 2009) whilst larvae of *An. gambiae s.s.* live in pools and temporary puddles created by rainfall (della Torre *et al*., 2005). *An. gambiae s.s.*, *An. coluzzii* and *An. funestus s.s.* adult females are mostly anthropophilic and endophilic (prefer resting inside human habitations) while *An. arabiensis* feeds on either humans or cattle, and are either endophilic or exophilic (Knox *et al*., 2014).

Anopheles mosquitoes are the main vectors of lymphatic filariasis in West Africa (Subramanian *et al*., 1998; Appawu *et al*., 2001) and the leading *Anopheles* vector includes *Anopheles gambiae s.l.* and the *Anopheles funestus* group (Boakye *et al*., 2004).

Lymphatic filariasis and malaria share similar vectors in most countries in Africa and the methods for controlling the malaria vectors (the use of insecticide treated bed nets, indoor residual spraying) in both personal and community levels can also be effective against lymphatic filariasis (Zielke and Chlebowsky, 1980; Pedersen and Mukoko, 2002).

2.3.1. Life cycle of *Anopheles* genera

Anopheles gambiae is a holometabolous insect, which undergoes a complete metamorphosis through four stages namely; the egg, larval, pupa and adult stages (Figure 1). All adult (male and female) mosquitoes, feed on plant nectar to build up their energy reserves, while just the female feeds on blood of vertebrates, from which it derives nutrients, specifically proteins for egg development (Foster and Walker, 2009). In captivity, adults *Anopheles* mosquitoes can live for up to one month, while in the wild, they live for one to two weeks (CDC, 2010).

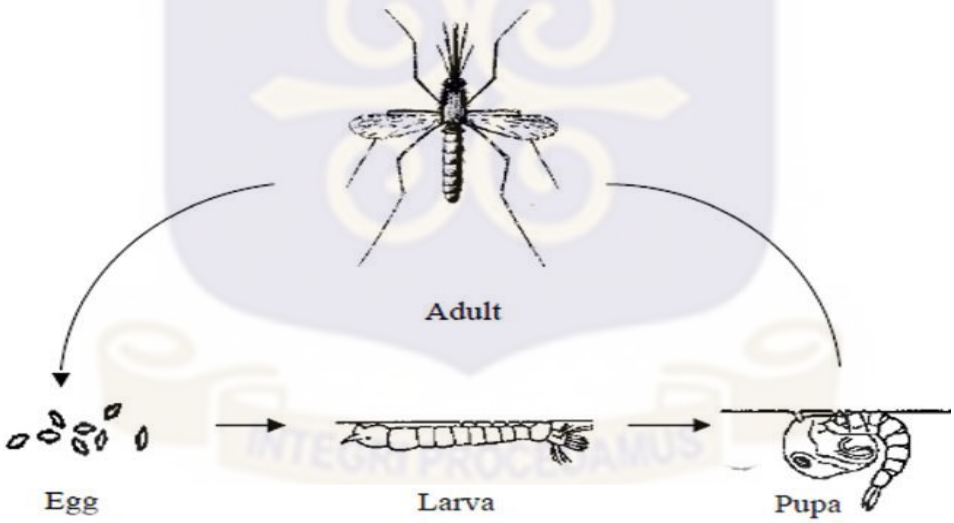


Figure 1: Life cycle of Anopheles spp.

Eggs: The eggs of *Anopheles* mosquitoes are between 0.47 and 0.48 mm long, curved outwards below and curved inwards above (Gillies and de Meillon, 1968). *Anopheles gambiae* eggs have floats on each of the sides and it is laid singly on water surfaces in a variety of habitats like

other *Anopheles* species (Foster and Walker, 2009) (Figure 1). The eggs of *Anopheles* mosquitoes are not impervious to drought (CDC, 2010). The eggs develop to adult in 10-11 days, but it is strongly influenced by temperature and can take a longer time under lower temperatures (Gillies and de Meillon, 1968).

Larvae: *Anopheles* larvae lie parallel to the water surface in order to breathe (Figure 1), because they lack the respiratory siphons used as breathing tubes in most other mosquito genera (Foster and Walker, 2009). The larvae develop through four larval instars before pupating (Foster and Walker, 2009) and feed on organic matter and algae (Garros *et al*., 2008).

Larvae live and develop within the aquatic habitat. Research reveals that larvae of *Anopheles gambiae* can thrive in persistent artificial structures, for example, solid tanks and waste canals (Mala *et al*., 2011) and natural pools like swamps, hoof prints and marshes (Mala *et al*., 2011; Kweka *et al*., 2012).

Pupae: When viewed from an angle, pupae of *Anopheles* mosquitoes are comma-shaped (CDC, 2010) (Figure 1). Unlike the pupae of many other insects, mosquito pupae are exceptionally mobile and they move through their aquatic habitat using the paddle at the terminal end of their abdomen (Foster and Walker, 2009). *Anopheles* pupae move frequently to the water surface in order to breathe through the respiratory trumpets on their cephalothorax. The pupae stage is a non-feeding stage amid which the adult mosquito is formed.

Adults: Adult female *Anopheles* can be identified by the presence of the palps (appendages found near the mouth) which are of equal length as their proboscis (feeding tube) (Foster and Walker, 2009). Adult *Anopheles* has a distinct resting position of an angle 180 ^{o} to the surface (Foster and Walker, 2009). The body color of *Anopheles gambiae* mosquitoes varies, but it ranges from light brown to grey with pale spots of yellow, white or cream scales, and dark

areas on their wings (Gillies and de Meillon, 1968). Adults are regarded as small to medium-sized mosquitoes with average wing length ranging from 2.8 to 4.4 mm when compared to other species (Gillies and de Meillon, 1968).

Adults are active at night, with peak hours of activity from 12:00 pm to 4:00 am, with activeness being sustained until just before day break (Gillies and de Meillon, 1968). The females are preferably endophilic although some feed outdoors (exophilic) and they are anthropophilic (White, 1974; Tuno *et al*., 2010). This makes them exceptionally effective vectors of malaria parasites in humans and adds to their importance as amongst the most critical vectors of malaria worldwide (CDC, 2010).

The seasonal abundance of *Anopheles gambiae* differs with respect to location, however, for the most part, the population declines in the dry season and increases in the rainy season (Gillies and de Meillon, 1968, Yaro *et al*., 2012). Mosquito populations starts to rise as the rainy season begins, tops in mid-season, and decreases as levels of water balance out and aquatic predators build up (Gillies and de Meillon, 1968).

2.4. Vegetable cultivation and pest control in Accra, Ghana

The food and agricultural sector is a major influence on the economy of most African countries, including Ghana. From cultivation to the final consumers, agriculture serves as a source of living for people engaged in its practice (Sinnadurai, 1992). In Ghana, the agricultural sector added an average of 41.3 % of gross domestic product and 12.2 % of national tax revenue, from 1990 to 1999, according to the Ghana Export Promotion Council (GEPC). This probably shows that the source of living of an average Ghanaian largely depends on either farming or farming-related business (Owusu-Boateng and Amuzu, 2013).

As a result of the increasing migration of rural dwellers into urban areas, urban agriculture has now become the predominant means to ensure food sustainability for the increasing population in the urban areas. According to Smith *et al*. (1996), urban agriculture is said to be the growing of crops and rearing of livestock, including the processing and marketing services and activities related to it, within the confines or boundaries of cities and towns.

Urban cultivation involving vegetable production is well practiced in Accra because it serves as a means of supplementing family income and improving the standard of living of people involved in its practice. According to Weinberger and Lumpkin (2007), the production and consumption of vegetables can likely generate income and reduce unemployment in developing countries. It also provides the body with the necessary micronutrients, antioxidants and phytochemicals that may boost the immune systems and protect people against diseases (Yang and Keding, 2009).

Cabbage (*Brassica olearaceae var. capitata*) is an important global vegetable (Rice *et al*., 1993) and the most common vegetable grown in Accra; others include, spring onions (*Allium fistulosum*), lettuce (*Lactuca sativa*), amaranth (*Amaranthus spp*.), garden egg (*Solanum melongena*), shallot (*Allium ascalonicum*), pepper (*Capsicum spp.*), okra (*Abelmoschus esculentus*). The cultivation of the vegetables within the Accra metropolis is a major commercial activity as a full time occupation or on a subsistence basis. The high market expectation of non-blemished fruits and vegetables with zero tolerance of pest damage has made farmers resort to increased pesticide use as the major means of crop protection. The farmers employ insecticides indiscriminately in high concentrations, spraying at high frequencies and mixing haphazardly. Associated with this practice is the risk it poses to the applicators themselves, inhabitants of houses in close proximity to farms and the various

environmental hazards as well as hazards of insecticide residues in the vegetables, soil, water and the atmosphere.

2.5. Agriculture and vector resistance

The input of insecticides in agriculture has been generally recorded to be on the expansion in late decades with roughly 90 % of all insecticides worldwide being utilized for the purposes of agriculture (Overgaard, 2006). Insecticides that have been used for a considerable length of time in agriculture are same as those used in public health for vector control, thus they all have the same effects. Aside the effects they have on target organisms in agriculture, non-target organisms including insect disease vectors are also affected when they occur in vicinities of the agricultural areas.

During pesticide application in agriculture, just around 15 % of the composition reach the target, the remainder are dispersed in the soil and air (Varca, 2002). It is realized that a large portion of the pesticides applied undergo many transport and change processes. Thus, they do not persist at their target site but infiltrate aquatic environments through soil percolation, air drift or surface run-off, affecting abundance and diversity of non-target species, and produce complex effects on the ecosystems, thereby, altering trophic interactions (Rand *et al*., 1995).

In Ghana, the cultivation of vegetables is considered to be a major factor that contributes to environmental contamination with pesticides in urban areas. Due to the growing population and high food demand to meet this growing population, particularly in urban areas, farmers in Ghana now practice monoculture and extensive cultivation. This action has prompted the increase in insect pest populations and manifestation, thereby, affecting optimal crop yield. Pest outbreaks occur with huge damage on cabbage farms because of the stable environmental conditions which are suitable for their survival and development (Afun *et al.*, 1992). In a bid

to protect their crops and produce, farmers resorted to control insect pests with the use of large quantities of insecticides. For instance, Brempong-Yeboah (1992) reported that cabbage farmers in the Accra Plains used substantial quantities of insecticides, particularly pyrethroids and organophosphates, and concoctions to control the Diamondback moth. High frequency of application of the insecticides was also recorded.

The preferred habitats in the urban areas where these mosquito vectors breed are in and around farming areas. According to a study conducted by Vanek *et al.* (2006), it was reported that habitats with farming activities were more inclined to favour the breeding of *Anopheles* larvae than areas where these activities are lacking. Likewise, Muriu *et al.* (2008) considered inundated rice agro-ecosystems as critical "hotspots" for mosquito-borne diseases because they serve as breeding sites to the various mosquito species.

The effects and contribution of breeding habitats in and around agricultural vicinities to the selection of resistance in malaria mosquitoes are distinct. Pesticides used in agriculture in these areas, apply pressure on mosquito vectors by draining into their breeding sites during rainfalls or as a result of accidental spillage. The indiscriminate utilization of pesticides in farms prompted the development of resistance in mosquito vectors breeding in those areas. A study conducted in Burkina Faso by Diabaté *et al.* (2002) reported that the use of insecticides in agriculture was implicated in the development of resistance to Permethrin and DDT in wild populations of mosquitoes. Klinkenberg and Boelee (2002) likewise outlined that urban agriculture, through open-space irrigated vegetable farms, may steer the creation and augmentation of breeding habitats for the malaria vectors, hence, impacting malaria transmission in Ghana.

Insecticide resistance is more prevalent in West Africa, where it is related with the utilization of same insecticides in agriculture for pests control and in public health for vector control (Diabate *et al*., 2002; Yawson *et al*., 2004).

2.6. Insecticide resistance mechanisms

Insecticide resistance is a heritable attribute in an insect population that leads to a consistent failure of an insecticide product to give the level of control when utilized as recommended with all negative factors removed (Insecticide Resistance Action Committee (IRAC), 2007). In other words, it is a reduction in the susceptibility of an insect population to an insecticide. Hemingway *et al*. (2004) reported that insecticide resistance is a characteristic trait that is hereditary and it involves some changes in one or more genes in an insect.

Additionally, insecticide resistance is an alteration in the genetic makeup of a pest population allowing individuals which were previously susceptible within a population to survive. Decreased susceptibility to individual pesticides or groups of pesticides is an inherited trait in resistant pest populations.

Generally, four types of resistance mechanisms have been described and they are, metabolic, target site insensitivity, reduced penetration (physiological) and behavioural resistance.

2.6.1. Metabolic resistance

The most common form of resistance mechanisms occurring in insects is the metabolic resistance (IRAC, 2007). This resistance mechanism depends on the enzyme frameworks which all insects have to enable them to detoxify or degrade foreign materials. It deals with the sequestration, and/or detoxification of insecticides, mostly through the overproduction of specific enzymes There are three groups of enzymes involved in this mechanism and they

include; esterases, monooxygenases and glutathione-S-transferases (Brogdon and McAllister, 1998). The activities of these enzymes are often highly elevated in resistant insect strains allowing them to detoxify insecticides before they are able to exert a toxic effect (IRAC, 2007). The most prevalent form of metabolic resistance mechanism is that of elevated activity levels of esterase, which hydrolyse ester bonds or sequester insecticides (IRAC, 2007).

Gluthathione-S-transferase is recorded to influence DDT resistance in *Anopheles* and *Aedes* mosquitoes in addition to other insects. Esterases are mostly implicated in organophosphate, carbamate resistance and to a lesser degree, pyrethroid resistance. Monoxygenases play a role in pyrethroids and organophosphates resistance and less commonly to carbamate resistance (Hemingway and Ranson, 2000). Metabolic resistance systems have been established in vector populations for every significant class of insecticide now utilized for vector control, including organophosphates, carbamates, pyrethroids and DDT.

2.6.2. Target site resistance

Insecticides basically target the nervous system within the insect. The target site can be altered in the resistant insect strain resulting in the insecticide becoming incapable of binding adequately. With the outcome that the insects become less affected or not affected at all by the insecticide than susceptible insects. Target site resistance is induced by modifications in the actual target site of an insecticide rendering it less sensitive to the active ingredients (Hemingway and Ranson, 2000; Weill *et al*., 2003). Most insecticides are neurotoxic and have the following target sites; sodium channels, gamma-aminobutyric acid (GABA) receptors, or acetylcholinesterases (AChEs) and alterations in these target sites can confer resistance in the insects (Hemingway and Ranson, 2000; Rivero *et al*., 2010).

Voltage gated sodium channels are channels accountable for raising the action potential in the neurones. These channels are the site of action for pyrethroids and organochlorines (DDT). They are transmembrane proteins in charge of the initial action potential in excitable cells (Catterall, 2000). The mutation in this site results in the insects not being sensitive or responding to effects of the insecticides, this situation is termed knockdown resistance. This resistance was first observed in the house fly *Musca domestica*. In this phenomenon, when insects are exposed to doses of the insecticide, they will not suffer paralysis which usually ends in death (knockdown), but will present a temporary paralysis which will afterwards lead to complete recovery (Busvine, 1951; Harrison, 1951; Milani, 1954). Research conducted demonstrates that *kdr* originated from a leucine to phenylalanine (L1014F) substitution in transmembrane segment 6 of domain II of the sodium channel (Williamson *et al*., 1996).

The GABA receptors act as chloride ion neurotransmission channel in the nervous system of insects, and it is the target site for cyclodiene such as dieldrin. Cyclodiene resistance is induced by single nucleotide changes within the same codon of a gene for γ- amino butyric acid (GABA) receptor (Ffrench-Constant *et al*., 1993).

Acetycholinesterase is the site of action for organophosphates and carbamate insecticides in the nerve cell synapses. Several modified forms of acetylcholinesterase (MAChE), have been reported which leads to decreased sensitivity to inhibition by these insecticides. For instance, this mechanism result in resistance to organophosphates recorded in *Culex* spp. (IRAC, 2007).

2.6.3. Reduced penetration

Reduced penetration is a form of physiological resistance which involves the development of barriers or the alterations in the cuticle or digestive tract linings in the cuticle of an insect that inhibits or slow down the penetration or absorption of insecticides. For instance, the additional

waxy layers found in some resistant insect strains. A wide range of insecticides can be influenced by this resistance mechanism (IRAC, 2007).

2.6.4. Behavioural resistance

This resistance mechanism is brought about by behavioural changes in response to delayed or prolonged exposure to an insecticide. It depicts any alteration in the behaviour of insects that enables them circumvent the deadly impacts of insecticides. This resistance mechanism does not have a distinguishable significance as physiological resistance but rather thought to be a contributing element, prompting the circumventing of lethal doses of an insecticide (IRAC, 2007).

For instance, in Mazoa Valley of Southern Zimbabwe, Muirhead-Thompson (1960) found that *Anopheles* mosquitoes originally are abundant both outdoor and in human dwellings. The indoor mosquitoes eventually disappeared from both treated and untreated dwellings after eight (8) years of benzene hexachloride (BHC) treatments in human dwellings and this condition persisted even after insecticidal treatments were discontinued. This suggests that during the period of the insecticide treatment, an outdoor strain evolved that lived and fed outdoors.

In some cases, the mechanism of resistance may be presented as an enhanced ability of the pest/vector to detect a toxicant and initiate a response to avoid it. This phenomenon has been studied by Kirkpatrick and Schoof (1958) for house flies and Gerold and Laarman (1964) for mosquitoes.

2.7. Cross resistance and multiple resistances

Resistance mechanisms are broadly divided into two categories: crossed and multiple resistances. Cross resistance occurs when a mechanism of resistance, that enables insects to

withstand one insecticide, also gives resistance to other insecticides within a similar class. This may also occur between different chemical classes, with respect to the mechanism (IRAC, 2007). Cross resistance is comparatively common in vector populations. For instance, DDT and pyrethroid insecticides both act on the voltage gated sodium channel, this led to insect populations developing resistance to DDT and pyrethroids, as a result of the *kdr* alteration at the sodium channel. When the acetylcholinesterase (AChE) has been altered or modified cross resistance between organophosphates (OP) and carbamate insecticides can also occur.

Multiple resistance is another common occurrence in insect populations and arises when various different mechanisms of resistance are present concurrently in resistant strains. The several different mechanisms of resistance may come together to confer resistance to multiple classes of insecticide products. Consequently, a single insect population becomes resistant to various compounds using diverse mechanisms. This may be the result of intensive and indiscriminate use of different chemical groups without following resistance management principles (Oppenoorth and Welling, 1976). Multiple resistance is usually a result of the synchronous utilization of different insecticides. Nonetheless, it is difficult at times to differentiate between cross resistance and multiple resistance, since genetic linkage may lead to different cross resistances, especially during selection in the laboratory (Oppenoorth and Welling, 1976). It is also somewhat common for the effect of different resistance mechanisms to adjust over time as selection processes develop (Oppenoorth and Welling, 1976).

2.8. Insecticide resistance management

Management of resistance is an attempt to delay or intercept the resistance development and it depends on proper pest management and pesticide-use strategies to prolong the efficacy of pesticides (IRAC, 2007).

In resistance management, a good understanding of the components that impacts its development is highly required. Resistance stability is a basic element in managing resistance and it involves an evaluation of how well resistance to a pesticide prevail in a pest population long after its use. Determination of resistance stability enables the restrained use of compounds prone to resistance. The rate at which susceptibility is restored greatly differs, but when pesticide utilization stops, selection for resistance is eliminated, and eventually results in reduced resistance (IRAC, 2007).

Insecticide resistance management can be initiated using insecticide-based strategies together with other vector control methods not requiring insecticides. The main kind of resistance management is insecticide-based, and this could take a few structures (IRAC, 2007).

2.8.1. Rotation

This approach involves the rotation over a period of time of two or more insecticide classes with distinct modes of action. This approach assumes that if resistance to each insecticide is uncommon then multiple resistance will be highly uncommon. It enables any resistance developed to the first insecticide to decrease over time when the next insecticide class is presented. The time allotment for rotation should be adequately short to intercept high levels of resistance being developed to any of the rotated insecticides. In agriculture, the rotation of different classes of insecticides having different modes of action, within a growing season is usually practiced while in most vector control programmes, yearly rotation is conceivable (IRAC, 2007).

2.8.2. Mixtures

In this context, a mixture is the co-utilization of at least two or more insecticides and can appear as a single formulation containing more than one insecticide, two or more insecticide formulations present together and being applied in the same spray tank, or insecticide treated nets (ITNs) treated with two or more insecticides (IRAC, 2007).

The use of mixtures to circumvent the build-up of resistance to fungicide in plant pathogens is a commonplace in agriculture. The use of tank mixes is a comparably easy resistance management strategy to apply and is practiced in agriculture. Nevertheless, for mixtures to be effective, the two insecticides should be utilized at their full application rate, and the effectiveness and constancy of the two insecticides should be extensively homogeneous. Product mixtures are seldom embraced in vector control programmes on account of cost, logistics, safety and due to the restricted number of approved compounds available. Nevertheless, with the evolution of recent vector control insecticides, this approach may be likely achievable (IRAC, 2007).

2.8.3. Fine scale mosaic

Isolated uses of various mixtures against the same insect represents a “mosaic” strategy to management of resistance. In vector control programmes, this can be accomplished for instance, by utilizing two insecticides in different dwellings in a single village. This enables insects within the same generation to encounter both insecticides, and would decrease the resistance selection rate, given that multiple resistance within the vector population was highly uncommon (IRAC, 2007). Supposing that a fine scale mosaic approach is to be adopted, cautious records of which insecticide was used in each dwelling are fundamental. Larger scale mosaics have been reported to be successful in the *Anopheles albimanus* trial in Mexico

(Penilla *et al*., 1998). Whilst there are some pragmatic impediments in executing a mosaic approach in a vector control programme, it renders the benefits of a mixture strategy with reduces input of insecticides and consequently cost. Mosquito bed nets created from panels treated with different insecticides accomplishes a comparable mosaic impact to treating houses with various mixes yet on a significantly finer scale.

2.9. Pesticide residue analysis

Pesticides utilized in pest control programs in agriculture and in public health for vector control can get into the environment through various routes (accidentally or through the unauthorized dumping of unwanted pesticide products or their containers), depending on the method and efficiency of application.

As a result of their extensive use, pesticides are detected presently in the environment by determination of their residues in several environmental matrices. such as air, soil and water (Watts *et al*., 1989). Pesticide residues are the accumulation of active ingredients of pesticide, its metabolites in some elements of the environment after its utilization, discharge or discarded.

According to Pesticide Action Network (PAN (1998)), pesticide residues can arise from the following ways; the utilization of legally approved pesticides according to good agronomic practices on a crop, indiscriminate use of legally permitted pesticide, or its use too near harvest and the illegal use of a pesticide that is not authorized for that crop or for post-harvest treatment.

Residue analysis gives an estimate of the quantity and nature of any chemical pollution in the environment and of its constancy. It is usually hard to relate pesticide residues in the environment with effects on fauna and/or ecological processes. However, they reveal whether a plant, animal or site has been exposed to chemicals and ascertain the possibility for future issues.

All pesticides are liable to degradation once discharged into the environment and this varies with different pesticides. The goal of residue analysis is to detect and identify the pesticide residues present at the time of collection and care must be taken to preserve the quality or state of the sample while transporting them to the laboratory. When sampling for residue analysis, care is taken to limit errors and in this manner boost the relationship between the result obtained from the analyzed sample and the actual residue levels present at the site.

2.9.1. Methods of residue analysis

Residues are present in very small quantities in heterogeneous compounds including biological materials. The process of residue analysis can be summarized according to the following steps:

i. **Sampling:** This procedure aims at obtaining a final sample representative of the whole sample, in order to determine its average pesticide content. The samples must be properly managed so as to avoid the loss of volatile or unstable pesticide residues and to also avoid pollution of the sample with other pesticides or interfering chemicals.

ii. **Extraction:** Insecticides are soluble in both polar and non-polar solvents. Extraction is normally carried out with appropriate solvent to separate the interested pesticide residue from other parts of the sample. Acetone or acetonitrile is utilized in most analytical laboratories to extract pesticides from food samples to be analysed. Hexane has also been exclusively used for extraction of chlorinated hydrocarbon and organophosphorus insecticides (Matsumura, 1985). Salts, for example, sodium chloride or anhydrous sodium sulphate, can be added to absorb water from the extract.

iii. **Clean up:** Clean up removes extraneous materials that are co-extracted from the analytical sample. Methods involved in general clean up include liquid-liquid partitioning, adsorbent

column chromatography, gel permeation chromatography and solid phase extraction (SPE) (Olson, 1988; Hetzel, 2000; Yeboah, 2001). The basic principle of SPE (accumulator or concentrator) is based on other clean up techniques such as adsorbent column chromatography, which depends on the interaction between a chemical dissolved in a solvent and an absorbing surface. Where the clean up is either achieved by the extractives or the pesticides being absorbed onto the absorbent i.e. the solvent passes through the column, dissolving and removing residues (eluting) leaving extractives attached to the absorbent or the co-extractives pass through the column and pesticides are eluted with appropriate solvent systems. Using the later principle therefore, the SPE cartridges absorb the pesticides when the extract is passed through without retaining co-extractives which are then eluted into the collecting containers. Conversely, SPE can retain the co-extractives and allow the pesticides to pass through. The column materials commonly used are florisil, alumina, silica gel, magnesium oxide and carbon (Yeboah, 2001).

iv. **Sample concentration:** This is aimed at reducing the volume of the solvent carrying the insecticide residues without losing residues, thus reducing the residues to a detectable level (Hetzel, 2000). This is done by using either nitrogen gas evaporator or rotary evaporator.

v. **Identification and quantification:** After providing the residue containing extract with or without clean up, the steps for resolution, detection, measurement, quantification and confirmation are performed sometimes after deprivation. Advances in the technologies available in these areas have been responsible for the improvements in both selectivity and detection limits over the past half century.

There are two basic methods in use for the detection and quantification of residues and they include:

a) Bioassay method:

Bioassay is the measurement of the potency or concentration of a substance by its effect on living cells or tissues. Bioassay has been used to determine pesticide residue levels (Sarode and Rattan, 1981). Laboratory bioassay involves the exposure of certain test samples to different doses of an insecticide after which it will be examined to either be dead or alive and recorded after a designated period of time. Insect bioassay systems are specific for separating insecticide residues from any other group of toxins and contaminants. The selection of test animals are based on their pesticide sensitivity and the ease with which large number of them can be reared (Matsumura, 1985; Aboagye, 2002). Brine shrimp, *Artemia salina*, a test organism is commonly used in the bioassay to detect and estimate levels of insecticide residues (Grosch, 1967). This is because it is found to be sensitive to a broad range of compounds at concentrations of 0.01 ppm in about 45 minutes to two (2) hours (McLaughlin, 1991). Other test organisms used in the measure of toxicants in water samples include the larvae of mosquitoes and *Daphnia* which provide very sensitive measurement of pesticide residues in water. Although bioassay is rapid, inexpensive and convenient, it is unable to distinguish metabolites, impurities and alteration product of pesticides from the parent compound, that is, it lacks specificity. Another limiting factor to this method is the difficulty in maintaining colonies of test animals. The principal function of this method is directed more towards the measurement of toxicity rather than the detection of pesticide residues.

b) Chromatographic methods:

These methods are for the routine analysis of pesticide residues contained in food products and found in the environment. Gas chromatography (GC), high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) are the most common methods used in this approach (Hetzel, 2000; Aboagye, 2002).

Gas chromatography is being used often to determine the quantity and confirm the identity of the residue detected. It can be fitted with Electron Capture Detector (ECD), Flame Ionizing Detector (FID) or Nitrogen Phosphorus Detector (NPD) depending on the type of compounds being analyzed (GTZ, 1979). The methods used for analysis varies, it could be a Multiple Residue Method (MRM), which can be used to determine various residues of different pesticides, Selective Multiple Residue Method (SMRM), which is used on small numbers of chemically related pesticides, and Single Residue Method (SRM), which is used to determine the residue of only one pesticide (FDA, 1994).

CHAPTER THREE

3.0. MATERIALS AND METHODS

3.1. Field studies

3.1.1. Study area

The study was conducted within the Accra Metropolitan area (5°32'59.99''N and 0°12'60.00''E) at four sites. A 3D global positioning system (GPS) was used to determine the specific geographical coordinates or geo-location of the sample sites (Figure 2). The sites were Korle-Bu (Latitude 5°32'20.124''N and Longitude 0°14'10.115''E), Mataheko (Latitude 5°34'0.4''N and Longitude 0°15'13.8''E), Opeibea (Latitude 5°35'54.54''N and Longitude 0°10'53.60''E) and Achimota (Latitude 5°36'59.99''N and Longitude 0°13'60.00''E) respectively.

Accra Metropolis is the largest, most assorted and sophisticated city in Ghana, with an estimated populace of over 1.8 million people. Accra is situated in the coastal savanna, which is defined by dry climatic conditions. The lowest mean monthly temperature of around 26 °C is recorded amid August and the highest of about 30 °C amid March and April. There are two peak rainfall periods, the first occurring from April to June and the second from September to October. The amount of rainfall is between 740 and 890 mm each year. The major rainy season falls between the months of June and July and after which a long dry season ensues (Yawson *et al*., 2004). The relative humidity ranges between 65 % and 75 % throughout the year.

The mosquito sampling or collection was conducted in the minor rainy season, from September to November, 2016 and in March 2017.

The sites were selected based on previous research conducted there which revealed the agro-ecological zone, the different types of agro-economic practices, and the dominance of

the different molecular forms, *Anopheles coluzzii* (M) and *Anopheles gambiae s.s.* (S) of *Anopheles gambiae s.s.* present.

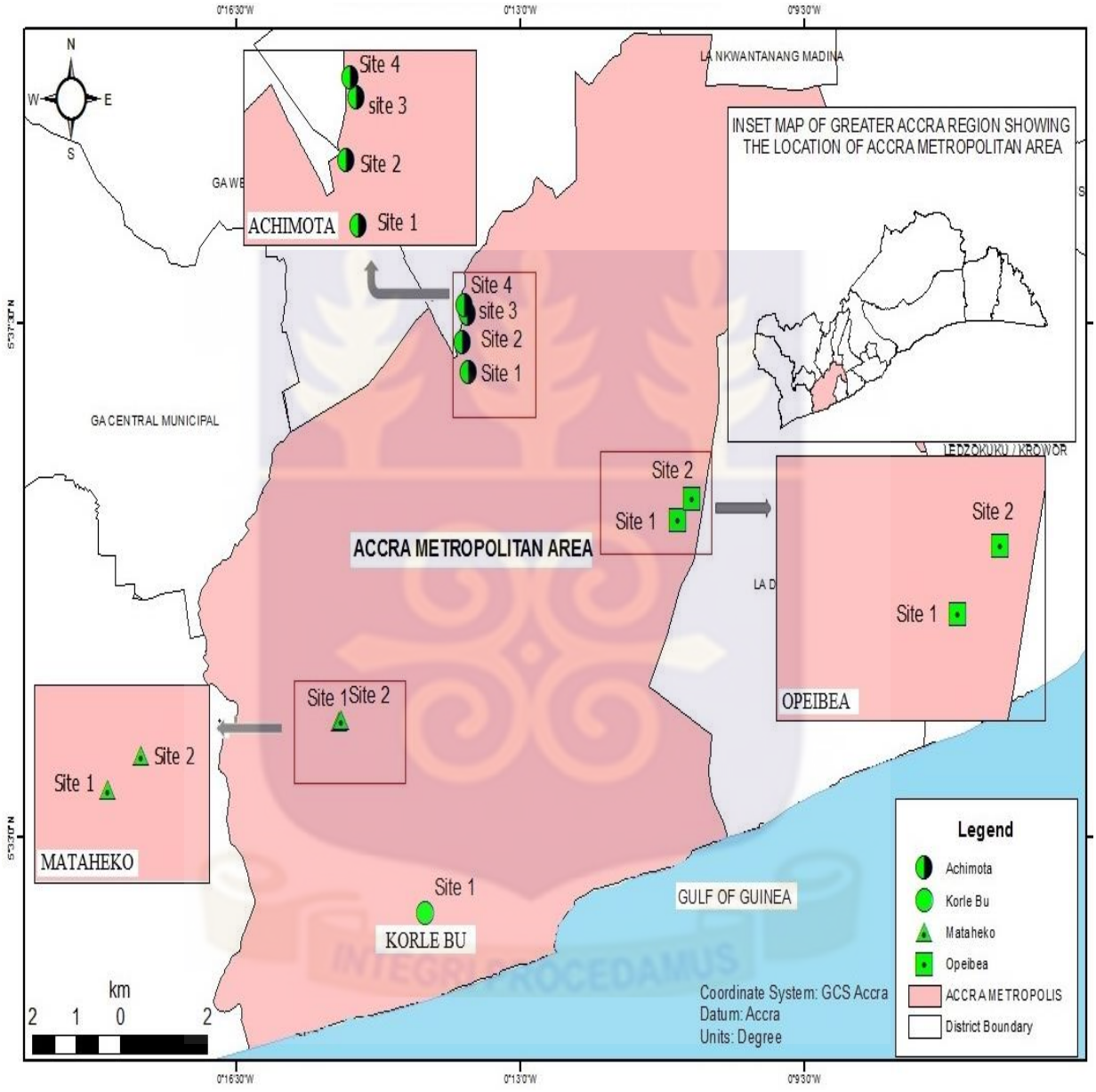


Figure 2: Map of Greater Accra showing the selected study areas and indicating the different collection sites within the study areas (Inset- Map of Greater Accra region).

3.1.2. Coordinates of the different sampling sites within the study areas and the hydrological variables estimated.

3.1.2.1. Opeibea site

The Opeibea site consists of two major vegetable farms. The GPS coordinates of the sites are; Site 1 (Latitude 5º35`54.54``N and Longitude 0º10`53.609``E), 40.00 m above ellipsoid, and Site 2 (Latitude 5º35`46.86``N and Longitude 0º11`2.694``E), 35.80 m above ellipsoid. The hydrological variables of the site are given in Table 3.1.

3.1.2.2. Korle-Bu site

The Korle-Bu site is also a vast expanse of vegetable farm with the farm lands more in clusters than that found in Opeibea site. The GPS co-ordinates is given as Latitude 5º32'20.124''N and Longitude 0º14'10.115''E, with an altitude of 22.30 m above ellipsoid and a height of 22.30 m. The hydrological variables of the site are given in Table 3.1

3.1.2.3. Achimota site

Sample collection at Achimota site was conducted at four different sites all of which were near building construction sites. The coordinates of the four sites include; Site 1 (Latitude 5º37`4.428``N and Longitude 0º13`38.381``E), Site 2 (Latitude 5º37`34.856``N and Longitude 0º13`39.035``E), Site 3 (Latitude 5º37`39.678``N and Longitude 0º13`41.328``E), Site 4 (Latitude 5º37`20.28``N and Longitude 0º13`42.531``E). The hydrological variables of the site are given in Table 3.1

3.1.2.4. Mataheko site

Here, mosquito sampling was conducted in two major sites, all of which were residential areas, in front of people's homes. The coordinates for the sites include; Site 1 (Latitude

5°34'1.3''N and Longitude 0°15'12.4''E) and Site 2 (Latitude 5°34'0.4''N and Longitude 0°15'13.8''E). The hydrological variables of the site are given in Table 3.1

Table 3.1: Hydrological variables at the study sites

Site	Sunlight intensity	Vegetable cover	Substrate type	Habitat type	Water current	Nature of breeding site
Opeibea	High	Present- less dense	Less muddy	Artificial; dug by farmers to store water during rainfall	Low	Rain-dependent pools dug in the farm
Korle-Bu	High	Present- less dense	Very muddy	Artificial; created by farmers unintentionally when watering	Very low	Between vegetable beds and in foot prints
Achimota	High	Absent	Less muddy	Artificial	Very low	Building construction sites
Mataheko	High	Absent	Less muddy	Artificial	Very low	Residential areas, in front of people's homes

3.1.3. Preliminary survey

A preliminary survey to determine the insecticidal use patterns of the farmers in the vegetable growing areas was conducted by administering questionnaires to the farmers at the different sites. The questionnaire was designed to obtain information on the following: the main methods of pest control, the type of chemical used for pest control and the active ingredients, frequency of use, the mode of application, the dosage applied, type of spraying equipment used and the interval between insecticide application and watering.

The data was analysed with the aim of knowing the commonly used insecticides by farmers in order to guide the selection of insecticides for the study. The questionnaire was also structured to obtain information on the type of vegetables planted on their farmland, the planting season

and interval for each vegetable to guide in analysing the time of the year control measures are at its peak.

The questionnaire was administered to correspond to the farmer's understanding and with the assistance of a translator who simplified and explained the questions to the farmers in their local dialect (Twi) and in French because not all the farmers were Ghanaian. In each vegetable growing area, a random representative number of growers were selected to partake in the survey. Of the farmers interviewed, six (6) were from Opeibea whereas the other three (3) was from Korle-Bu.

3.1.4. Water sample collection

Water samples of 2.5 litres each were obtained in duplicates from the four sites in Winchester bottles and transported immediately to the laboratory for filtration. This was to prevent the water from putrefaction. The filtration was done using filter paper and the filtrates were collected in conical flasks which were labelled and then refrigerated.

The samples were concentrated to about 100 ml using a Buchi rotary vacuum evaporator at 40 ^{o}C. The residue was stored at 4 ^{o}C for further use.

3.1.5. Sampling larvae and pupae of *Anopheles* mosquitoes

Mosquito larvae and pupae were sampled from ponds and pools of water in the farms. *Anopheles* larvae were identified by their characteristic body position in water which is parallel to the surface of the water or immediately below the water surface film. *Culex* larvae were also identified in the water body because of their characteristic vertical position which is as a result of the presence of a siphon which is lacking in *Anopheles* mosquitoes.

The larvae were carefully collected using a ladle which is made up of a wooden handle with a plastic container at its tip or an aluminium ladle depending on the volume of the larval breeding water (Figure 3a) and then transferred into white transparent containers. Afterwards, the samples collected in the containers were passed through a set of sieves with different mesh sizes avoid losing the larvae. They were then labelled and transported to the insectary in Vestergaard-NMIMR vector laboratory at Noguchi, in small plastic transparent containers which had holes punctured on the lid to allow for ventilation while being transported (Figure 3b).

Figure 3: (a) Larval sampling between beds at Korle-Bu; (b) Plastic containers for transporting mosquito larvae to the insectary.

3.2. Laboratory activities

3.2.1. Rearing of the mosquitoes

In the insectary, the *Anopheles* larvae were sieved and transferred into rectangular white plastic bowls containing about 1.5 litres of clean tap water. The samples were sorted to remove excess

debris, dirt and other mosquito larvae like *Culex* species which often shares breeding sites with *Anopheles* mosquitoes although *Anopheles* mosquitoes breed in cleaner water bodies. The bowls were then labelled to indicate the date of collection, species present, the mosquito generation and name of site (Figure 4a).

The larvae were fed daily on a fish diet (Tetramin). Much care was taken while feeding the larvae to avoid over feeding and to also prevent the water from being too clouded which reduces the oxygen in the water and eventually kills the larvae. Also overcrowding was also avoided by not placing too much larvae in a bowl (approximately 50 larvae per bowl). Larval feeding was done daily by putting a pinch of feed (about 2 mg) into the rearing water. The temperature and relative humidity during the rearing process were 25 - 33 ^{o}C and 55 - 80 % RH respectively.

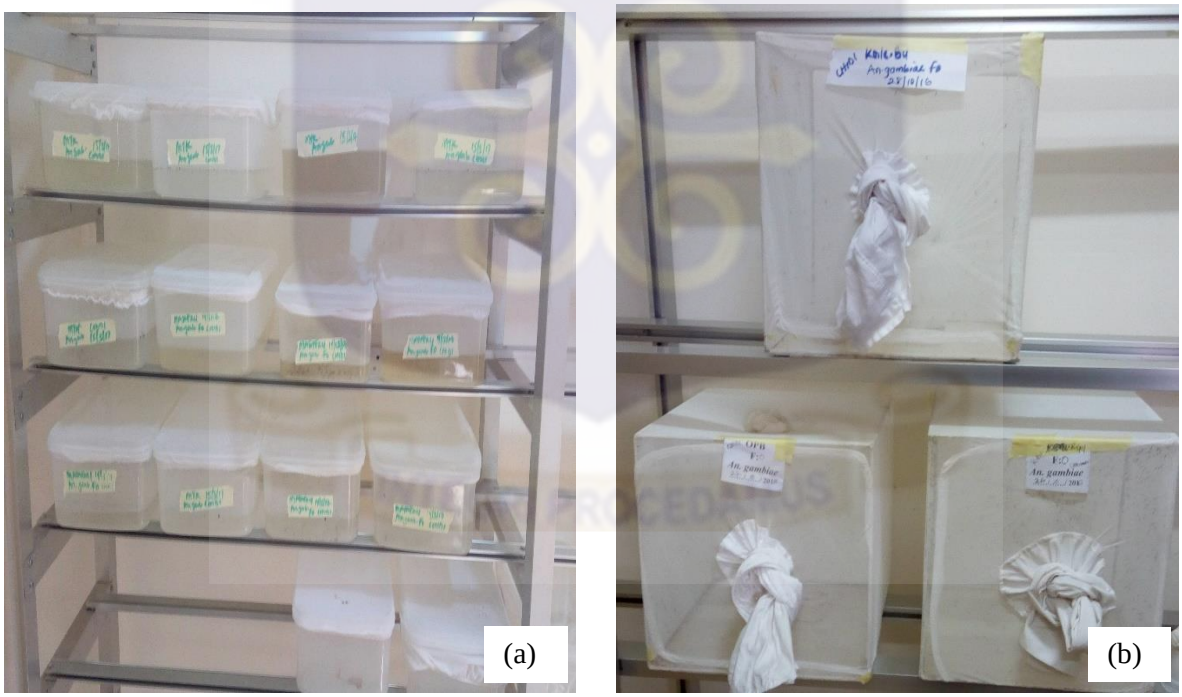


Figure 4: (a) Larval rearing bowls; (b) Some of the adult rearing cages.

The pupae were identified based on their comma shape when viewed from the side and were mostly found at the corners of the plastic bowl. The pupae were picked daily from the tray

using a pipette and transferred into 50 ml white plastic cups which were put into a 0.27 m^3 screened cage for adult emergence. The screened cages were labelled to reflect the following information: the date for the next day (which will be useful in calculating the age of the mosquito for the bioassay because their ages are counted usually after emergence), the site and the generation (Figure 4b).

Emerged adults were fed with 10 % sucrose solution soaked in cotton wool within 12 hrs of post emergence because the bioassays require non-blood fed female mosquitoes (F1 generation) within the range of 2-5 days old. The cotton wool was soaked in the sugar solution in a way to prevent the solution from dripping into the cage and to avoid attracting ants to the cage and also avoid the formation of moulds.

3.2.2. Bioassays

WHO insecticide susceptibility tests were carried out using susceptibility test-kits supplied by the Universiti Sains Malaysia, Penang Malaysia and standard protocol (WHO, 2013).

The protocol kit included insecticide-impregnated papers (used for the test) and non-insecticide impregnated papers (used for the controls), aspirators, 12 wire clips (6 copper and 6 steel) and plastic tubes (marked red for exposure, yellow for control and green for holding). The steel wire clips are used in fitting the holding tubes while the copper wire clips are used to fit the exposure tubes (Figure 5).

Figure 5: WHO test tubes for susceptibility tests.

The choice of WHO insecticide papers used for the susceptibility test (bioassay) were dependent on the results from the preliminary survey with one insecticide selected from each insecticide class. The five insecticides used for the tests were: 0.05 % Deltamethrin, 0.75 % Permethrin, 0.1 % Bendiocarb, 5.00 % Malathion and 4.00 % DDT. Additionally, synergist assay was conducted using 5.0 % Piperonyl Butoxide (PBO) with Deltamethrin and Permethrin.

The holding tubes were prepared by lining papers in the tube which were held in place by steel wire clips (two (2) clips per tube) and then covered with a lid at one end and a slide unit with the part marked green at the other end. The exposure tubes were prepared in the same way but lined with insecticide-impregnated papers and fitted with copper wire clips and joined to the other end of the slide unit marked red.

In each test, 150 non-blood fed 2 - 5 days old female adults were aspirated from the cages in batches of 25 with an aspirator and transferred into six holding tubes through the filling hole in the slide unit to give six replicate samples (four replicates of insecticide-impregnated paper tubes and two controls) containing 20 - 25 mosquitoes in each tube. After the mosquitoes were

transferred, the slide unit is closed immediately and carefully to prevent the mosquitoes from escaping and then the holding tubes were kept upright vertically for an hour. Afterwards, the empty exposure tubes were fitted to the vacant end of the slide unit, after which the slide units were opened and the mosquitoes blown gently into the exposure tubes and the timers were started. The holding tubes were then gently detached and set to one side.

The knockdown (KD) counts were recorded cumulatively in five minutes for the first 20 minutes and then 10 minutes interval for the remaining forty minutes (i.e, 5, 10, 15, 20, 30, 40, 50 and 60 minutes respectively). The knocked down mosquitoes were identified by their uncoordinated way of flying and standing which usually fell to the bottom of the exposure tubes. After 60 minutes, the slide units were opened and the mosquitoes were gently blown back into the holding tubes. Pads of cotton wool soaked in 10 % sucrose solution were then placed on the mesh-screen end of the exposure tubes (cover lid) for the mosquitoes to feed on for the next 24 hours before mortality was recorded. This ensured that mortality was recorded based on just the test procedure and not hunger and weakness. After 24 hours post exposure (the recovery period), the mortality was counted and recorded. Dead mosquitoes were identified by their inability to fly, bent posture and slender abdomen. They were counted and recorded. The live mosquitoes were knocked down using chloroform to enable easy counting.

The mortality of test sample was calculated by adding the number of dead mosquitoes across all the four exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes}}{\text{Total sample size}} \times 100$$

A similar calculation was also made in order to obtain a value for the control mortality. When control mortality was greater than 5 % but less than 20 %, the observed mortality was corrected using Abbott's formula, as follows:

$$\text{Corrected observed mortality} = \frac{(\%\ \text{observed mortality} - \%\ \text{control mortality})}{(100 - \%\ \text{control mortality})} \times 100$$

The mosquitoes were transferred into well labelled Eppendorf tubes reflecting the site, date, whether they were dead or alive, and the insecticide used. They were then stored in the refrigerator till molecular assay was carried out on them.

For the synergist assay, the PBO papers were lined in tubes and afterwards the mosquitoes were gently blown into the tubes and exposed for an hour, after which they were transferred into another tube lined with the insecticide papers. All assays were performed in four (4) replicates. The remaining procedure then continued as indicated above.

The temperature and relative humidity of the procedure were between the range of 25 ± 2 °C and 70 % ± 10 %. The starting and ending temperature and relative humidity for the exposure and observation periods were recorded accordingly.

3.2.3. DNA extraction and molecular assays

The stored mosquito samples from the insecticide susceptibility tests (bioassay) were used for the molecular assay. This was carried out by selecting forty (40) mosquitoes each from the four sites, ten (10) from each insecticide class; five (5) of which were alive and the other five (5) dead. They were transferred into 1.5 ml Eppendorf tubes with one mosquito per tube, labelled accordingly and placed in the freezer.

DNA was extracted from the adult *An. gambiae* mosquitoes following the protocol outlined by Wagner *et al*. (1987). The mosquitoes in the 1.5 ml Eppendorf tube were ground in 200 µl of

2 % CTAB using a motorized pestle to increase the effectiveness of grinding and to produce a homogenized solution. After grinding, the samples were heated at 65 ^{o}C for five (5) minutes in the blocks heater. Two hundred microliters (200 µl) of chloroform was then added and mixed per inversion which helps to separate insoluble proteins and other materials from nucleic acids. The samples were centrifuged for 5 mins at 1200 rpm at room temperature. After centrifuging, the upper phase (supernatant) were siphoned and transferred into another batch of labelled tubes where 200 µl portions of isopropanol were added and mixed per inversion to precipitate the DNA and centrifuged again for 15 minutes at 1200 rpm at room temperature. The isopropanol was removed with care to prevent discarding the pellet formed at the bottom of the tubes. Then 200 µl of 70 % ethanol was added to wash out the excess salt present in the solution. The samples were centrifuged again at 1200 rpm for 5 minutes at room temperature. Afterwards, the ethanol was also poured out with care to prevent discarding the DNA pellets. The tubes were then dried overnight by being inverted on the bench. The next day, 20 µl of DNase free water was added and allowed to stand for an hour after which the samples were vortexed and centrifuged for 2 minutes. After centrifuging, the samples were diluted by transferring 97 µl each of DNase free water into another set of labelled tubes and then 3 µl of DNA each was transferred into the same new tubes, vortexed and centrifuged for another 2 minutes and stored at -84 ^{o}C until ready for use. All molecular assays were performed using these extracted mosquito DNA samples.

3.2.3.1. Polymerase chain reaction (PCR) identification of *An. gambiae* complex

The method described by Fanello *et al*. (2002) was used. The primers included, universal primer (UN), *An. gambiae s.s.* (AG), *An. arabiensis* (AR) and *An. melas* (AM) (Table 3.2).

Table 3.2: Oligonucleotide primer sequences and band sizes for identification of *An. gambiae* species complex.

Primer	Sequence (5`-3`)	Band size (bp)
UN	GTGTGCCGCTTCCTCGATGT	468
AG	CTGGTTTGGTCGGCACGTTT	390
AR	AAGTGTCCTTCTCCATCCTA	315
AM	GTGACCAACCCACTCCCTTGA	464

Firstly, the master mix was prepared in the hood. This was done by pipetting 2.1 µl of DNase free water into a new Eppendorf tube (1.5 ml) labelled master mix. The primers were added in their appropriate volumes into the tube containing the water, afterwards, the Gotaq was added into the mix. It is lastly added to prevent it from reacting with the primers if added earlier. After this was done, the mix was vortexed for about a minute and then spun for about three minutes, this was to enable the reagents mix properly in the tube. The master mix was transferred into the wells and then 0.5 µl of the DNA was distributed into the appropriate wells in addition to three controls which included confirmed DNA samples of *An. gambiae s.s.* and *An. arabiensis* (positive controls) and water which is the negative control. The wells were covered and labelled accordingly using the various sample codes and then amplified in the thermocycler by polymerase chain reaction.

The PCR thermal cycling profile was as follows: an initial denaturation step of 3 minutes at 94 ^{o}C, followed by 35 cycles with denaturation at 94 ^{o}C for 30 seconds, annealing at 50 ^{o}C for 30 seconds and extension at 72 ^{o}C for 60 seconds and ending with an extension at 72 ^{o}C for 5 minutes.

The amplified products were analysed by gel electrophoresis. The gel was prepared by dissolving 3 g of agarose powder in 150 ml of 1x Tris acetate- EDTA (TAE) buffer and mixed with a wooden stick and heated in the microwave for 10 minutes. It was allowed to cool and then 2 µl of Ethidium bromide was added to the mixture, stirred and then poured into the gel casting tray and allowed to solidify for about 15 - 20 minutes. After solidification, 10 µl of the 100 bp DNA ladder and each PCR product were transferred into the wells in the gel and allowed to run for about one hour or until the bands were seen to be separated in the gel. The gel was visualized and photographed in the UVP dual trans-illuminator at short wavelength. The identity of the sibling species was established by comparison with the DNA size and the mobility of the 100 bp DNA ladder and also with the controls.

3.2.3.2. Identification of the molecular forms of *An. gambiae s.s.*

The identification of *An. gambiae s.s.* molecular forms were done using the SINE PCR method according to Santolamazza *et al.* (2008). This method is based on the detection of powerful genetic markers such as short interspersed nuclear elements (SINEs) which are present in all *An. gambiae* M forms but absent in the S forms. The primers used for the reaction were, F6.1a and R6.1b (Table 3.3).

Table 3. 3: **Primer sequences used for the identification of molecular forms of *Anopheles gambiae s.s.***

Primers	Sequence
F6.1a	TCGCCTTAGACCTTGCGTTA
R6.1b	CGCTTCAAGAATTCGAGATAC

The master mix was prepared in the same manner as described under section 3.2.3.1. but in the required volumes and transferred into the respective wells, after which 4 ul of the DNA

templates were distributed into the wells with the addition of four controls. The controls included: confirmed *An. gambiae* M forms, S forms and MS forms and then water which is the negative control. The samples were amplified following the PCR conditions as described under section 3.2.3.1. and the products electrophoresed through Ethidium bromide-stained 2 % agarose gel and the resulting bands visualized under UV transilluminator. The molecular forms of the mosquito population from each site were then recorded.

3.2.3.3. PCR detection of the *kdr* alleles in the *Anopheles gambiae s.l.*

PCR detection of the *kdr* alleles in *An. gambiae* complex was done using the method of Martinez-Torres *et al*. (1998). This resistance mechanism is caused by two amino acids substitutions at the same codon position 1014 in the voltage-gated sodium channel. The primers used for this assay were AgD1, AgD2, AgD3 and AgD4 (Table 3.4).

Table 3.4: Primer sequences for the detection of *kdr* alleles in the *An. gambiae* complex

Primer	Sequences	Melting temp. (ºC)
AgD1	ATAGATTCCCCGACCATG	54
AgD2	AGACAAGGATGATGAACC	64
AgD3	AATTTGCATTACTTACGACA	40
AgD4	CTGTAGTGATAGGAAATTTA	52

The master mix was prepared from the forty selected DNA samples from each site and used for detection of the *kdr* genotypes of the resistant and susceptible population. The samples were amplified in the thermocycler by the following PCR conditions at 94 ºC for 3 minutes, initial denaturation followed by 40 cycles at 94 ºC for 30 seconds, 50 ºC for 1 minute 50 seconds and

72 ^{o}C for 2 minutes. There was a final cycle at 72 ^{o}C for 10 minutes followed by cooling at 4 ^{o}C. The products were electrophoresed through Ethidium bromide stained 2 % agarose gel and the resulting bands visualized under UV transilluminator as described previously under section 3.2.3.2. The *kdr* genotypes of both the susceptible, resistant and the heterozygous resistant populations were then recorded (Table 3.5).

Table 3.5: Expected band sizes of the *kdr* genotypes of the *An. gambiae s.l.*

Genotype	Band size (bp)
SS (Susceptible)	293
	137 (diagnostic band)
RR (Resistant)	293
	195 (diagnostic band)
RS (Heterozygous resistant)	293
	195 (resistant band)
	137 (susceptible band)

3.2.3.4. PCR detection of the *ace-1* gene mutations in *An. gambiae s.l.*

The method of Weill *et al*. (2003) was used to detect acetylcholinesterase (*ace-1*) gene mutations in *An. gambiae* complex. It involved a PCR-RFLP method, which is the amplification of the DNA with the PCR and then the digestion of the PCR products with restriction enzymes. Single nucleotide substitution in the *ace-1* gene, confers a glycine to serine (G119S) amino acid substitution causing resistance to organophosphate and carbamate insecticides in *An. gambiae.* The primers used were EX3AGdir and EX3AGrev (Table 3.6).

Table 3.6: Table 3.6: Primer sequence for the detection of *ace-1* gene mutation in *An. gambiae s.l.*

Primer	Sequence
EX3AGdir	GATCGTGGACACCGTGTTCG
EX3AGrev	AGGATGGCCCGCTGGAACAG

The sample master mix was prepared and amplified in the thermocycler following the PCR cycling conditions at 94 ºC for 3 minutes, followed by 35 cycles at 94 ºC for 30 seconds, 62 ºC for 30 seconds and 72 ºC for 30 seconds. Then a final extension cycle at 72 ºC for 5 minutes. For the second step which is the enzymatic digestion of the PCR products, a restriction digest reaction mix was prepared with the following reagents; PCR grade water, enzyme buffer 10X, acetylated BSA, restriction enzyme (Alu 1) in addition to the PCR products in their appropriate volumes. Afterwards the sample master mix was amplified in the thermocycler again following the PCR cycling conditions at 37 ºC for 60 minutes, 37 ºC for 60 minutes, 37 ºC for 60 minutes and another 37 ºC for 60 minutes. This step runs for a duration of four (4) hours. The products were electrophoresed through Ethidium bromide stained 2 % algarose gel and the resulting bands visualized under UV transilluminator as described previously under section 3.2.3.2. *Ace-1* gene mutations of both susceptible, resistant and heterozygous resistant populations were then recorded (Table 3.7).

Table 3.7: Expected band sizes of the *ace-1* gene mutations of the *An. gambiae s.l.*

Genotype	Band size (bp)
SS (Susceptible)	403 (diagnostic band)
	138
RR (Resistant)	253 (diagnostic band)
	150
	138
RS (Heterozygous resistant)	403 (susceptible band)
	253 (resistant band)
	150
	138

3.2.4. Biochemical assays

This assay is used to quantify the levels of the activities of the various enzymes in individual mosquitoes. Fifty (50) fresh mosquitoes each were selected from the four different sites, immobilized by freezing and then transferred into labelled 1.5 ml Eppendorf tubes (one per tube) and then stored at -82^{o} C before used for the assays. One hundred and twenty (120) Kisumu susceptible strain (30 per site) were also selected for the assay and used as controls.

The mosquitoes were homogenized thoroughly in 100 µl of potassium phosphate buffer on ice and afterwards diluted further with 900 µl of the same buffer to obtain a total volume of 1000 µl. This homogenate was used for all the assays for each individual mosquito.

3.2.4.1. Protein assay

This assay quantifies the amount of protein in each mosquito. In preparation of the solutions for the protein assay, 60 mg of BSA was dissolved in 30 ml of distilled water to get the stock

(2 mg/ml), then ten (10) two-fold serial dilutions in potassium phosphate buffer (pH 7.2) were performed to arrive at the tenth concentration standard which was 0.0039 mg/ml.

The standard curve was determined by transferring 100 µl of the protein standards of concentrations 0.125, 0.063, 0.031, 0.016, 0.008, 0.004, 0.002 and 0.00 mg/ml into respective wells on a microtitre plate in duplicates, after which 200 µl of protein dye was added. The absorbance was read immediately at 620 nm in a Biotek ExL800 microplate reader. The calibration curve derived was then used to extrapolate the total protein per mosquito.

Coomassie plus protein assay reagent was used in determining the protein content of the individual mosquitoes. Twenty microliter (20 µl) of mosquito homogenate samples were placed in appropriate wells on labelled microtitre plates in duplicates after which 200 µl of protein dye reagent was added with 80 µl of potassium phosphate buffer (K_3PO_4) used as the negative control. The plate was read immediately (T0) with Biotek ExL800 microplate reader using 620 nm filter.

3.2.4.2. Oxidase assay

The solution for this assay was prepared by dissolving 10 mg of cytochrome-C in 100 ml of sodium acetate buffer, pH 5 to give a stock concentration of 0.1 mg/ml. Afterwards, a two-fold serial dilution was also performed in potassium phosphate buffer (K_3PO_4) of pH 7.2, to the tenth concentration to arrive at 0.0002 mg/ml. Then tetramethyl-benzidine (TMBZ) was prepared by dissolving 20 mg of 3, 3`,5,5`-tetramethyl-benzidine in 25 ml of methanol and then 75 ml of 0.25 M sodium acetate buffer pH 5 was added.

The standard curve was determined by transferring 100 µl of the cytochrome-C standards of concentrations 0.0250, 0.0125, 0.0063, 0.0031, 0.0016, 0.0008, 0.0004 and 0.0000 mg/ml into appropriate wells in a microtitre plate in duplicates after which, 25 µl of 3 % hydrogen peroxide

was added and incubated for 5 minutes. The absorbance was then read after five minutes at 620 nm in the plate reader. The calibration curve obtained was used in extrapolating the results from the assay.

Hundred microliter (100 µl) of mosquito homogenate samples were loaded into appropriate wells on microtitre plates in duplicates. Afterwards, 200 µl of 3, 3`.5,5`-tetramethyl-benzidine (TMBZ) and one drop (25 µl of 3 % hydrogen peroxide (H_20_2) was added onto the same wells and incubated for 5 minutes. The plate was read with Biotek ExL800 microplate reader using 620 nm filter. Cytochrome C and potassium phosphate (K_3PO_4) buffer were used as the positive and negative controls respectively.

3.2.4.3. Glutathione-S-transferase assay

Hundred microliter (100 µl) of mosquito homogenate were transferred into the appropriate wells followed by 100 µl of reduced glutathione and 100 µl of cDNB (1-chloro-2,4`-dinitrobenzene). The plates were read immediately (T_0) with a Biotek ExL800 microplate reader using 340 nm filter. The plates were incubated at room temperature and readings were taken again at 340 nm after five minutes (T_5). The difference between the two readings was used for the calculation of the GSTs activity levels.

3.2.4.4. Insensitive acetylcholinesterase assay

This assay determines the presence of altered acetylcholine site. Here, 100 µl of mosquito homogenates were transferred into appropriate wells and 100 µl each of acetylthiochloride iodide (ATCH) and dithio-bis-nitrobenzoic acid (DTNB) were added to each well. The plates were read immediately (T_0) using 414 nm filter and read again after 10 minutes (T_{10}) using the same wavelength. The difference between the two readings was used for the calculation of the results obtained.

3.2.4.5. Non-specific esterase assay

This assay measures the levels of non-specific esterase in each mosquito using two substrates of the enzyme, alpha and beta-naphthyl acetate. The solutions were prepared by dissolving 50 mg of α-naphthyl acetate in 10 ml of acetone, 90 ml of potassium phosphate (K_3PO_4) buffer was added to give a stock solution concentration of 0.5 mg/ml. Afterwards, two-fold serial dilutions of the solution were performed in potassium phosphate buffer (pH 7.2) to the tenth concentration to arrive at 0.0009 mg/ml; same procedure was followed for the preparation of β-naphthyl acetate standard concentrations.

The calibration curve was determined by transferring 100 µl of alpha- and beta-naphthyl acetate standard concentrations, 0.250, 0.125, 0.063, 0.031, 0.016, 0.008, 0.004 and 0.000 mg/ml into microtitre plate wells in duplicates, after which 100 µl of freshly prepared dianisidine was added and then incubated for two minutes. After incubation, the absorbance readings were taken at 540 nm. The calibration curve obtained was used in extrapolating the results obtained from the assay. Hundred microliter (100 µl) of mosquito homogenate were again transferred into each wells on the microtitre plates, then 100 µl of α-naphthyl acetate was added to each well and then incubated at room temperature for 10 minutes. Afterwards, 100 µl of dianisidine was added to each well and incubated for 2 minutes and then read with a Biotek ExL800 microplate reader using a 620 nm filter. The same procedure was used in preparing the plates using β-naphthyl acetate, but after incubation for 2 minutes, it was read using a 540 nm filter. Alpha and beta-naphthyl acetates were used as the positive controls while potassium phosphate (K_3PO_4) served as the negative control.

3.3. Pesticide residue analysis on the water samples

3.3.1. Extraction

The method of extraction used was that of Handa *et al*. (1999) with slight modification. 50 ml of the water concentrate was taken in a measuring cylinder and transferred into a separating funnel in duplicates and shaken with 50 ml of hexane: ethyl acetate mixture (9:1) for 5 minutes. The organic phase was carefully transferred into clean 250 ml conical flask. The process was again repeated three times with fresh solvents and then all three extracts were pooled together and transferred into a clean 250 ml flask containing 5 g of anhydrous sodium sulphate to remove the moisture present.

3.3.2. Concentration of extracts

The extracts were concentrated to dryness using the Buchi rotary vacuum evaporator at 40 ^{o}C. They were transferred into pre-weighed vials and dried using a desiccator. The dry extracts were redissolved in 0.5 ml of hexane and stored at 4 ^{o}C for further use.

3.3.3. Clean up of water extracts

The extracts were cleaned up using methanol, ethyl acetate and hexane solvents through C-18 solid phase extraction (SPE) columns. The C-18 cartridge SPE columns were pre-conditioned with 2 ml of methanol. Each of the extracts (0.5 ml) were applied to the top of the tube using a pipette. Eluting solvents (2 ml) in the order, ethyl acetate, methanol (polar solvents) were passed through the column to elute the sample which was collected in a vial, and afterwards, hexane (non-polar) was also passed through the column to obtain non-polar eluates in another vial making duplicates of the samples per site (one batch eluted in polar solvents, the other in a non-polar solvent). The effluents were collected into pre-weighed 5 ml vials and evaporated to dryness in a desiccator. The weight of the fractions recovered after the clean-up was determined and the extract was reconstituted in appropriate volume of solvent to obtain a

concentration of 5 mg/ml for samples eluted in polar solvents and 1 mg/ml for samples eluted in a non-polar solvent. The samples were then submitted for gas chromatography analysis.

3.3.4. Analysis of insecticide residues using gas chromatography (GC) method

A Varian CP3800 gas chromatograph ECD and PFPD were used to detect and quantify organochlorines and pyrethroids and organophosphates residues respectively, in the water from mosquito breeding sites at the Pesticide Residue Analysis Laboratory of the Ghana Standards Authority (GSA). For the organochlorines and pyrethroids, samples were injected into the column of the GC with an autosampler at a temperature of 270 ^{o}C. The compounds were partitioned through the stationery phase, a capillary column (30 m + 10 m EZ Guard, internal diameter (i.d.) 0.25 mm, fused with silica coating VF- 5 ms, 0.25 µm film) carried by solvents. The oven temperature was programmed at 70 ^{o}C for 2 min, then 25 ^{o}C/min till 180 ^{o}C followed by 5 ^{o}C/min up to 300 ^{o}C. The carrier gas was nitrogen maintained at a constant flow rate of 1 ml/min.

However, for the organophosphates, the samples were injected into the column of the GC with an autosampler at a temperature of 270 ^{o}C. The compounds were partitioned through the stationery phase, a capillary column (30 m x 0.25 mm internal diameter (i.d.) fused with silica capillary coating with VF- 1701 ms, 0.25 µm film) carried by solvents. The oven temperature was programmed at 70 ^{o}C for 2 min, then 25 ^{o}C/min till 200 ^{o}C followed by 20 ^{o}C/min up to 250 ^{o}C. The carrier gases were nitrogen (1 ml/min), Air 1 (17 ml/min), Hydrogen (14 ml/min) and Air 2 (10 ml/min).

The molecules of the insecticide residues were differentiated at different rates through the gas. The electron capture detector (ECD) and the pulsed flame photometric detector (PFPD) was used to detect the compounds present. The detection temperatures were 300 ^{o}C for the ECD

and 280 ^{o}C for the PFPD. These detectors were linked to a computerized integrated system which counts and records the signals as peaks, which were used to quantify insecticide residues present.

3.4. Data analysis

Microsoft Windows Excel (version 2010) was used for constructing tables and plotting graphs to illustrate the trends in the various parameters under consideration. The data from the questionnaire on the insecticide use pattern was analysed using SPSS 20.0. Mortality observed in the susceptibility test was calculated as percentages, in cases where the mortality was between 5- 20 %, it was corrected using the Abbott's formula (Abbott, 1925). WHO guidelines for interpreting susceptibility test results (WHO, 2013) which include: 98 - 100 % (susceptible), 90- 97 % (suspected resistance) and < 90 % (resistant) was used to interpret the susceptibility status of the mosquitoes to the insecticides.

The values for the knockdown times KDT_{50} and KDT_{95} were estimated from the time-mortality regression using probit analysis software (SPSS). T-test was used to compare the percentage mortalities between the vegetable growing areas and the non-vegetable growing areas. It was also used to compare the *kdr* and *ace-1* gene mutations frequencies between the vegetable growing areas and the non-vegetable growing areas. The frequency is usually between the range of 0 and 1 and was calculated using the Hardy Weinberg formulae below:

$$Fo = \frac{2nRR + n\,RS}{2(nRR + nRS + nSS)}$$

Where, RR - Resistant,

RS - Heterozygous resistant,

SS - Susceptible,

n - Total number and

Fo - Frequency.

Enzyme activities were analysed and compared using GraphPad Prism 5. Kruskal-wallis H test was used to compare the levels of pesticide residues detected from each of the sites.

CHAPTER FOUR

4.0. RESULTS

4.1. Insecticides and their use pattern

This survey was conducted on two vegetable farms at Korle-Bu and Opeibea in Accra. The survey sought to provide information on the agrochemicals (brand names), class and active ingredients used by the farmers (Table 4.1). From the survey, pyrethroids were the most commonly used insecticides in the two vegetable areas with a percentage usage by 9 (100 %) farmers, followed by organophosphates with percentage usage by 7 (78 %) farmers and biopesticides 7 (78 %) farmers. The least used were neonicotinoids 2 (22 %), fungicides 1 (11 %) and avermectin 1 (11 %).

Table 4. 1: Agrochemicals (brand names) used by the farmers, class and active ingredients.

Agrochemical (Brand name)	Class	Active ingredients
Proclaim 1.9 E.C	Avermectin	Emamectin benzoate 1.92 % EC
Confident 532 EC	Pyrethroid + Organochlorine	Cypermethrin + Endosulphan
Bypel 1 (PrGV-Bt)	Biopesticide	*Pierix rapae darnulosis* virus 10000 pib/mg +*Bacillus thuringiensis* 15000 µ/mg
Attack (EC)	Organophosphate + pyrethroid	Pirimiphos-methyl 475 g/L Permethrin 25 g/L
Lambda super 2.5 EC	Pyrethroid	*Lambda* Cyhalothrin 5 % EC
Akape (Anti-Attah)	Neonicotinoids	Imidacloprid
Porselen	Avermectin	Emamectin benzoate 5 % SG
Agricombi	Organophosphate + Pyrethroid	Fenitrothion 30 % + Fenvalerate 10 %
Agrithane 80 WP	Fungicide	Mancozeb 800 g/kg
Cydimsuper	Pyrethroid + Organophosphate	Cypermethrin + Dimethoate
Dursban	Organophosphate	Chlopyrifos
Goland (SL)	Neonicotinoid	Acetamiprid

4.2.1. Method of insecticide application

Four (44.4 %) of the growers were able to give the various volumes of insecticides used per sprayer. They used the lid of the chemical containers as standard measure of dosages while for some farmers it was difficult to estimate because the application equipment was not calibrated before use.

The equipment used for the application of the insecticides was mainly the knapsack sprayer to which were attached different nozzles. But all the farmers in Korle-Bu (66.7 %) and only one (11.1 %) in Opeibea made use of improvised channels in place of the nozzle. Of the farmers that use nozzles in Opeibea, 5 (100 %) use polyjet green, 2 (40 %) use polyjet yellow and green and 1 (20 %) use polyjet red and green.

It was also observed that only 3 (33.3 %) farmers wear protective clothing during insecticide application, 4 (44.4 %) wear protective clothing sometimes, while 2 (22.2 %) do not wear any protective clothing during spraying.

4.2.2. Frequency of insecticide application

Information on the interval between insecticide application and watering of the fields was also obtained from the survey (Figure 6). Out of the nine (9) farmers interviewed, 1 (11.1 %) of the respondents, watered within same day of insecticide application (watering in the morning, spraying in the afternoon), 2 (22.2 %) watered the next day after application, 1 (11.1 %) watered two days after application while another 1 (11.1 %) watered only when the need arises, there is no specific time interval. No information was gathered from 4 (44.4 %) farmers due to their unwillingness to continue participating in the survey (Figure 6).

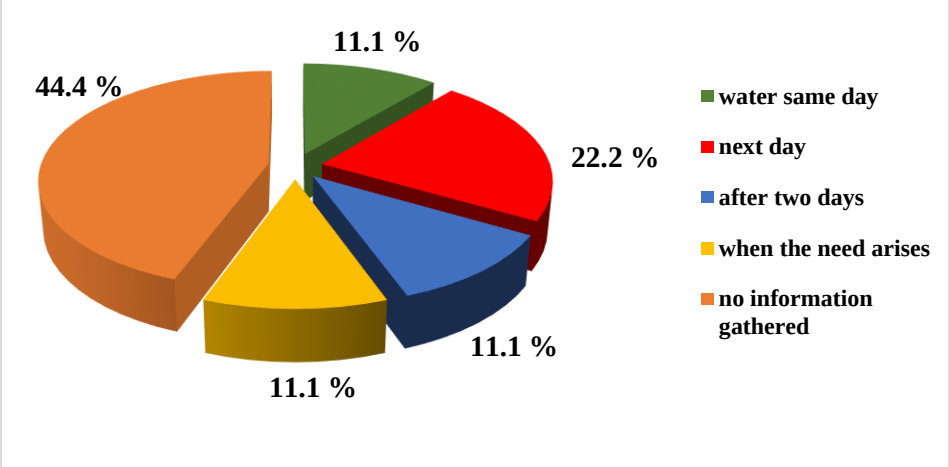


Figure 6: Interval between insecticide application and watering by the farmers.

On the frequency of pesticide application on each vegetable per planting season, out of the nine (9) farmers interviewed, 5 (55.6%) applied pesticides weekly on the vegetables till harvest while 2 (22.2 %) applied once in two weeks, no information was gathered from the remaining 2 (22.2 %) growers because they became uninterested in the survey (Figure 7).

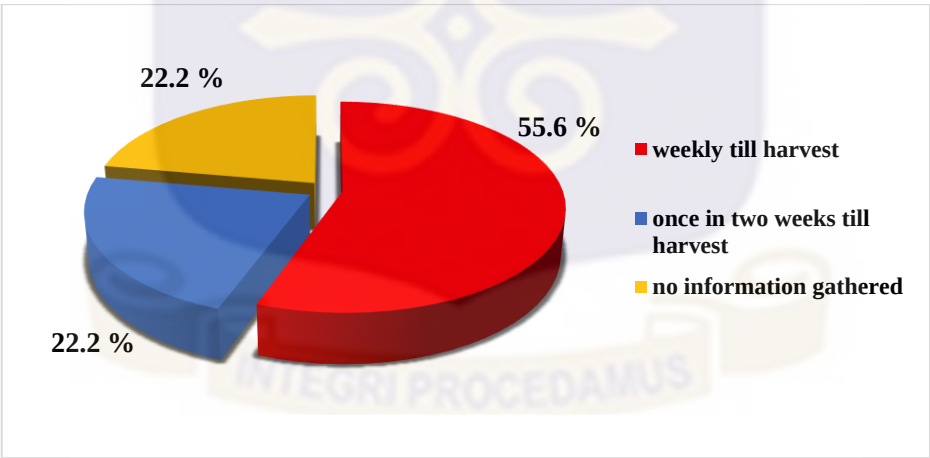


Figure 7: Frequency of insecticide application by the farmers.

This high frequency of application practiced by majority of the growers could probably be as a result of the sources of professional advice on proper insecticide use and handling. Only 2

(22.2 %) receive advice from Extension Workers, the other 7 (77.8 %) get advice from pesticide sale points, and their fellow farmers.

With regards to knowledge on the problems associated with insecticide residues in foods especially vegetables, only 2 (22.2 %) of the farmers are aware of the dangers of insecticide residues in food, while the other 7 (77.8 %) growers have no idea about it.

4.2. Bioassays on *Anopheles gambiae s.l.*

A total of 2,684 adult female *Anopheles* mosquitoes of about 2-5 days old, were used for the bioassays for the four sites. Of these, about 1,617 were exposed to the five different insecticides, with 632 exposed to the synergist (PBO) prior to Deltamethrin and Permethrin insecticides whereas 435 were used as control. The values for the controls were not included in the graph because, they were not exposed to insecticides, it only helped to provide an assessment or measure of natural mortality during the test and to report for all factors that may instigate mortality aside the insecticides being tested. The percentage mortalities recorded between vegetable and non-vegetable growing areas are shown in Figures 8 and 9 respectively.

4.2.1. Bioassays on *Anopheles* mosquitoes from Achimota and Opeibea

Using WHO susceptibility test, 685 adult female *Anopheles* mosquitoes were exposed to the five insecticides at Achimota. Malathion had the highest percentage mortality rate of 41.82 % (n = 85) on *Anopheles gambiae s.l.*, while DDT recorded the lowest mortality of 6.98 % (n = 86) (Figure 8). For the synergist assay, PBO + Deltamethrin had a higher mortality of 76.47 % (n = 85) than 44.05 %(n = 84) of PBO + Permethrin. Pyrethroids (Deltamethrin and Permethrin) showed percentage mortalities of 36.17 % (n = 78) and 13.05 % (n = 81) respectively (Figure 8). Generally, the percentage mortalities were high in these mosquitoes compared to Opeibea.

There was a significant difference in the percentage mortalities with respect to the insecticides ($p = 0.010$). A summary of the percentage mean total mortalities, values of KDT_{50} and KDT_{95} and resistance status are given in Appendix I.

At Opeibea, 723 female *Anopheles* mosquitoes were assayed for susceptibility to the insecticides. Percentage mortalities of *Anopheles gambiae s.l.* for the insecticides ranged from 2.50 % (n = 81), for Permethrin to 33.33 % for (n = 76) Malathion (Figure 8). For synergists assay, PBO + Deltamethrin has a higher mortality of 56.50 % (n = 77) on *Anopheles gambiae s.l.* than PBO + Permethrin which has a mortality of 46.80 % (n = 74). Amongst the pyrethroids, mortalities on *Anopheles gambiae s.l.* ranged from 2.50 % for Permethrin to 2.60 % (n = 93) for Deltamethrin (Figure 8). Here, there was no statistically significant difference ($p = 0.054$), between the different percentage mortalities observed in the mosquito population with respect to the different insecticides. A summary of the percentage mean total mortalities, KDT_{50} and KDT_{95} values and resistance status are given in Appendix II, but the values of KDT_{50} and KDT_{95} for Permethrin were not included because of the very low knockdown effects recorded.

By way of comparison, the percentage mortalities of *Anopheles gambiae s.l.* mosquitoes to the various insecticides used, between Achimota and Opeibea, was not statistically significant ($p = 0.360$).

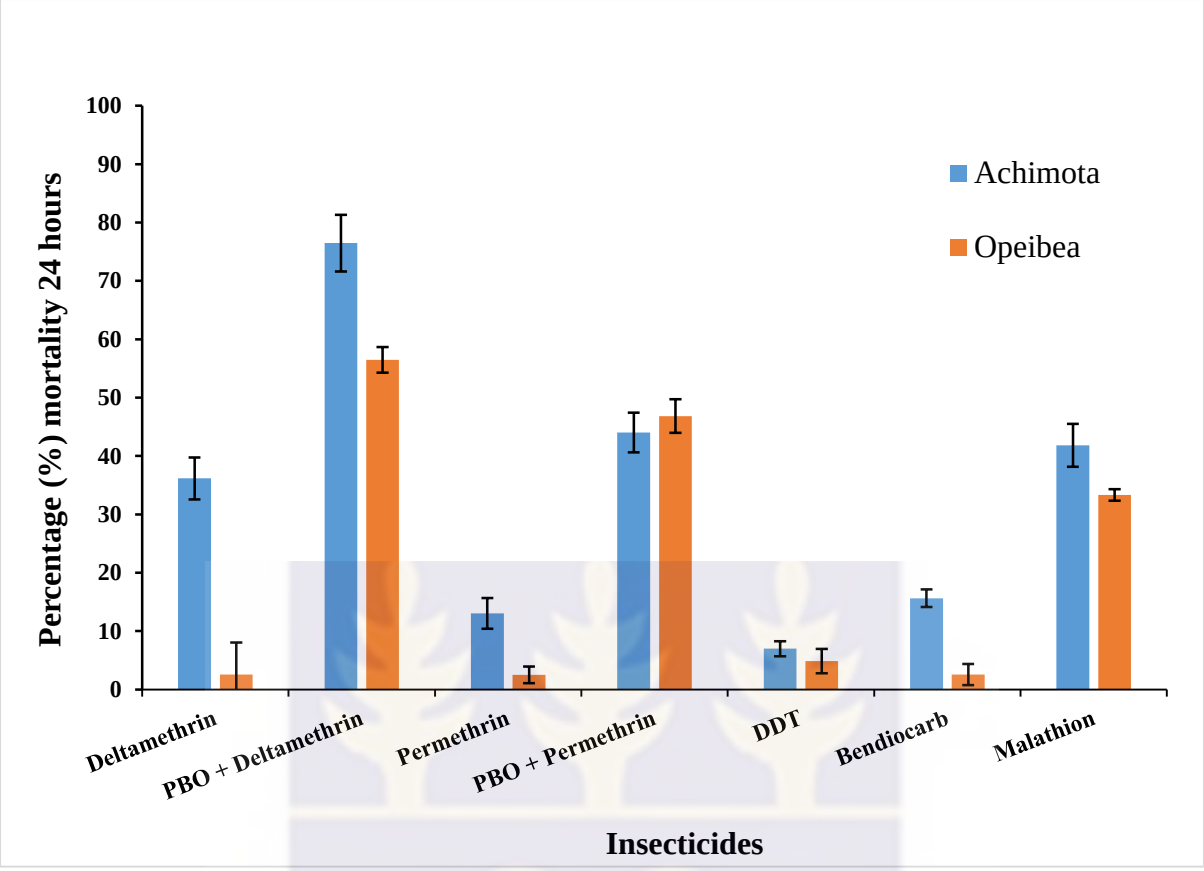


Figure 8: Percentage mortalities of *Anopheles gambiae s.l.* mosquitoes from Achimota and Opeibea.

4.2.2. Bioassays on *Anopheles* mosquitoes from Mataheko and Korle-Bu

A total of 566 female *Anopheles* mosquitoes were exposed to the insecticides at Mataheko. Percentage mortalities of *Anopheles gambiae s.l.* caused by the different insecticides in this site ranged from 5.26 % (n = 76) DDT to 40.51 % (n = 79) Malathion (Figure 9). For the synergist assay, PBO + Deltamethrin also had a higher mortality of 93.15 % (n = 73) than 66.22 % (n = 74) of PBO + Permethrin. Amongst the pyrethroids, Deltamethrin had a higher mortality of 38.89 % (n = 72) on *Anopheles gambiae s.l.* than Permethrin with a mortality of 7.79 % (n = 77) (Figure 9). The observed difference between the mortalities across the different insecticides was statistically significant (p = 0.027). A table with the summary of the percentage mean total mortalities, values of KDT_{50} and KDT_{95} and resistance status is given in Appendix III, but the

values of KDT_{50} and KDT_{95} for DDT were not included because it had a very low knockdown effect on the mosquitoes.

For Korle-Bu, a total of 710 female *Anopheles* mosquitoes were exposed to the various insecticides. Percentage mortalities of *Anopheles gambiae s.l.* as a result of the insecticides ranged from 1.19 % (n = 84) for DDT to 64.58 % (n = 96) for Malathion (Figure 9). Results for the synergist assay showed that, PBO + Deltamethrin had a higher mortality of 61.45 % (n = 83) against *Anopheles gambiae s.l.* compared to that of PBO + Permethrin which caused a mortality of 13.95 % (n = 86) on the mosquitoes (Figure 9). *Anopheles gambiae s.l.* exposed to Deltamethrin had a higher mortality of 12.50 % (n = 88) amongst the pyrethroids, compared to those exposed to permethrin with 5.68 % (n = 88) (Figure 9). A significant difference was observed ($p = 0.046$) in the percentage mortalities of the mosquitoes with respect to the different insecticides. A summary of the percentage mean total mortalities, KDT_{50} and KDT_{95} values and resistance status are given in Appendix IV, but the KDT_{50} and KDT_{95} values of PBO + Permethrin and DDT were not included because of the very low knockdown effects observed.

In comparison of the percentage mortalities to the insecticides in *Anopheles gambiae* mosquitoes between Mataheko and Korle-Bu, the difference recorded was also not statistically significant ($p = 0.478$).

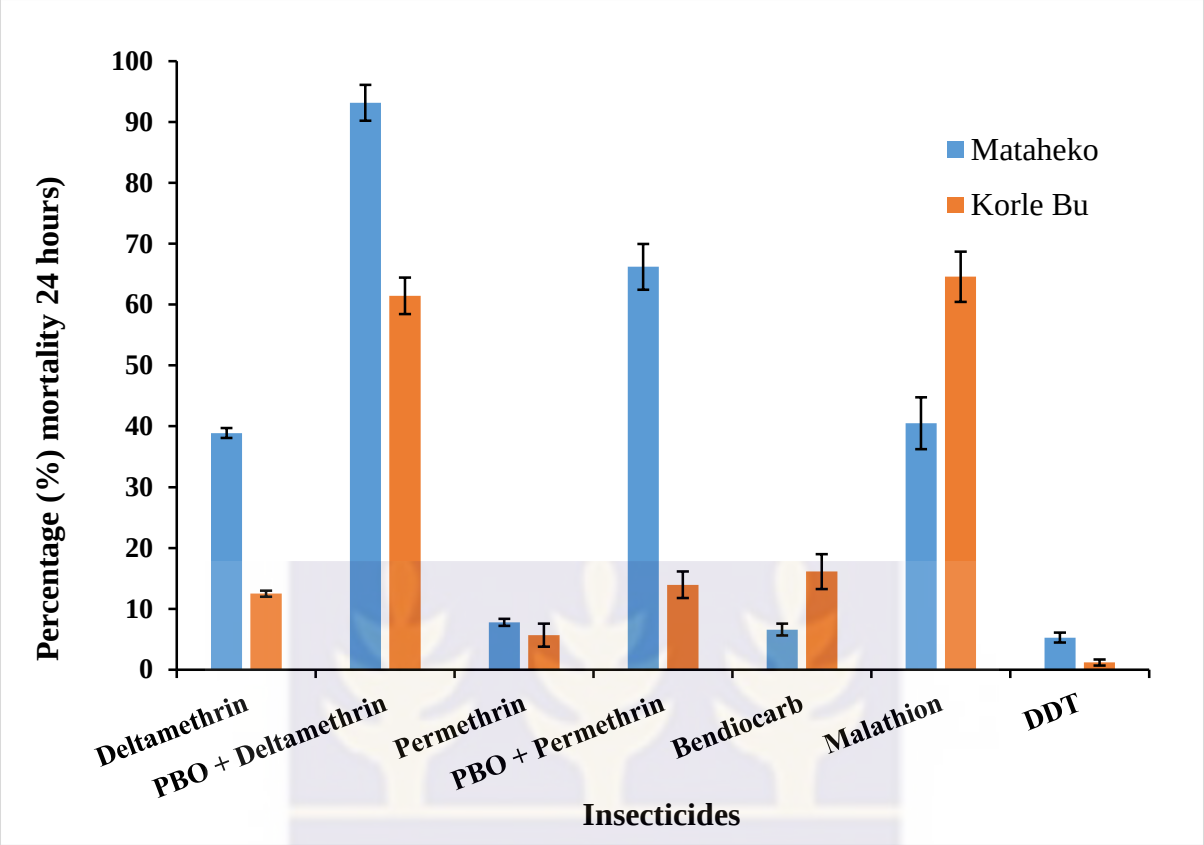


Figure 9: Percentage mortalities of *Anopheles gambiae s.l.* mosquitoes from Mataheko and Korle-Bu.

Generally, no statistically significant difference was observed (p = 0.493) in the comparison of the percentage mortalities of *Anopheles gambiae s.l.* exposed to insecticides between vegetable growing areas (Opeibea and Korle-Bu) and non-vegetable growing areas (Achimota and Mataheko).

4.3. Molecular characterization of *Anopheles gambiae s.l.*

A total of forty (40) mosquitoes each per the four sites were used for this characterization and all the mosquitoes were previously used for the susceptibility tests. The summary of the results is given in Appendix V.

4.3.1. Identity of *An. gambiae s.l.* mosquitoes found in the study sites

All mosquitoes identified from the four sites were *Anopheles gambiae s.s.* An example of the gel electrophoregram showing the diagnostic PCR product band size of *Anopheles gambiae s.s* is shown in Figure 10.

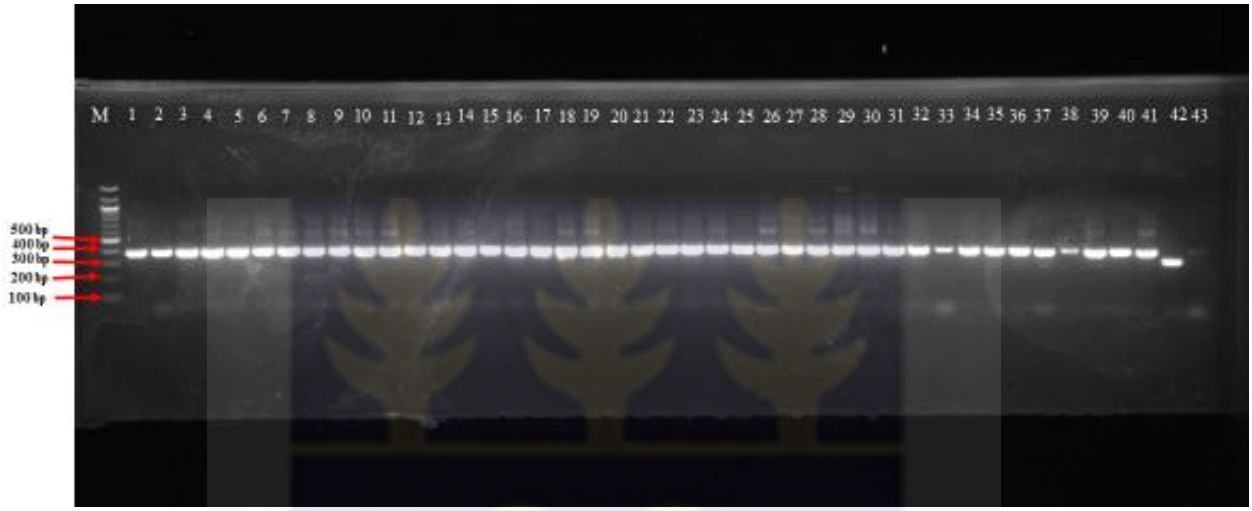


Figure 10: Ethidium bromide-stained agarose gel electrophoregram of PCR products showing *An. gambiae s.s.* identification. M: 100 bp ladder; Lanes 1-40: *Anopheles gambiae s.s.*; Lane 41: *An. gambiae s.s.* positive control; Lane 42: *An. arabiensis* positive control; Lane 43: DNase free water negative control.

Overall, among the *An. gambiae s.s.* mosquitoes identified, *An. coluzzii* constituted 53.75 % of the total, followed by *An. gambiae s.s.* (43.13 %) while M/S had a total of 3.13 % (Figure 11). The M/S forms were only present in Mataheko. An example of the gel electrophoregram is shown in Figure 12.

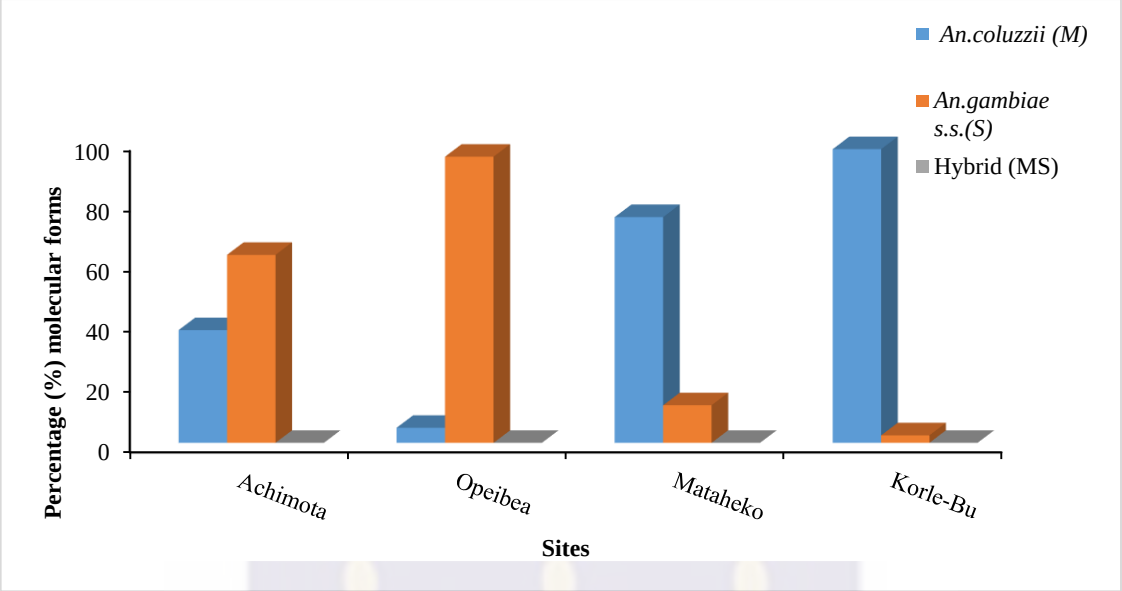
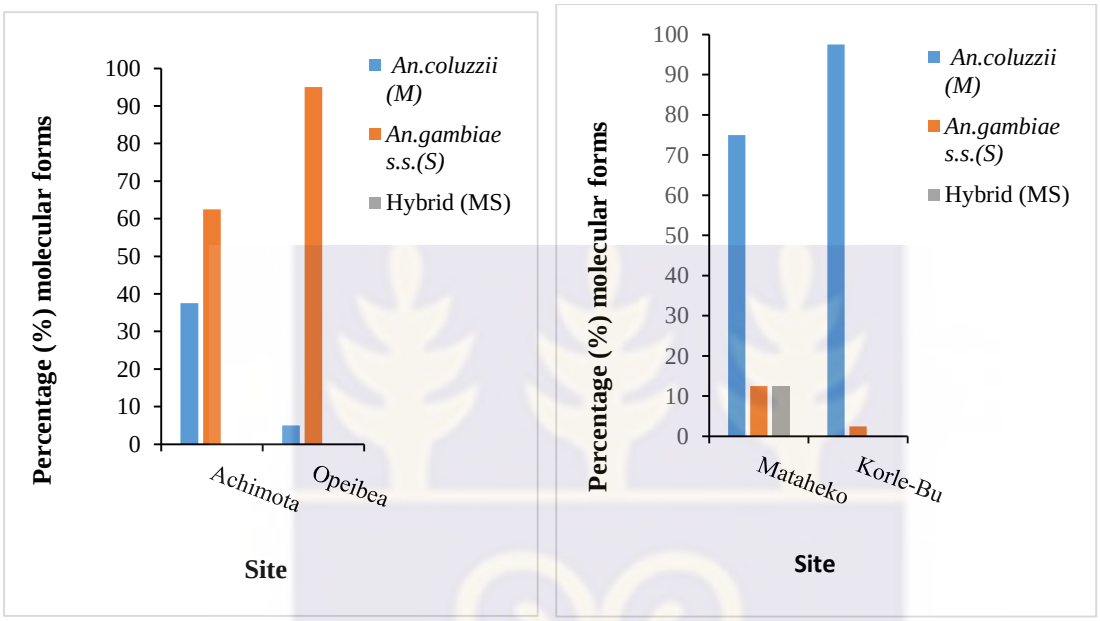


Figure 11: The percentage distribution of *An. coluzzii* (M), *An. gambiae s.s.* (S) and Hybrid (M/S) mosquitoes identified per site.

Figure 12: Ethidium bromide-stained agarose gel electrophoregram of PCR products showing the identities of the molecular forms of *An. gambiae s.s.* found in the study sites: M: 100 bp ladder; Lanes 1-13, 15-34, 36-38: *Anopheles gambiae s.s.*; Lane 14 and 35: *An. coluzzii*; Lane 39: *An. coluzzii* positive control; Lane 40: *An. gambiae s.s.* positive control; Lane 41: Hybrid (M/S) positive control; Lane 42: DNase free water negative control.

The association between the dominant molecular forms of *Anopheles gambiae s.s.* present at the different study sites were statistically significant with p = 0.000. The association between the molecular forms present and the status of the sites (vegetable growing or non-vegetable growing) was also found to be significantly significant (p = 0.04), as shown in Figure 13.



(a) **(b)**

Figure 13: Comparison of the distribution of *Anopheles gambiae s.s.* molecular forms between (a) Opeibea and Achimota (b) Korle-Bu and Mataheko.

4.3.2. Allelic frequency distribution of *kdr* gene in *Anopheles gambiae s.s.*

The total frequencies of *kdr* gene of *Anopheles gambiae s.s.* for the various study sites were given as 0.88 for Achimota; 0.91 for Opeibea; 0.83 for Mataheko and 0.89 for Korle-Bu (Figure 14).

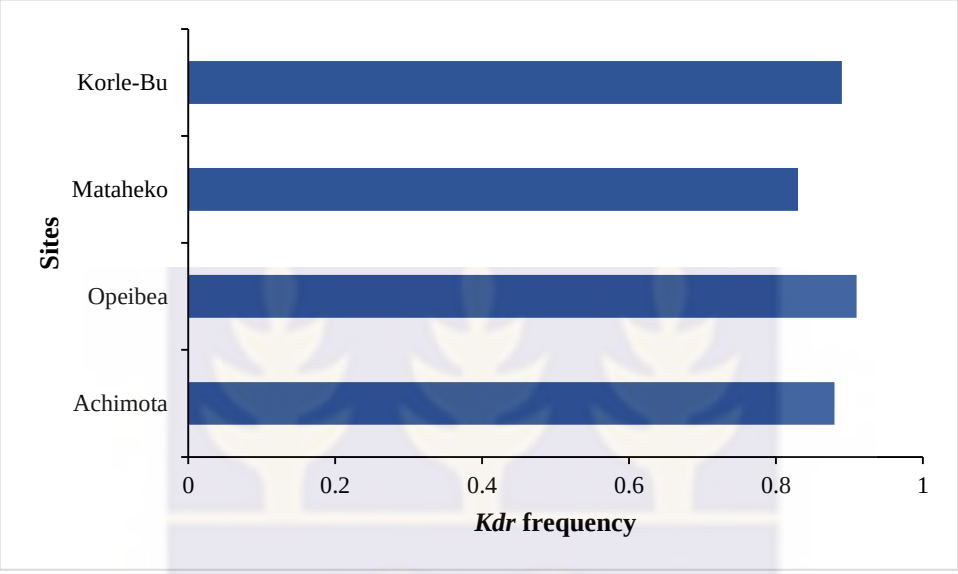


Figure 14: Overall *kdr* gene frequencies of *Anopheles* mosquitoes at the study sites.

There was no significant difference between the frequency of *kdr* distribution of *Anopheles gambiae s.s.* ($p = 0.811$) in the four sites. There was also no significant difference ($p = 0.237$) between the status of the site (vegetable growing or non-vegetable growing) and the frequency of *kdr* distribution in *Anopheles gambiae* mosquitoes breeding there.

There was however, an observed significant difference ($p = 0.003$) between the *kdr* frequencies for the different molecular forms (*An. coluzzii*, *An. gambiae s.s* and M/S) as given in Figure 15.

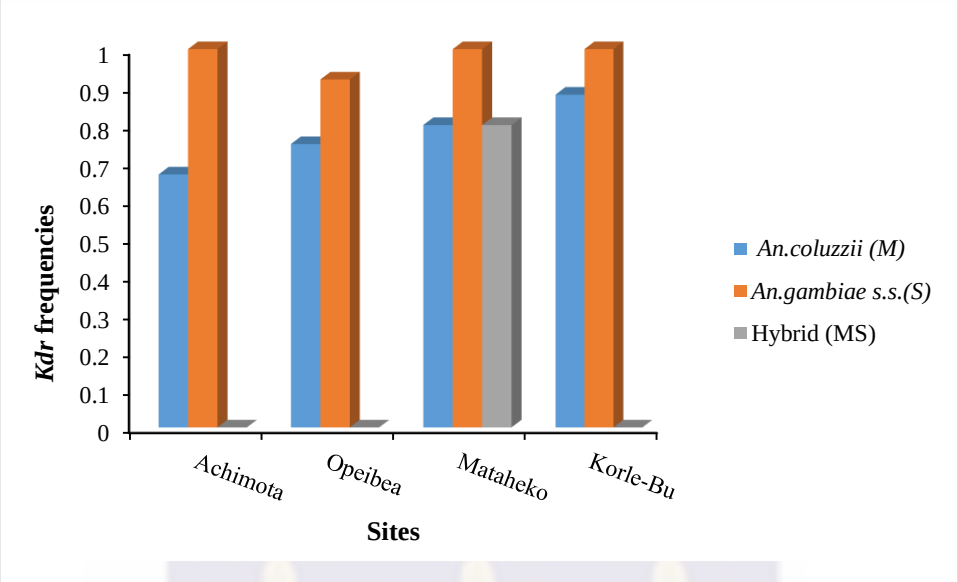


Figure 15: Comparison of the distribution of *kdr* frequencies between *An. coluzzii, An. gambiae s.s.* and Hybrid (M/S) per site.

4.3.3. Allelic frequency distribution of *ace-1* mutation in *Anopheles gambiae s.s.*

Out of the 160 mosquitoes from all the sites profiled for *ace-1* gene mutation, 158 were successfully scored, in other words, all 40 mosquitoes each were scored in Achimota, Opeibea and Korle-Bu while only 38 mosquitoes were scored in Mataheko. An example of the gel electrophoregram is shown in Figure 16.

Figure 16: Ethidium bromide-stained agarose gel electrophoregram of PCR products showing the detected *ace-1* gene mutation in *An. gambiae s.s.* M: 100 bp ladder; Lanes 1 and 30: Resistant *An. gambiae s.s.*; Lanes 2-8, 10-14, 16-19, 21, 23-26, 28-29, 31-39: Heterozygous resistant *An. gambiae s.s.*; Lanes 9, 15, 22 and 27: Susceptible *An. gambiae s.s.*; Lane 40: Resistant *An. gambiae s.s.*, positive control; Lane 41: Heterozygous resistant *An. gambiae s.s.* positive control; Lane 42: Susceptible *An. gambiae s.s.* positive control.

The overall frequency of the *ace-1* gene mutation obtained was; 0.63 for Achimota; 0.79 for Opeibea; 0.42 for Mataheko and 0.45 for Korle-Bu (Figure 17).

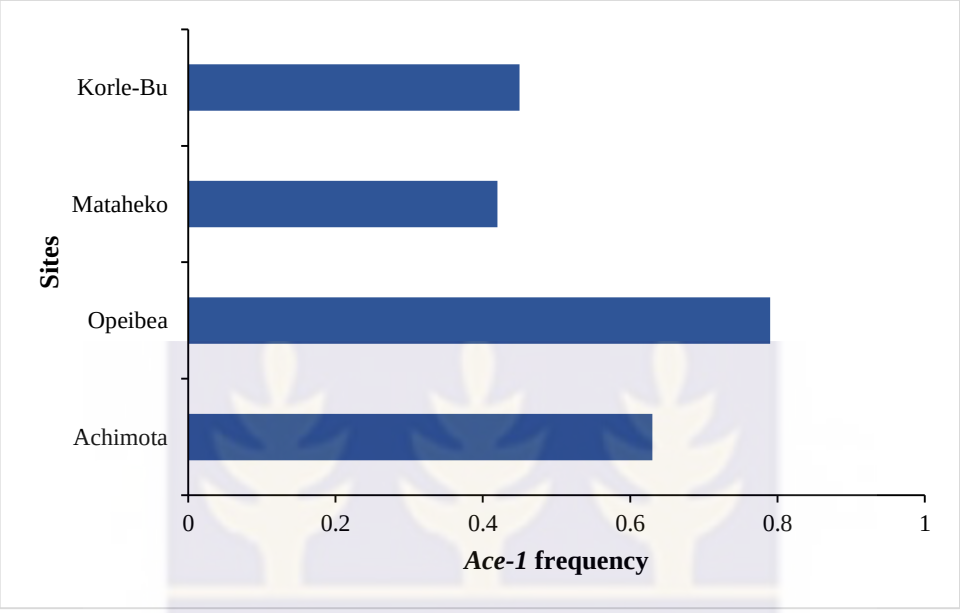


Figure 17: Overall *ace-1* gene mutation frequencies of Anopheles mosquitoes per site.

No significant difference was observed between the frequency of *ace-1* gene mutation at the different sites (p = 0.991). There was also no significant difference (p = 0.681) between the nature of sites (vegetable growing or non-vegetable growing areas) and the frequency of *ace-1* gene mutation in *Anopheles gambiae* mosquitoes breeding there.

Based on molecular forms, significant difference was observed (p = 0.003) in the frequency of *ace-1* gene mutation across the molecular forms of *Anopheles gambiae s.s.* (Figure 18).

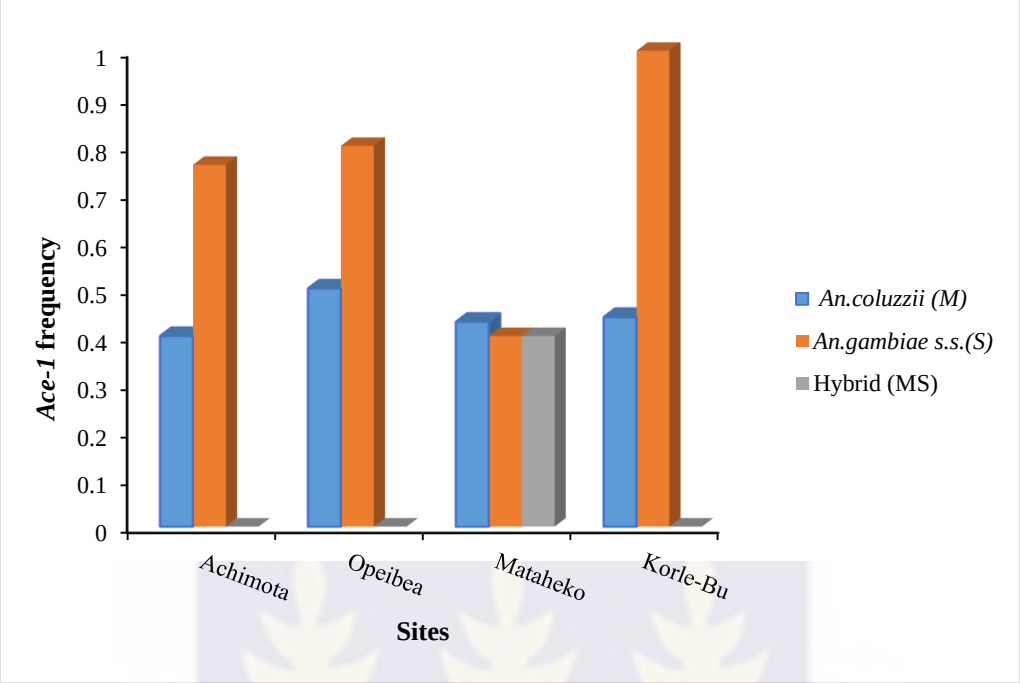


Figure 18: Comparison of the distribution of ace-1 gene mutation frequencies between *An. coluzzii*, *An. gambiae s.s.* and Hybrid (M/S) per site.

4.4. Biochemical assay

A total of 320 fresh female *Anopheles* mosquitoes were used; 200 wild mosquitoes (50 selected from each site) and 120 Kisumu susceptible strain. The enzyme activities of oxidase, non-specific esterases (alpha and beta), glutathione-S-transferase and acetylcholinesterase were determined (Figure 19). The activity for each enzyme is represented in Figures 20 - 24 while tables summarising the details of activities are given in Appendices VI-X. The level of significance was determined by comparing with the Bonferroni corrected level of significance ($\alpha = 0.005$).

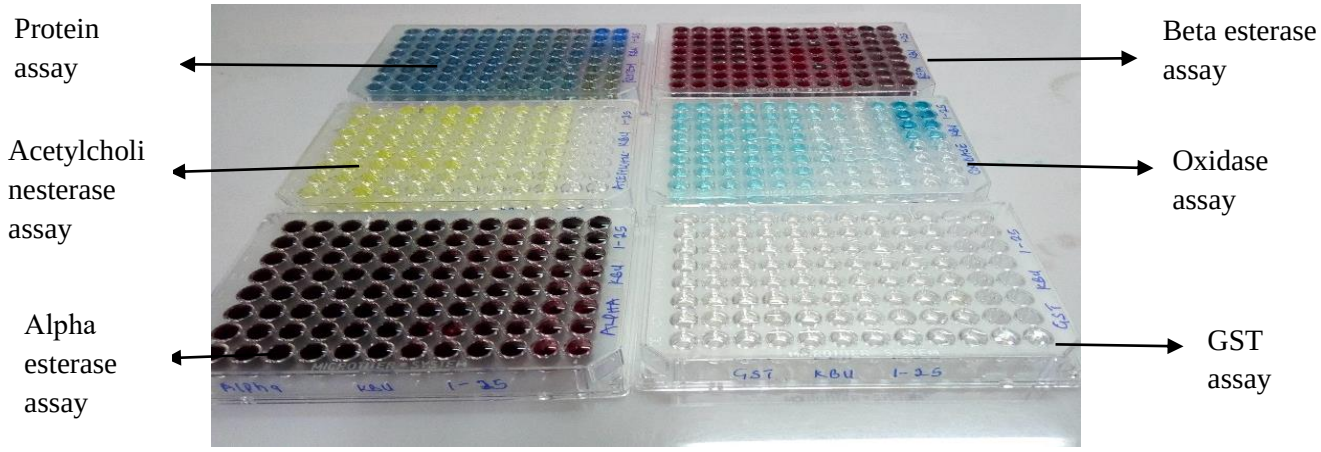


Figure 19: Microtitre plates showing layout of the assays for enzymes.

4.4.1 Oxidase

The distribution of oxidase activities of *Anopheles* mosquitoes in the different sites are shown in Figures 20a and 20b respectively. The wild strain had higher oxidase activities in comparison to the Kisumu susceptible strain ($p < 0.005$), and Opeibea mosquitoes had the highest oxidase activity levels (Figure 20a). Comparison between Achimota and Opeibea (Figure 20a), shows the oxidase activities in Opeibea mosquitoes were significantly higher than that recorded in Achimota mosquitoes ($p = < 0.000001$), while for the comparison between Korle-Bu and Mataheko mosquitoes (Figure 20b), there was no significant difference in the oxidase activities ($p = 0.448$).

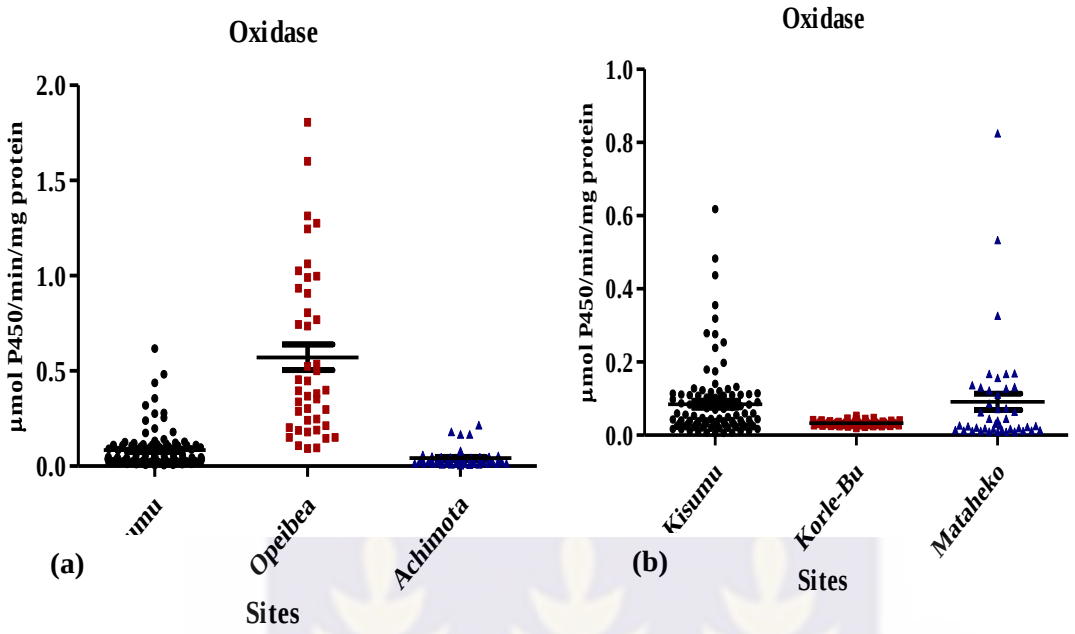


Figure 20: Comparison of oxidase activities in wild *Anopheles* mosquitoes from (a) Opeibea and Achimota and (b) Korle-Bu and Mataheko in relation to Kisumu susceptible strain.

4.4.2. Acetylcholinesterase

The activities of this enzyme in mosquitoes from the different sites are shown in Figures 21a and 21b. There was a significant difference between the acetylcholinesterase activities in the wild strain of *Anopheles* mosquitoes from Opeibea relative to the Kisumu susceptible strain ($p = < 0.0001$), while there was no significant difference in acetylcholinesterase activities in wild strain mosquitoes from Achimota, Korle-Bu and Mataheko relative to Kisumu susceptible strain ($p > 0.005$). Comparison between Achimota and Opeibea (Figure 21a), shows that acetylcholinesterase activities in Opeibea mosquitoes were significantly higher than that in Achimota mosquitoes ($p = 0.001$), while the comparison between Korle-Bu and Mataheko mosquitoes (Figure 21b), shows no significant difference in the acetylcholinesterase activities ($p = 0.010$).

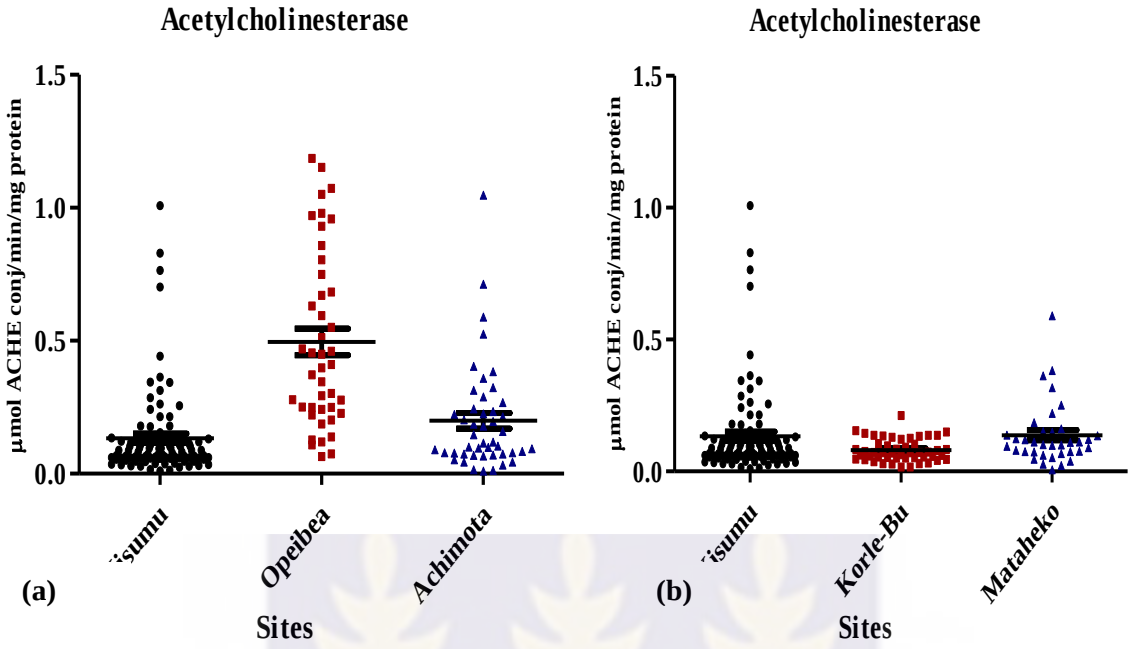


Figure 21: Comparison of acetylcholinesterase activities in wild *Anopheles* mosquitoes from (a) Opeibea and Achimota and (b) Korle-Bu and Mataheko in relation to Kisumu susceptible strain.

4.4.3. Glutathione-S-transferase

Glutathione-S-transferase activities in the mosquitoes in the individual sites are given in Figures 22a and 22b. There was no significant difference in the activities of glutathione-S-transferase in the wild populations relative to the Kisumu susceptible strain ($p > 0.005$). There was also no significant difference in the level of glutathione-S-transferase activities between Achimota and Opeibea mosquitoes ($p = 0.252$) (Figure 22a) and between Korle-Bu and Mataheko mosquitoes ($p = 0.197$) as shown in Figure 22b.

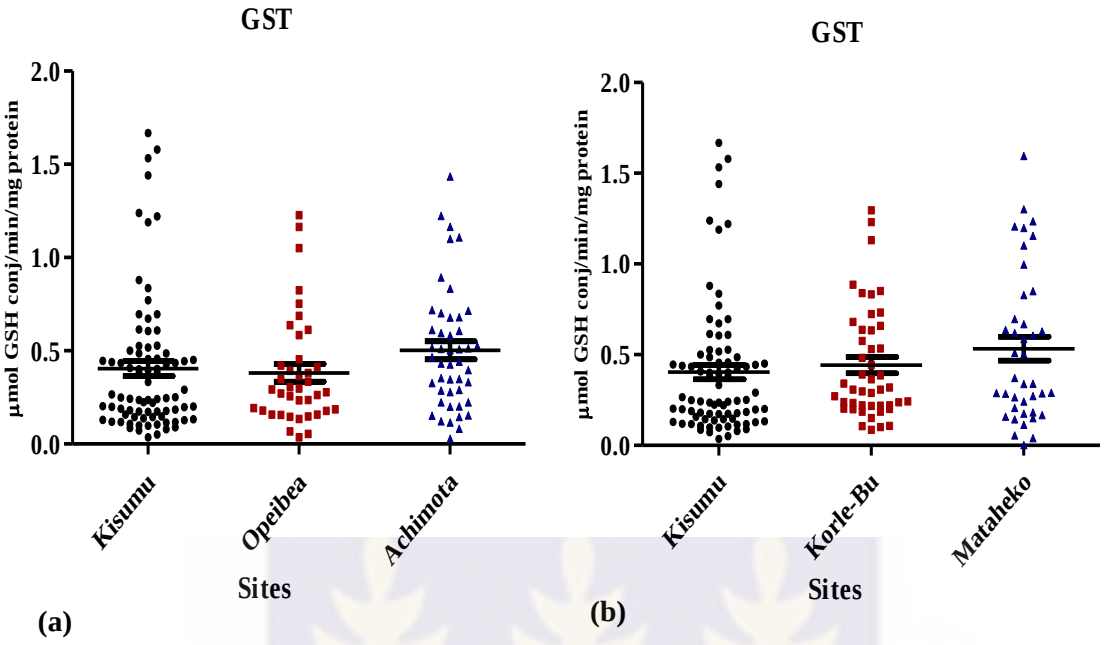


Figure 22: Comparison of activities of GSTs in wild *Anopheles* mosquitoes from (a) Opeibea and Achimota and (b) Korle-Bu and Mataheko in relation to Kisumu susceptible strain.

4.4.4. Non-specific esterases

The activities of these enzymes in the mosquitoes in the individual sites are given in Figures 23a, 23b, 24a and 24b respectively. For alpha esterases, there was a significant difference in the enzyme activities between the wild strains from Opeibea, Korle-Bu and Mataheko relative to the Kisumu susceptible strain ($p < 0.005$), while there was no significant difference between the Kisumu susceptible strain and Achimota mosquitoes ($p = 0.028$). A significant difference was observed ($p = < 0.0001$) between alpha esterase activities in Achimota and Opeibea mosquitoes (Figure 23a), while there was no significant difference ($p = 0.944$) in the alpha esterase activities between Korle-Bu and Mataheko mosquitoes (Figure 23b).

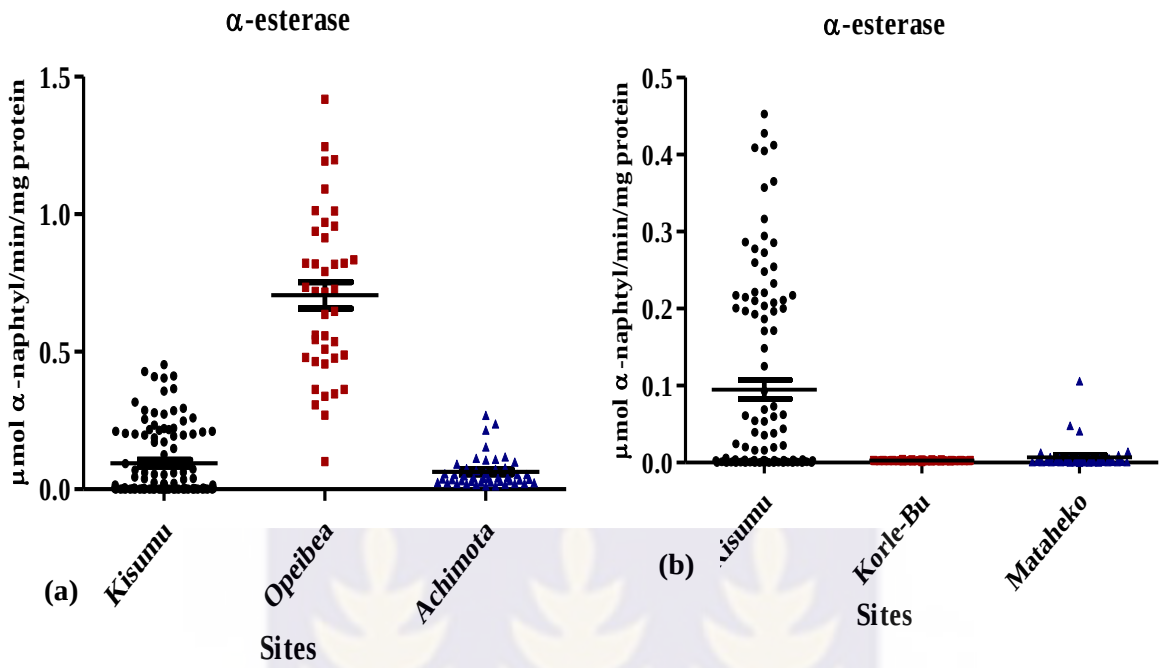


Figure 23:.. Comparison of α-esterase activities in wild *Anopheles* mosquitoes from (a) Opeibea and Achimota and (b) Korle-Bu and Mataheko in relation to Kisumu susceptible strain.

For beta esterase activity, there was a significant difference between the wild strains from Achimota, Korle-Bu and Mataheko relative to the Kisumu susceptible strain ($p < 0.005$), while there was no significant difference between the wild strains from Opeibea and the susceptible strain ($p = 0.073$). There was also a significant difference ($p = 0.002$) in the beta esterase activity between Achimota and Opeibea mosquitoes (Figure 24a) but no significant difference ($p = 0.720$) between Korle-Bu and Mataheko mosquitoes (Figure 24b).

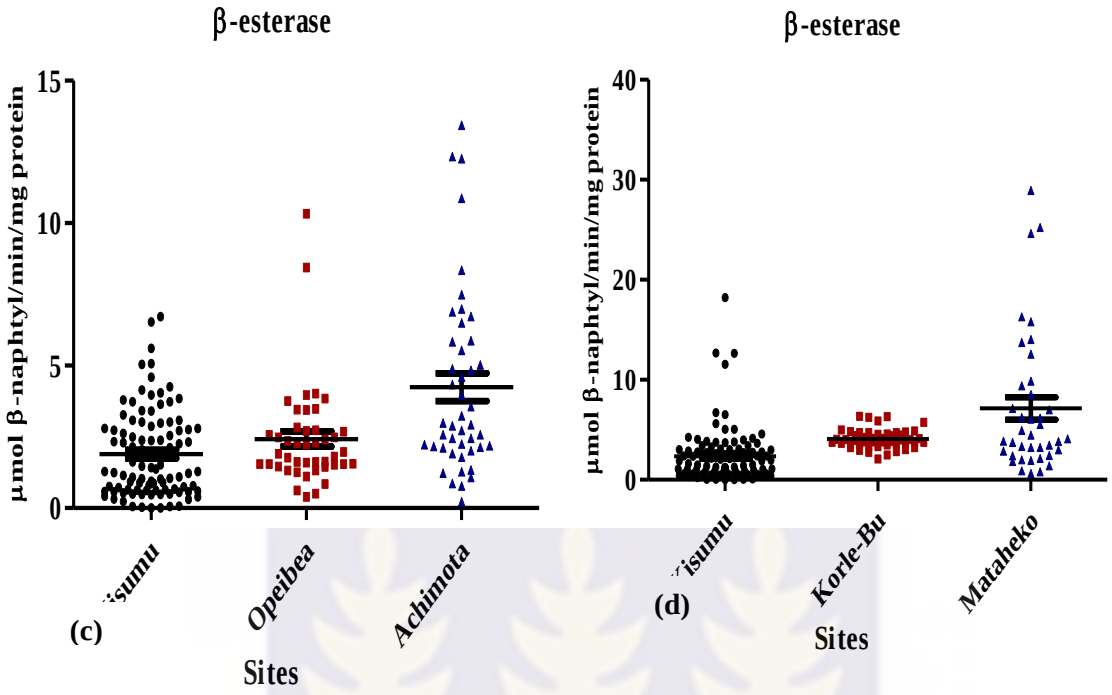


Figure 24: Comparison of β-esterase activities in wild *Anopheles* mosquitoes from (a) Opeibea and Achimota and (b) Korle-Bu and Mataheko in relation to Kisumu susceptible strain.

4.5. Residue levels of chlorpyrifos and dichlorodiphenyldichloroethylene (p, p`-DDE)

Residues of chlorpyrifos as an organophosphate was detected in all the samples (those dissolved both in polar and non-polar solvents) from the different sites (Table 4.2), while residues of dichlorodiphenyldichloroethylene (p, p`-DDE), an organochlorine were detected only in Korle-Bu and Opeibea samples dissolved only in polar solvents (Table 4.3). Residue levels detected ranged from as low as 0.05 mg/l (ACHnH) to as high as 6.00 mg/l (MTKME). Generally, chlorpyrifos residue was the highest residue detected in the samples. No significant difference was observed between the levels of chlorpyrifos and p, p`-DDE at the sites ($p > 0.05$ Kruskal-wallis H test). There was also no significant difference in the residue levels of chlorpyrifos ($p = 1.00$) and p, p`-DDE detected ($p = 0.34$) with respect to vegetable growing and non-vegetable growing areas.

Table 4.2: Chlorpyrifos residue levels in water from the four sites

Sample	Integrated peak area (A)	Analyte residue concentration (mg/l)	Residue level in water (mg/l)*	Total residue content per sample**
ACHME	148499	0.57	1.43	1.48
ACHnH	4791	0.02	0.05	
OPBME	525193	2.03	5.07	6.19
OPBnH	121953	0.47	1.12	
MTKME	621717	2.40	6.00	6.10
MTKnH	10967	0.04	0.10	
KBUME	174369	0.67	1.67	1.92
KBUnH	25633	0.09	0.25	

*Residue levels in water was the analyte residue concentration in 2.5 litres of the sample

**sum of Chlorpyrifos residue levels in water per site

Table 4. 3: Dichlorodiphenyldichloroethylene (p, p`-DDE) residue levels from Korle-Bu and Opeibea

Sample	Integrated peak area (A)	Analyte residue concentration (mg/ml)	Residue level in water (mg/l)*
KBUME	108586	0.05	0.12
OPBME	133512	0.06	0.15

* Residue levels in water was the analyte residue concentration present in 2.5 litres of the sample

KBUME: SPE fraction of water from Korle-Bu site eluted in methanol and ethyl acetate.

OPBME: SPE fraction of water from Opeibea site eluted in methanol and ethyl acetate.

ACHME: SPE fraction of water from Achimota site eluted in methanol and ethyl acetate.

MTKME: SPE fraction of water from Mataheko site eluted in methanol and ethyl acetate.

KBUnH: SPE fraction of water from Korle-Bu site eluted in n-hexane.

OPBnH: SPE fraction of water from Opeibea site eluted in n-hexane.

ACHnH: SPE fraction of water from Achimota site eluted in n-hexane.

MTKnH: SPE fraction of water from Mataheko site eluted in n-hexane.

CHAPTER FIVE

5.0. DISCUSSION

This cross-sectional study focused on determining the status of insecticide resistance of *Anopheles* mosquitoes in vegetable and non-vegetable growing areas in Accra and investigated the factors responsible for the selection of the resistant forms in the vegetable growing areas.

Results from the questionnaire survey revealed that farmers relied solely on the use of insecticides for the control of their crop pests, with the notion that they produce faster results than all other control measures. From the survey also, a greater proportion of the farmers (55.6 %) apply pesticides to their crops weekly until harvest. This high overdependence on insecticides and the fact that most of the farmers do not have a precise dosage for the pesticide application (they just use their discretion) has also contributed to the development of insecticide resistance in the mosquitoes breeding in their farmlands through the contamination of their breeding sites.

With respect to the resistant status of *Anopheles gambiae* in the vegetable growing and non-vegetable growing areas, the results of the susceptibility test on *Anopheles gambiae s.l.* showed that the mosquitoes from all the sites were resistant to Deltamethrin, Permethrin, Bendiocarb, DDT and Malathion according to the WHO definition of resistance (WHO, 2013). The use of PBO (synergist) showed an enhancement in the susceptibility of *Anopheles gambiae s.l.* from all the sites and mostly in the population from Achimota (93.15 %). The total mortalities recorded were between the range of 2.50 - 64.58 %. These results are similar to those reported by Diabate *et al*. (2002), Akogbeto *et al*. (2006), Yadouleton *et al*. (2009) and Edi *et al*. (2012). There was a significant difference ($p < 0.05$) in the mortalities with respect to the different insecticides used within the four study sites but no significant difference was observed in the percentage mortalities with respect to the farming and non-farming areas ($p > 0.05$). An

obvious reason for the general resistance phenotype recorded could be due to the intensive use of the same insecticides in the control of agricultural pests and also for public health purposes (treated bed nets and indoor residual spraying).

The frequency of *kdr* allele, which is implicated in the development of resistance to pyrethroids and organochlorines, was very high, in the range of 0.83 to 0.91, with Opeibea mosquitoes having the highest frequency (0.91), although not statistically significant ($p > 0.05$). PCR-RFLP method of identification of the *ace-1* gene mutation that confers resistance to organophosphates and carbamates, revealed a moderately high frequency in the range of 0.42 to 0.79 with Opeibea mosquitoes also having the highest frequency (0.79), but not statistically significant ($p > 0.05$).

In the course of the study, *kdr* allele was detected in conjunction with *ace-1* gene mutation (target site resistance) in some *An. gambiae* mosquitoes from the four sites, and also with elevated activity of detoxifying enzymes (metabolic resisitance), this confirms the presence of multiple resistance in *An. gambiae* from these study sites. This finding is in line with the reports from Edi *et al*. (2012) in Southern La Côte d'Ivoire, and Namountougou *et al*. (2012) in Burkina Faso.

Generally, *kdr* and *ace-1* gene mutation frequencies did not differ significantly ($p > 0.05$) with respect to the nature of the sites, whether vegetable growing or non-vegetable growing, even though the highest values were found in Opeibea. This may be attributed to the use of the same insecticides (pyrethroid, organophosphate and carbamate) for pest control in agriculture as well as in public health for vector control.

Biochemical assays conducted to determine the activities of the enzymes (oxidases, acetylcholinesterase, glutathione-S-transferase (GST) and non-specific esterases) responsible for conferring metabolic resistance to the insecticides generally revealed that the enzyme

activity levels in the mosquitoes from all the sites were highly elevated but much higher in mosquitoes from Opeibea ($p < 0.005$), except for the GSTs and beta esterase. This presumably suggests a higher insecticide pressure at Opeibea than in other study sites. This enhanced enzyme activity levels lend credence to the high resistance depicted by the mosquitoes to the insecticides at all the sites.

Pesticide residues in the breeding waters of the mosquitoes also serves as a selection pressure that selects for resistant phenotypes in the mosquitoes. The gas chromatography analysis performed on the water samples from the four sites revealed the presence of two pesticides; chlorpyrifos and dichlorodiphenyldichloroethylene (p, p`-DDE). Of these two pesticides, chlorpyrifos residue levels was the highest and was detected in all the sites. Generally, there was no significant difference in the pesticide residue levels present in both vegetable growing and non-vegetable growing areas ($p > 0.05$). This may be attributed to the general high usage of these insecticides by the farmers in Opeibea and Korle-Bu and also its use in insecticide-treated nets (ITN's) and indoor residual spray (IRS) in malaria vector control programmes.

With respect to resistance to pyrethroids in the mosquitoes, high resistance to Deltamethrin and Permethrin was observed, with mortalities ranging between 2.50 % for Permethrin to 36.17 % for Deltamethrin at the four sites which also affirmed the high KDT_{50} and KDT_{95} obtained. Similar findings were reported by N'Guessan *et al*. (2007), Yadouleton *et al*. (2009), Edi *et al*. (2012), Kwiatkowska *et al*. (2013) and also that of Ranson *et al*. (2009) who recorded Permethrin and Deltamethrin resistance in all the four sites in the susceptibility test conducted in Chad. In Ghana, these results agree with the research findings of Adasi *et al*. (2000), Achonduh *et al*. (2008) and Boakye *et al*. (2009). These high levels of pyrethroid resistance recorded can be attributed to the intensive use of pyrethroid insecticides for both the control of agricultural pest (Brempong-Yeboah, 1992; N'Guessan *et al*., 2007; Yadouleton *et al*., 2009)

and for vector control in public health (Boakye *et al*., 2009; PMI, 2012). This is due to its relatively low mammalian toxicity and rapid knockdown effect, and this intensive use may have led to its reduced efficacy. This is also consistent with the research findings that pyrethroid resistance was induced from resistance to DDT, because of their similar target sites which are the sodium channels, therefore, the resistance to DDT is invariably conferred to pyrethroids in the mosquito populations (Chandre *et al*., 1999).

The use of PBO enhanced the susceptibility of the mosquitoes to Deltamethrin and Permethrin by blocking the enzyme system (mixed function oxidases), thereby allowing more insecticides to reach the sodium channels, but did not terminate resistance to the insecticides, and this is suggestive of the fact that the activities of mixed function oxidases (MFO's) are highly implicated in pyrethroid resistance in mosquitoes.

The high frequency of the *kdr* allele observed in all the mosquitoes is also suggestive of resistance to Deltamethrin, and Permethrin insecticides.

Oxidase which is involved in the metabolism of pyrethroids and organophosphates insecticides, had higher activity levels in the wild *Anopheles* mosquitoes when compared with Kisumu susceptible strain ($p < 0.005$). In Ghana, a similar finding was reported by Ben-Mahmoud (2008). This higher activity levels in the oxidase enzyme in the wild strain of *Anopheles* mosquitoes indicates the increased pressure on the mosquitoes to metabolise pyrethroids and organophosphates insecticides because of its intensive exposure from the breeding sites. The mosquitoes from Opeibea had the highest oxidase activity level. The mean oxidase activity level observed was higher in Opeibea than Achimota, its control site ($p < 0.005$), this suggests the presence of a higher insecticide pressure in Opeibea than in Achimota. This result therefore, justifies the high level of resistance to Deltamethrin and Permethrin observed in the mosquitoes. The presence of increased activities of oxidases (metabolic resistance) in the wild

strain and the high frequency of *kdr* observed indicates the presence of multiple resistance in *An. gambiae* mosquitoes from the four sites.

Pyrethroid residues were not detected in samples of the larval breeding water from the various study sites and this observation is generally inconsistent with the fact of it being used more extensively by all the farmers (100 %) in Korle-Bu and Opeibea than other classes of insecticides. Prevailing conditions in the breeding water (pH, available oxygen, sunlight) and the organic matter content may account for the absence of pyrethroid residues in the samples. Another reason for its absence in the larval breeding water could be its high tendency to bind to soil particles making residue levels too low to be detected in the water sample. According to Thomson (1985) and Wagenet (1985), Permethrin decays quickly in water, notwithstanding, it can adhere firmly to soils particularly by organic matter and leaching seldom occur. Cypermethrin degrades rapidly in soils low in organic matter and has low persistence under sunlight this is according to the United States Environmental Protection Agency [USEPA, (1989)].

With respect to DDT (organochlorine) resistance, *Anopheles gambiae* from all the sites showed a very high level of resistance with mortalities ranging from 1.19 % to 6.98 % which also confirms the high KDT_{50} and KDT_{95} values obtained although not obtained in Korle-Bu and Mataheko due to the very low population of mosquitoes knocked down. DDT was not reported to be used by the farmers in the farming sites, however, the high level of resistance recorded could probably be as a result of cross resistance with pyrethroids in the mosquitoes. Other reasons could be attributed to, the long persistence of DDT in the environment, or the illegal use by the farmers in pest control. This result agrees with studies by Achonduh *et al*. (2008), Edi *et al*. (2012) and Kwiatkowska *et al*. (2013). The high frequency of the *kdr* allele in all the

Anopheles mosquitoes at the four study sites, also confirms the high resistance to DDT insecticides.

The mean activity levels of glutathione-S-transferase (GST) which is reported to play a role in DDT resistance, were relatively the same in wild strains and the Kisumu susceptible strain, with no significant difference being observed ($p > 0.005$). Ben-Mahmoud (2008), reported similar findings. With respect to vegetable and non-vegetable growing areas, there was also no significant difference in the activity levels of GST ($p > 0.005$). This result indicates that there is no difference in the resistance to DDT in both susceptible and wild strain of *An. gambaie* in vegetable growing areas and non-vegetable growing area. This is attributed to the high persistence of DDT insecticides in the environment and also the establishment of cross resistance to pyrethroids. This result is also confirmed by the high resistance to DDT observed in *An. gambaie* mosquitoes from all the sites from the susceptibility test conducted. The presence of increased activities of GSTs (metabolic resistance) in the wild strain and also the high frequency of *kdr* recorded is suggestive of multiple resistance in *An. gambiae* mosquitoes from the four sites.

p, p`-DDE is a breakdown product of DDT and a highly persistent insecticide that can remain in the environment for a longer time, but its residue was detected in low levels, and only at the vegetable growing areas, Korle-Bu (0.12 mg/l) and Opeibea (0.15 mg/l). It was absent in Achimota and Mataheko. Although this insecticide was not used by the farmers in Korle-Bu and Opeibea, yet the residues were detected probably because it had been used previously before its ban and thereby still persists in the environment. The low levels detected in Korle-Bu and Opeibea, could also be as a result of its non-use by the farmers in pest control or due to the excessive flooding of the breeding sites. This low levels of p, p`-DDE residues detected,

somewhat contrasts the high resistance to DDT observed in the bioassay and the high frequency of *kdr* gene allele reported in the mosquitoes from all the sites.

For Malathion, an organophosphate, low insecticide susceptibility was also recorded in the mosquitoes with percentage mortalities ranging from 33.30 % to 64.60 % for all the sites. High KDT_{50} and KDT_{95} values were obtained which also confirmed the reduced susceptibility observed. This result is similar to the findings of Edi *et al*. (2012). This may probably be as a result of the high usage of organophosphate insecticides especially by 78 % of the farmers in Korle-Bu and Opeibea, because the farmers regarded the insecticide to be very potent which also accounts for the high residue of chlorpyrifos detected in the water sample. The establishment of cross resistance to Bendiocarb insecticides is another contributing factor because of the similar target sites they share in the mosquitoes.

For Bendiocarb, a very high resistance was recorded in all the sites with mortalities ranging from 2.60 % to 16.13 % and also confirmed by high KDT_{50} and KDT_{95} values. This result agrees with the findings of Edi *et al*. (2012). Although, this insecticide was not reportedly used by the farmers in Korle-Bu and Opeibea, the reduced susceptibility observed could be as a result of its use in controlling public health pests especially in the non-crop growing areas and also as a result of cross resistance to organophosphates (Malathion) as earlier mentioned.

The high frequency of *ace-1* gene mutation reported from the sites is strongly linked to the reduced susceptibility of the mosquitoes to Malathion and Bendiocarb observed in the study. These insecticides are used both in the farms for pest control and in public health for personal protection.

Non-specific esterases which are known to be involved in the metabolism of organophosphate insecticides, revealed for alpha esterases, a higher enzyme activity level in wild strains from Opeibea, Korle-Bu and Mataheko than in the Kisumu susceptible strain ($p < 0.005$). It was only

in Achimota wild strain that there was no significant difference in the mean enzyme activity levels of this enzyme in comparison to the Kisumu susceptible strain ($p > 0.005$). *Anopheles* mosquitoes from Opeibea had higher alpha esterase activity levels than those from Achimota ($p = < 0.0001$). On the other hand, wild strains from Korle-Bu, Mataheko and Achimota demonstrated higher beta esterases activity levels than the susceptible Kisumu strain. In comparison between mosquitoes from Opeibea and Achimota, Opeibea mosquito populations showed a higher beta esterase activity levels ($p < 0.005$). These results indicate that the wild strain of *Anopheles gambiae s.s.* had a higher pressure from the organophosphate insecticides than the Kisumu susceptible strain because of the high level of exposure of the mosquito population to this insecticide in the environment through the contamination of their breeding sites. Mosquitoes from Opeibea were invariably more exposed than those from Achimota because of the intensive use of this insecticide by farmers in the area and this accounts for the higher activity levels of alpha and beta esterases in Opeibea mosquitoes (vegetable growing area) than in Achimota mosquitoes (non-vegetable growing area).

For the acetylcholinesterase assay, which is also involved in the metabolism of organophosphate and carbamate insecticides, a higher enzyme activity was only observed in the Opeibea wild strain of *An. gambiae* than the Kisumu susceptible strain ($p = < 0.0001$), but there was no significant difference in the enzyme activity levels between the wild strain from other sites and the Kisumu susceptible strain ($p > 0.005$). Additionally, it was also only between Opeibea and Achimota mosquito populations that significant differences ($p < 0.005$) were observed in their mean acetylcholinesterase activity levels. This higher enzyme activity levels in Opeibea mosquitoes, than in the Kisumu susceptible strain, indicates a higher level of exposure of the mosquitoes to the insecticides. This may also explain why the activity levels of the enzyme is higher in Opeibea mosquitoes (vegetable growing area) than in the mosquitoes from Achimota (non-vegetable growing area).

In the study, *Anopheles* mosquitoes from all the sites were identified as *Anopheles gambiae s.s.* using Polymerase Chain Reaction (PCR). *An. coluzzii, An. gambiae s.s*. and hybrids of the two species (M/S) were identified and recorded as 53.75 %, 43.13 % and 3.13 % respectively. There was a significant difference between the percentages of *An. coluzzii, An. gambiae s.s*. and hybrid (M/S) present and the nature of the sites ($p < 0.05$). The overall higher percentage abundance of *An. coluzzii* observed suggest that the dry climatic conditions which characterizes the coastal savannah ecological zone in Accra creates a more favourable habitat for their breeding. The study revealed that *An. coluzzii* and *An. gambiae s.s*. were sympatric in all the sites as was also recorded by della Torre *et al*. (2005) in 24 African countries, de Souza *et al*. (2010) in Ghana and Kamdem *et al*. (2012) in Cameroon.

Korle-Bu had a dominance of 97.5 % *An. coluzzii* and 2.5 % *An. gambiae s.s,* while Opeibea had a dominance of 95 % *An. gambiae s.s.* and 5 % *An. coluzzii* which were only slightly different from each of their control sites, Mataheko and Achimota respectively. This is in agreement with studies of Achonduh *et al*. (2008) and Bilali *et al*. (2011) in Ghana. The high selection by these different species could attest to the nature of the vegetable areas; at Korle-Bu the larvae were collected in between beds where water from irrigation of the crops formed a pool in which the mosquitoes bred, while at Opeibea, the larvae were collected from temporary rain dependent pools. This is similar to the findings of Kamdem *et al*. (2012) and Tene Fossog *et al*. (2015) in Cameroon.

Another reason could be the ecological distributions of the molecular forms: *An. coluzzii* generally breeds in arid zones or drier areas and in habitats created by irrigation/flooding or human activities that are more stable on the other hand, *An. gambiae s.s.* occurs in more humid areas and temporary rain dependent pools, while the hybrids being found in between (that is in habitats not too dry and not humid like Mataheko) (Toure *et al*., 1994 and 1998; Sogoba *et al*.,

2008; de Souza *et al*., 2010; Kamdem *et al*., 2012; Tene Fossog *et al*., 2015). The larval breeding water at Korle-Bu was more turbid than that at Opeibea, and this is in line with the findings of Kamdem *et al*. (2012) and Tene Fossog *et al*. (2015) in Cameroon, who reported that *An. coluzzii* larvae exhibits greater tolerance to environment stressors in their habitat than *An. gambiae s.s*. which generally breeds in open, sunlit puddles created by accumulation of rain.

From the study, Korle-Bu had an elevation of 22.1 m while Opeibea had an elevation of 40.0 m above ellipsoid, this may also suggest the selection of these dominant forms in these areas. This result agrees with those of Kamdem *et al*. (2012) in Cameroon and de Souza *et al*. (2010) in Ghana, who reported that *An. coluzzii* showed a negative correlation with elevation which is directly opposite that of *An. gambiae s.s.*

According to the molecular forms, *An. gambiae s.s*. had the highest *kdr* allele frequency ($p < 0.05$) than other molecular forms from all the sites and this agrees with the results of Chandre *et al*. (1999) in La Cote d'Ivoire, Brooke *et al*. (1999) in Nigeria and La Cote d'Ivoire, della Torre *et al*. (2001) in several parts of West Africa and Yawson *et al*. (2004) and Bilali *et al*. (2011) in Ghana. This high frequency of the *kdr* allele in *An. gambiae s.s*. reveals that the resistant gene was initially present in high frequencies in *An. gambiae s.s*. and strongly associated with it before genetic introgression of the gene into *An. coluzzii* (Chandre *et al*., 1999; Diabate *et al*., 2004; Main *et al*., 2015 and Vincente *et al*., 2017). The high dominance of *An. gambiae s.s*. at Opeibea probably also suggests why the *kdr* gene frequency was higher there than at Korle-Bu.

There was also a significant difference in *ace-1* gene frequencies ($p < 0.05$) with respect to molecular forms, with *An. gambiae s.s*. also having the highest frequency in all the sites apart from Mataheko. This agrees with the findings of Diabate *et al*. (2004) and Djogbénou *et al*.

(2008) where it was reported that the distribution and frequency of this mutation in Burkina Faso was more dominant in *An. gambiae s.s*. but in lower frequency in *An. coluzzii*. This lends credence to the fact that the gene was previously dominant in *An. gambiae s.s*. before its introgression to *An. coluzzii*.

5.1. Conclusion

The high resistance observed in the WHO susceptibility tests, high frequencies of the *kdr* and *ace-1* gene mutations in the mosquitoes from the four study sites, the presence of elevated levels of enzyme activities in the mosquitoes and the detection of pesticide residues in the larval breeding water in both vegetable and non-vegetable growing areas, is suggestive of the fact that, *Anopheles gambiae* mosquitoes from all the study areas were resistant to insecticides.

However, the resistance status of *Anopheles gambiae* was only relatively higher in vegetable growing areas than non-vegetable growing areas, although not statistically significant ($p > 0.05$). This was probably driven by the intensive application of insecticides by the farmers to control agricultural pest.

This proposition is instigated by the fact that not only agricultural activities but also public health practices like the extensive use of insecticide-treated nets and indoor aerosol sprays contribute to the development of resistance in *Anopheles gambiae* mosquitoes in both vegetable growing areas and non-vegetable growing areas in Accra, Southern Ghana.

5.2. Recommendations

Based on the research findings gathered from this study, the following recommendations are made:

1. Enhancement of susceptibility to Deltamethrin and Permethrin in all the sites indicated the involvement of cytochrome P450's in the development of resistance in the mosquitoes. Therefore, preparation of vector control tools such as long lasting insecticide-treated nets (LLIN's) impregnated with synergist (PBO) can be effective even in the presence of pyrethroid resistance.
2. Resistance was only relatively higher in vegetable growing areas (Korle-Bu and Opeibea) than non-vegetable growing areas (Mataheko and Achimota) due to the excessive use of insecticides in agricultural activities. Therefore, more studies are recommended in other urban vegetable growing areas to understand the full scale of the impact of agricultural pesticides use in the development of resistance in *An. gambiae s.l.*
3. Inasmuch as the insecticide residues detected from the study sites is suggestive of resistance to insecticides, however, consistent monitoring and sampling of the larval breeding water, possibly in both rainy and dry seasons should be conducted, to provide a broader picture of the residues present, their levels and in what seasons, to help draw better conclusions.
4. Since, it has been confirmed from earlier research that insecticides used for both pest control in agriculture and for public health control of mosquitoes are similar, it is recommended that new active ingredients for public health applications be developed, to facilitate slow development of resistance in *An. gambiae* mosquitoes, then good progress can be made in the control of malaria in Ghana and Africa at large.

5.3. Limitation encountered during the study

Getting the farmers in each vegetable field to participate in the survey was very difficult. They were initially unwilling to partake in the survey. Some of them were of the opinion that they should be given some incentives (money) to encourage their participation. This is because other researchers they encountered previously who promised them some incentives, failed to fulfil their promises. This accounted for the low participation amongst the farmers at Korle-Bu and Opeibea.

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APPENDICES

APPENDIX 1

Mortality at 24 hrs, Mean, SD, SE and Resistance status of *Anopheles* mosquitoes from Achimota

Insecticide and Concentration (%)	Number of exposed mosquitoes		Mean Total Mortality (%)		Mean corrected (%) mortality	SD	KDT_{50} (mins)	KDT_{95} (mins)	Resistance Status
	Test	Control	Test	Control					
Deltamethrin (0.05)	78	17	36.17	3	17.65	3.59	70.06	203.26	R
PBO and Deltamethrin (5.05)	85	47	76.47	0	-	4.86	39.19	70.02	R
Permethrin (0.75)	81	17	13.05	3	17.65	2.63	11350.35	103902.09	R
PBO and Permethrin (5.75)	84	47	44.05	0	-	3.40	659.22	10062.08	R
Bendiocarb (0.10)	79	43	15.63	3	6.98	1.29	363.40	4493.84	R
Malathion (5.00)	85	43	41.82	3	6.98	1.50	189.72	1649.77	R
DDT (4.00)	86	47	6.98	0	-	3.69	356.47	2014.39	R

df = 6, t = 3.68, p = 0.010, SD- standard deviation, KDT (mins)- Knockdown time, R-resistant.

APPENDIX II

Mortality at 24 hrs, Mean, SD, SE and Resistance status of *Anopheles* mosquitoes from Opeibea

Insecticide and Concentration (%)	Number of exposed mosquitoes		Mean Total Mortality (%)		Mean corrected (%) mortality	SD	KDT_{50} (mins)	KDT_{95} (mins)	Resistance Status
	Test	Control	Test	Control					
Deltamethrin (0.05)	93	47	2.6	0	-	5.48	4481.28	302668.93	R
PBO and Deltamethrin (5.05)	77	39	56.5	0	-	2.22	51.16	95.43	R
Permethrin (0.75)	81	44	2.5	0	-	1.41	-	-	R
PBO and Permethrin (5.75)	74	39	46.8	0	-	2.87	338.04	2844.71	R
Bendiocarb (0.10)	73	35	2.6	0	-	2.08	809.80	26163.77	R
Malathion (5.00)	76	39	33.3	0	-	1.83	128.31	284.59	R
DDT (4.00)	72	35	4.9	0	-	1.00	128.32	287.92	R

df = 6, t = 2.39, p = 0.054, SD- standard deviation, SE- standard error, KDT (mins)- Knockdown time, R-resistant.

APPENDIX III

Mortality at 24 hrs, Mean, SD and Resistance status of *Anopheles* mosquitoes from Mataheko

Insecticide and Concentration (%)	Number of exposed mosquitoes		Mean Total Mortality (%)		Mean corrected (%) mortality	SD	KDT_{50} (mins)	KDT_{95} (mins)	Resistance Status
	Test	Control	Test	Control					
Deltamethrin (0.05)	72	39	38.89	0	-	0.82	61.17	106.67	R
PBO and Deltamethrin (5.05)	73	39	93.15	0	-	2.94	23.91	55.42	SR
Permethrin (0.75)	77	39	7.79	0	-	0.58	-	-	R
PBO and Permethrin (5.75)	74	39	66.22	0	-	3.77	1235.82	124461.18	R
Bendiocarb (0.10)	76	39	6.58	0	-	0.96	424.56	2944.58	R
Malathion (5.00)	79	39	40.51	0	-	4.24	90.97	209.64	R
DDT (4.00)	76	39	5.26	0	-	0.82	-	-	R

df = 6, t= 2.89, p = 0.027, SD- standard deviation, KDT (mins)- Knockdown time, R-resistant, SR- suspected resistance.

APPENDIX IV

Mortality at 24 hrs, Mean, SD, and Resistance status of *Anopheles* mosquitoes from Korle-Bu

Insecticide and Concentration (%)	Number of exposed mosquitoes		Mean Total Mortality (%)		Mean corrected (%) mortality	SD	KDT_{50} (mins)	KDT_{95} (mins)	Resistance Status
	Test	Control	Test	Control					
Deltamethrin (0.05)	88	41	12.50	0	-	0.50	84.76	203.39	R
PBO and Deltamethrin (5.05)	83	41	61.45	0	-	2.99	38.63	63.70	R
Permethrin (0.75)	88	41	5.68	0	-	1.89	-	-	R
PBO and Permethrin (5.75)	86	41	13.95	0	-	2.16	-	-	R
Bendiocarb (0.10)	93	50	16.13	0	-	2.87	470.38	3920.57	R
Malathion (5.00)	96	50	64.58	0	-	4.12	165.40	867.97	R
DDT (4.00)	84	41	1.19	0	-	0.50	2.14E+24	5.00E+40	R

df = 6, t = 2.51, p = 0.046, SD- standard deviation, KDT (mins)- Knockdown time, R-resistant.

APPENDIX V

The molecular forms, *kdr* and *Ace-1* frequencies of *Anopheles gambiae* mosquitoes from the different study sites

Sites	**Molecular forms**	***Kdr***							***Ace-1***				
		Total	%	Total	RR	RS	SS	**Freq.**	Total	RR	RS	SS	**Freq.**
ACHIMOTA	*An. gambiae s.s*	25	62.5	25	25	0	0	1.00	25	14	10	1	0.76
	An. coluzzii	15	37.5	15	8	4	3	0.67	15	0	12	3	0.40
	TOTAL	40		40	33	4	3	0.88	40	14	22	4	0.63
KORLE-BU	*An. gambiae s.s*	1	2.5	1	1	0	0	1.00	1	1	0	0	1.00
	An. coluzzii	39	97.5	39	31	7	1	0.88	39	1	32	6	0.44
	TOTAL	40		40	32	7	1	0.89	40	2	32	6	0.45
OPEIBEA	*An. gambiae s.s*	38	95.0	38	32	6	0	0.92	38	25	11	2	0.80
	An. coluzzii	2	5.0	2	1	1	0	0.75	2	1	0	1	0.50
	TOTAL	40		40	33	7	0	0.91	40	26	11	3	0.79
MATAHEKO	*An. gambiae s.s*	5	12.5	5	5	0	0	1.00	5	0	4	1	0.40
	An. coluzzii	30	75.0	30	21	6	3	0.80	28	2	20	6	0.43
	MS	5	12.5	5	3	2	0	0.80	5	0	4	1	0.40
	TOTAL	40		40	29	8	3	0.83	38	2	28	8	0.42

APPENDIX VI

Alpha esterase activities of *Anopheles gambiae* mosquitoes from the different study sites

Study sites	Kisumu	Opeibea	Achimota	Korle-Bu	Mataheko
Number of mosquitoes	107	41	46	49	45
Minimum	0.0001	0.1002	0.0129	0.0011	0.0001
25 % Percentile	0.0014	0.4778	0.0261	0.0023	0.0010
Median	0.0160	0.7194	0.0411	0.0026	0.0018
75 % Percentile	0.2008	0.9473	0.0761	0.0029	0.0048
Maximum	0.4526	1.7240	0.2697	0.0043	0.1061
Mean	0.0940	0.7299	0.0629	0.0026	0.0071
SD	0.1273	0.3380	0.0578	0.0006	0.0177
SE	0.0123	0.0527	0.0085	0.0001	0.0026
Lower 95 % CI of mean	0.0706	0.6232	0.0458	0.0025	0.0017
Upper 95 % CI of mean	0.1193	0.8366	0.0801	0.0028	0.0124
p-value*		0.0050	0.0280	<0.0001	<0.0001
Sum	10.1600	29.9300	2.8940	0.1293	0.3182

p value*- showing the comparison between Kisumu mosquitoes and the mosquitoes from the other study sites, SD - standard deviation, SE - standard error, CI - confidence interval. Bonferroni corrected significance level: 0.005.

APPENDIX VII

Beta esterase activities of *Anopheles gambiae* mosquitoes from the different study sites

Study sites	Kisumu	Opeibea	Achimota	Korle-Bu	Mataheko
Number of mosquitoes	102	44	47	49	39
Minimum	0.0031	0.3912	0.2320	2.0800	0.6417
25 % Percentile	0.6379	1.5230	2.1390	3.5470	2.4380
Median	1.4910	1.9400	2.9900	3.9690	4.1100
75 % Percentile	3.0210	2.7230	6.5080	4.6380	9.4260
Maximum	18.2100	10.3300	23.4700	6.3400	28.9600
Mean	2.3580	2.4240	5.0240	4.0860	7.1370
SD	2.8040	1.8040	4.9000	0.9454	7.0000
SE	0.2777	0.2720	0.7147	0.1351	1.1210
Lower 95 % CI of mean	1.8070	1.8750	3.5850	3.8150	4.8680
Upper 95 % CI of mean	2.9090	2.9720	6.4630	4.3580	9.4060
p-value*		0.0730	<0.0001	<0.0001	<0.0001
Sum	240.5000	106.6000	236.1000	200.2000	278.3000

p value*- showing the comparison between Kisumu mosquitoes and the mosquitoes from the other study sites, SD - standard deviation, SE - standard error, CI - confidence interval. Bonferroni corrected significance level: 0.005.

APPENDIX VIII

Oxidase activities of *Anopheles gambiae* mosquitoes from the different study sites

Study sites	Kisumu	Opeibea	Achimota	Korle-Bu	Mataheko
Number of mosquitoes	105	42	46	49	44
Minimum	0.0054	0.0927	0.0062	0.0192	0.0095
25 % Percentile	0.0289	0.2101	0.0166	0.0274	0.0143
Median	0.0449	0.3976	0.0257	0.0327	0.0299
75 % Percentile	0.1088	0.9145	0.0457	0.0367	0.1265
Maximum	0.6179	1.8060	0.2153	0.0528	0.8251
Mean	0.0850	0.5716	0.0417	0.0331	0.0910
SD	0.1011	0.4398	0.0469	0.0071	0.1491
SE	0.0020	0.0678	0.0069	0.0010	0.0225
Lower 95 % CI of mean	0.0654	0.4345	0.0278	0.0311	0.0457
Upper 95 % CI of mean	0.1045	0.7086	0.0556	0.0352	0.1363
p-value*		0.0010	<0.0001	<0.0001	0.0010
Sum	8.9200	24.0100	1.9180	1.6230	4.0030

p value*- showing the comparison between Kisumu mosquitoes and the mosquitoes from the other study sites, SD - standard deviation, SE - standard error, CI - confidence interval. Bonferroni corrected significance level: 0.005.

APPENDIX IX

Activities of GST's in *Anopheles gambiae* mosquitoes from the different study sites

Study sites	Kisumu	Opeibea	Achimota	Korle-Bu	Mataheko
Number of mosquitoes	82	39	46	46	40
Minimum	0.0355	0.0363	0.0286	0.0853	0.0020
25 % Percentile	0.1468	0.1768	0.2651	0.2188	0.1884
Median	0.2501	0.2919	0.4539	0.3136	0.3571
75 % Percentile	0.4895	0.4539	0.6802	0.6421	0.7968
Maximum	1.6680	1.2280	1.4350	1.2950	1.5950
Mean	0.4042	0.3814	0.5028	0.4420	0.5331
SD	0.3691	0.2945	0.3250	0.3072	0.4154
SE	0.0408	0.0472	0.0479	0.0453	0.0657
Lower 95 % CI of mean	0.3231	0.2859	0.4063	0.3508	0.4003
Upper 95 % CI of mean	0.4853	0.4768	0.5993	0.5332	0.6660
p-value*		0.3900	0.0450	0.2520	0.0150
Sum	33.1500	14.8700	23.1300	20.3300	21.3300

p value*- showing the comparison between Kisumu mosquitoes and the mosquitoes from the other study sites, SD - standard deviation, SE - standard error, CI - confidence interval. Bonferroni corrected significance level: 0.005.

APPENDIX X

Activities of ACHE in *Anopheles gambiae* mosquitoes from the different study sites

Study sites	Kisumu	Opeibea	Achimota	Korle-Bu	Mataheko
Number of mosquitoes	95	43	46	49	36
Minimum	0.0100	0.0639	0.0109	0.0168	0.0075
25 % Percentile	0.0557	0.2422	0.0742	0.0505	0.0744
Median	0.0727	0.4101	0.1179	0.0707	0.1058
75 % Percentile	0.1218	0.7496	0.2500	0.1143	0.1515
Maximum	1.0080	1.1860	1.0480	0.2115	0.5908
Mean	0.1330	0.4958	0.1989	0.0802	0.1374
SD	0.1694	0.3283	0.2007	0.0426	0.1170
SE	0.0174	0.0501	0.0296	0.0061	0.0195
Lower 95% CI of mean	0.0985	0.3948	0.1393	0.0680	0.0978
Upper 95% CI of mean	0.1675	0.5968	0.2585	0.0965	0.1770
p-value*		<0.0001	0.0190	0.3780	0.0910
Sum	12.6300	21.3200	9.1490	3.9300	4.9460

p value*- showing the comparison between Kisumu mosquitoes and the mosquitoes from the other study sites, SD - standard deviation, SE - standard error, CI - confidence interval. Bonferroni corrected significance level: 0.005.

APPENDIX XI

Preliminary survey to determine the insecticide use patterns in the vegetable growing areas of Korle-Bu and Opeibea in Southern Ghana.

Questionnaire for Vegetable farmers in Korle-Bu and Opeibea

1. Survey agent _____
2. Name of farmer _____
3. Date _____
4. Site _____
5. What is the size of your farm per planting season?
    a. Less than ½ ha
    b. 1 ha
    c. 1-2 ha
    d. Greater than 2 ha
6. How long have you been cultivating vegetables on your farmland?
    _____
7. What is the planting season and interval for each vegetable?
    _____
8. What irrigation equipment do you use?
    a. Sprinkler
    b. Watering can
    c. Water hose
9. If watering can, what type of nozzle is attached? _____
10. Are insect pests a problem on your farm? Yes { } No { }
11. Which is the most serious pest on your farm?
    a. Diamond back moth (*Plutella xylostella*)
    b. Cabbage aphids (*Hellula undalis*)
    c. Cabbage web worm (*Brevicoryne brassicae*)
    d. Cabbage looper (*Trichoplusia ni*)
    e. Flea beetles
    f. Leaf webber
    g. All of the above
    h. Others (specify) _____
12. In what season is the pest attack more serious?
    a. Rainy season
    b. Dry season
13. What methods are used in controlling these pests?
    a. Chemical control methods (spraying insecticides)
    b. Cultural control methods
    c. Biological control methods

d. IPM
e. Other (specify) _____

14. If chemical methods are used, what type of insecticides do you use currently? _____
15. Which others have you ever used? _____
16. Why did you stop using them? _____
17. How do you use the various insecticides?
    a. Alternatively (one after another)
    b. As a mixture
18. State the precise dosage of the insecticide you apply _____
19. What insecticide equipment do you use?
    a. Knapsack sprayer
    b. Other (specify) _____
20. Give reasons for your choice of equipment _____
21. If knapsack sprayer is used, what nozzle type do you use?
    a. Polyjet red
    b. Polyjet yellow
    c. Polyjet green
    d. Polyjet blue
    e. Cone
22. Do you wear protective clothing during insecticide application? Yes { } No { } Sometimes { }
23. What is the interval between planting seasons? _____
24. What is the frequency of pesticide application on each vegetable? _____
25. What is the interval between insecticide application and watering? _____
26. Where do you get professional advice on proper insecticide use and handling?
    a. From extension officers
    b. Pesticide sale points
    c. Fellow farmers
    d. Other (specify) _____
27. Do you keep records of your insecticide use patterns? Yes { } No { }
28. Do you have any knowledge on the problems associated with insecticide residues in foods? Yes { } No { }
29. If yes, please explain _____