Dissertation
zum Erwerb des Doctor of Philosophy (Ph.D.)
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

Doctoral Thesis for the awarding of a Doctor of Philosophy (Ph.D.)
at the Medical Faculty of
Ludwig-Maximilians-Universität, Munich

vorgelegt von
submitted by

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Flensburg, Germany

am (Tag an dem die Dissertation abgeschlossen wurde)
submitted on (day of finalization of the thesis)

30.09.2014

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Date of Oral Defence:	19.03.2015

Insecticide resistance status of Culex species in Urban areas in Ghana and efficacy of long lasting nets against them

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Key Words

Culex, insecticide, insecticide-treated-material, insecticide-resistance, malaria control, nuisance, urban

Abstract

Background

Current strategic plan for malaria control in Ghana includes the attainment of 80% of the general population sleeping under insecticide treated materials (ITM) by 2015. This coverage may not be attained if there is non-compliance in the use of bed nets. Failure of ITM to protect users from nuisance mosquitoes, particularly *Culex* mosquitoes has been cited as one of the major threats to the sustained use of ITM. A nationwide survey was therefore carried out to determine insecticide resistance status of *Culex* species and efficacy of ITM against them.

Methods and materials

Mosquito larvae were sampled from various land use and ecological settings and at different seasons. These were reared to adults and used for the various tests. In adults, insecticide susceptibility tests to eight insecticides as well as cone and tunnel bioassays were performed. Biochemical and molecular analyses were also conducted to determine the resistance mechanisms in the study populations.

Results

Culex quinquefasciatus and *C. decens* were the *Culex* species that were identified in the study area. DDT and deltamethrin resistances were evident across the country. A

strong relationship between resistance status and urban size was observed in the study population (Pearson χ^2 =48.2; df = 1; P<0.0001). Not only *kdr* and *ace*1 mutations but also elevated levels of three detoxifying enzymes were found in the study populations. New ITMs evaluated had reduced efficacy against pyrethroid-resistant *Culex* mosquitoes.

Conclusions

Insecticide resistance status of *Culex* species in urban areas of Ghana was determined. Insecticide resistance level was high in large urban areas. Urbanization and its associated problems as well as ecology and different land use were observed to have some impact on level of insecticide resistance in the *Culex* population. ITM with synergist and organophosphate insecticides were seen as a possible resistance management tool against pyrethroid-resistant *Culex* mosquitoes.

Table of Content

i) List of Figures

ii) List of Tables

iii) List of appendices

1) Appendix 1: Pictures of different mosquito breeding habitats where larvae were collected. a)Accra residential area category (Kaneshie), b) Accra, urban agriculture category (Korle bu), c) Kumasi swampy area category (Bohyen) d) Sunyani residential area category (Penkwasi)
2) Appendix 2: The output of Primer 3 software showing the sequence of Cytochrome c oxidase of *Culex decens* and the position where primer was design from.
3) Appendix 3: Species identification (A) and *kdr* assays (B). Lane 1 1kb ladder, (A) lanes 2-6 *Culex quinquefasciatus* species, (B) lanes 2-10 heterozygote resistance, lane 11-12 susceptible
4) Appendix 4: Multiple comparisons of the mean absorbance from α-esterase, β-esterase and oxidase using ANOVA

iv) Abbreviations

AChE	Acetyl cholinesterase
ACT	Artemisinin-based combination therapy
Bp	Base pair
DDT	Dichlorodiphenyltrichloroethane
GABA	Gamma-Amino butyric acid
gDNA	Genomic deoxyribose nucleic acid
GST	Glutathione S-transferase
h	Hour
ITM	Insecticide treated material
IRS	Indoor residual spraying
kdr	Knockdown resistance gene
MDG	Millennium Development Goal
OR	Odds ratio
P	95% Probability value
P450	Cytochrome P450 enzymes
PBO	Piperonyl butoxide
PCR	Polymerase chain reaction
RBM	Roll Back Malaria
UV	Ultraviolet
WHO	World Health Organization

1) Introduction

Over the past decade, estimated malaria morbidities and mortalities in many African countries are falling and this success has been linked to intense malaria control interventions, including the use of insecticide treated materials (ITM) supported by indoor residual spraying of insecticide (IRS) (WHO, 2013). Nevertheless, acceptance and utilization of ITM still remains a problem and if not properly addressed can jeopardize malaria control efforts and elimination in Africa. Among many factors, failure of ITM to protect users from the nuisance of *Culex* mosquitoes due to a lack of insecticide efficacy against them has been cited as one of the major threats to the sustained use of ITM (Samuelsen *et al*., 2004). In this regard, for local communities to accept and maintain ITM use, it must be able to protect its users from bites from both vector and nuisance mosquitoes.

In Ghana, the main malaria vectors are *Anopheles gambiae* and *A. funestus.* The major nuisance mosquito is *Culex* mosquitoes, which are also vectors for different parasitic and viral anthroponoses. Occurrence and distribution of insecticide resistance and the mechanisms involved in the malaria vectors (*Anopheles* species) have been well investigated in the country (Yawson *et al.,* 2004; Okoye *et al*., 2008; Boakye *et al*., 2009; Hunt *et al*., 2011; Kabula *et al*., 2011). Different insecticide-resistance statuses have been observed across different ecological zones, seasons and even different breeding habitats among *Anopheles* species (Yawson *et al.,* 2004; Anto *et al*., 2009; Kabula *et al*., 2011). However, not much of such information is available for *Culex* mosquitoes in Ghana.

Culex mosquitoes are the most widely distributed and abundant mosquito in Ghana and in order to improve and maintain the use of ITM, particularly in areas without a tradition of bed net use, there is a need to attain a better understanding of the impact of *Culex* mosquitoes on ITM usage. A key determinant is the resistance status of *Culex* and the efficacy of ITM against pyrethroid-resistant populations. Moreover, with growing pyrethroid resistance in West Africa (Chandre *et al*., 1998; Corbel *et al*., 2007), the use of alternative classes of insecticides for vector control is becoming very popular. As a result, knowledge on the resistance status of vectors against insecticides found in the important classes of insecticides used in public health and the mechanisms involved as well as factors that influence the resistance has become important.

This study was therefore carried out to assess insecticide resistance status of *Culex* in Ghana and determine its impact on the efficacy of ITM. It is anticipated that the results would give an insight into the potential role *Culex* mosquitoes in malaria control and the possible insecticide resistance management strategy against them.

2) Literature review

2.1) Mosquitoes and human welfare

2.1.1) Biology and behaviour of mosquitoes

Knowledge of the life history of mosquitoes is as important as knowing that mosquitoes transmit diseases. Mosquitoes are among the best known groups of insects because of their role as vectors of some of the most serious human diseases. There are about 3400 species of mosquitoes, of which about 100 are vectors of human diseases. Mosquitoes are two-winged insects belonging to the family Culicidae of the order Diptera. They can be distinguished from most other diptera by a combination of the following characters: a long proboscis projecting forward from the head, the presence of scales on the thorax, legs, abdomen and wing veins, and a characteristic wing venation with the second, forth, and fifth longitudinal veins being branched (Service, 2008).

Important mosquito vectors can be grouped into anophelines (*Anopheles*) and culicines (*Aedes, Culex, Mansonia, Haemagogus* and *Sabethes*). The following characteristics are mostly used to distinguish *Anopheles, Culex and Aedes* mosquitoes: 1) the resting position of the larvae, 2) the length of the palps to that of the proboscis, and 3) the angle to the resting surface of the adult mosquito (Figure 2.1.1.1)

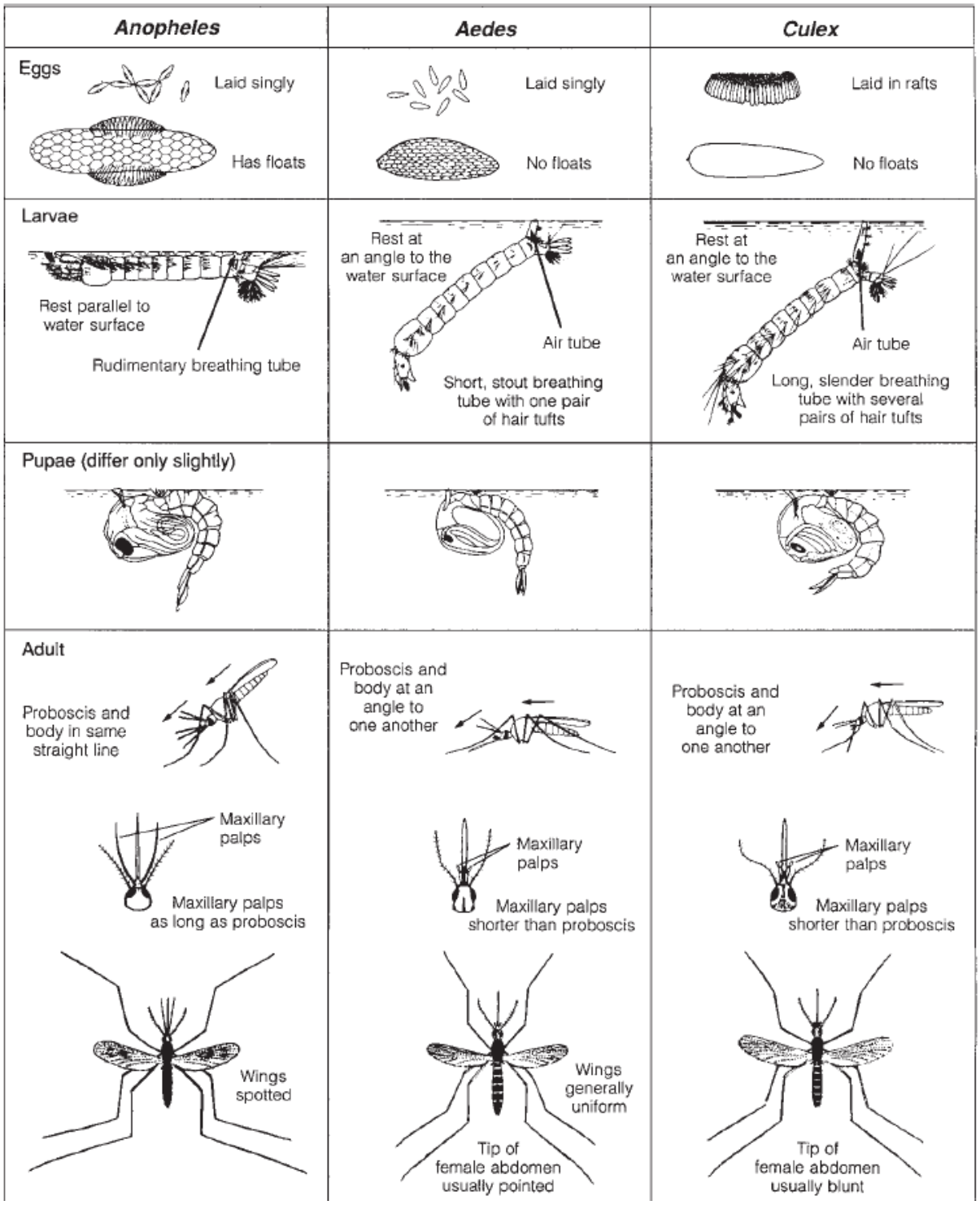


Figure 2.1.1.1: Characteristics for differentiating *Anopheles, Aedes* and *Culex* mosquitoes (Source: Rozendaal, 1997)

Mosquitoes undergo complete metamorphosis, passing through four distinct stages during their life cycle: the eggs, larva, pupa, and adult (Figure 2.1.1.2). Immature stages of mosquitoes require water to complete their life cycle. The growth period of the first three stages is dependent on the species and temperature. There are four larval instars and in tropical countries the development of juvenile stages may be as short as 5-7 days. Many species, however, require about 7-14 days. Female mosquitoes lay their eggs in various water habitats including natural and artificial, permanent and temporal water bodies with salinities varying from water produced from melting snow to salinities greater than sea water in evaporating tidal pools (Service, 2008; Kettle, 1995).

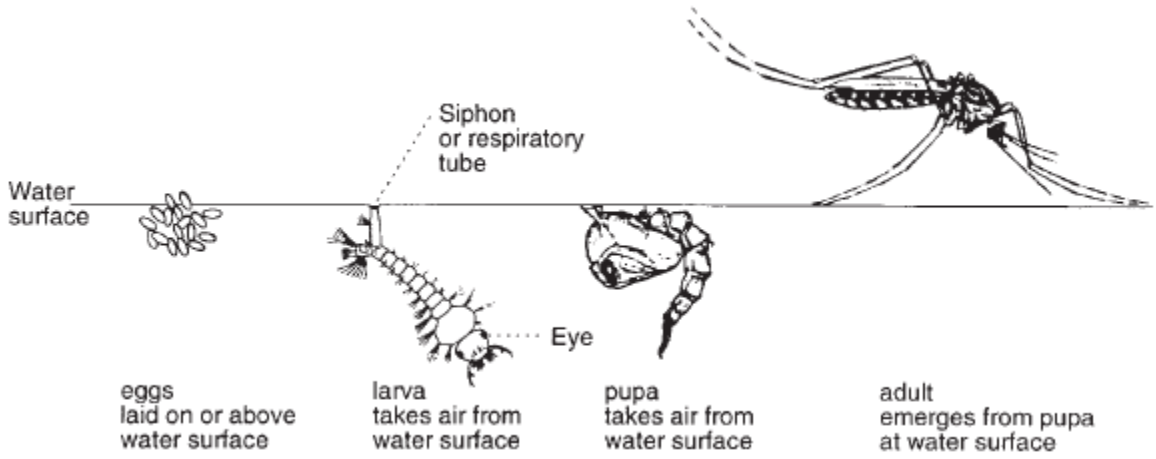


Figure 2.1.1.2: The life cycle of mosquitoes showing the four stages (Source: Rozendaal, 1997)

On average, females live approximately 2-3 weeks but the lifespan of males is generally shorter. Adult male and female mosquitoes feed on nectars and other naturally occurring sugary secretions to provide energy, but it is only the female that seeks a blood meal, a requirement to provide protein for egg development. Male

mosquitoes cannot bite and are unable to transmit diseases. During blood feeding many female mosquitoes can inject pathogenic micro-organisms into their host which can cause diseases such as malaria, yellow fever, Dengue, and filariasis.

The source of the blood meal is a major factor in determining the potential of a species as a vector of disease. While many mosquitoes are generalist feeders, some specialize in feeding on certain animals. About nine basic feeding habits have been recognised in mosquitoes (Tempelis, 1975). However, the most important mosquitoes in terms of public health are the ones that readily feed on mammals. Mosquitoes that readily feed on mammals can be further subdivided into those which are anthropophilic, feeding on humans, and those which are zoophilic, feeding on animals (Service, 2008).

The feeding and resting habits of mosquitoes are of great importance in disease control programmes. Most mosquito species bite immediately after sunset while others bite later, around midnight or early morning. Mosquitoes that prefer to enter houses to bite are described as being endophagic whereas mosquitoes that mostly bite outside are exophagic. After a blood meal, mosquitoes usually rest for a short period to digest the blood. During this period some mosquitoes that enter houses to bite remain indoor to rest; these are referred to as endophilic. Mosquitoes that rest outside after blood meal (e. g. on plants, in holes) are termed exophilic. The process of blood-feeding and egg maturation, followed by oviposition is repeated several times throughout the females' life. This cycle is termed gonotrophic (Service, 2008).

2.1.2) Impact of mosquitoes on human welfare

In general, mosquitoes may affect human welfare by direct annoyance and transmission of diseases. Mosquitoes are important vectors of several tropical diseases, including malaria, filariasis, and numerous viral diseases such as Dengue and yellow fever. Mosquito-borne diseases greatly affect public health and contribute significantly to disease burden, deaths, economical and developmental problems worldwide.

Anopheles species are the only vectors of malaria. Besides malaria, they can also transmit filariasis and some arboviruses. *Aedes* mosquitoes are important vectors for Dengue, yellow fever and many other arboviruses while *Culex* mosquitoes also transmit several diseases such as filariasis and many arboviruses as well (Table 2.1.2.1).

Table 2.1.2.1: Diseases transmitted by different genera of mosquitoes.

Mosquito	**Diseases**
Anopheles	Malaria, lymphatic filariasis,O'nyong-nyong (ONNV), other viral diseases
Culex	Lymphatic filariasis, St. Louis encephalitis, Japanese encephalitis, Eastern equine encephalitis, other viral diseases
Aedes	Yellow fever, Dengue, Dengue haemorrhagic fever, Chikungunya, other viral diseases, lymphatic filariasis
Mansonia	Lymphatic filariasis

Several mosquito species can also be a great nuisance without presenting a direct risk to health. Nuisance biting alone can have negative impact on the standard of living in the community (Webb and Russell, 2007). Halasa *et al.* (2014) quantified the impact of mosquito nuisance on quality of life and found out that mosquitoes prevented about 60% of residents in New Jersey, USA, from enjoying their outdoor activities, at least to some extent. Respondents rated the importance of enjoying outdoor activities without mosquitoes comparable to that of neighbourhood safety and higher than that of a clean neighbourhood. A considerable amount of money is spent on mosquito control, not because of their status as vectors of disease but because of their nuisance (Service, 2008). A study concluded that city residents in the USA were willing to pay an average of $147 per household per year to reduce

mosquito nuisance, compared to only $21 for programs targeting disease transmitting mosquitoes (Dickinson *et al*., 2012).

There is not much information on positive impacts of mosquitoes on humans or on the ecosystem. However, like many other aquatic invertebrates, mosquito larvae provide food for predators while assisting in nutrient recycling. Adult mosquitoes provide food for several terrestrial invertebrates, birds, mammals, amphibians and reptiles. It was estimated that the number of migratory birds that nest in the Arctic tundra could drop by more than 50% without mosquitoes to eat (Fang, 2010). Many species of insects, spiders, salamanders, lizards and frogs would also lose a primary food source. Poulin *et al*. (2010) tracked insect-eating house martins (*Delichon urbicum)* at a park in the French Camargue region after the area was sprayed with a microbial mosquito-control agent. They found that the birds produced an average of two chicks per nest after spraying, compared with three for birds at control sites (Poulin *et al*., 2010). This shows the importance of mosquitoes as a food source to birds. In addition, mosquitoes play a role in the pollination of some plants. For example, *Aedes communis* is an important pollinator of *Platanthera obtusata*, the blunt-leaf orchid (USDA, 2014).

2.1.3) Mosquito management strategies

Mosquito control measures can be directed at either the immature aquatic stages or the adult stages or at both stages simultaneously. In extensive breeding sites, control

of the juvenile stages is exercised mostly through the application of larvicides (insecticides). Larval control through source reduction and routine application of larvicides was a key intervention in eradicating malaria in many parts of the world (Utzinger *et al*., 2001, Killeen *et al*., 2002).

Apart from chemicals, living organisms or their products are also used to control mosquito juveniles, which is termed "biological control". The organisms used include viruses, bacteria, protozoa, fungi, plants, parasitic worms, predatory mosquitoes and fish. The aim is generally to kill larvae without polluting the environment. Physical control or environmental management methods such as land-fills, source reduction and environmental manipulation are also effective against mosquito larvae.

Control directed at adults includes personal protection (mosquito nets, repellents), window and door screens, and use of insecticide in the form of aerosol, indoor house residual spraying (Ogoma *et al*., 2009). The choice of a particular control strategy depends on the knowledge of the habitat ecology of the mosquitoes and their behaviour. For example, understanding the choice of oviposition sites of mosquito species is important for the design of successful larval control strategies. Likewise, knowledge on adult mosquito behaviour may help to choose appropriate control methods. For example, effective indoor residual spraying against malaria vectors depends on whether mosquitoes rest indoors. Furthermore, the effectiveness of insecticide treated nets depends on vectors biting at hours when most people are in bed (Pates and Curtis, 2005).

2.2) Malaria and malaria control

2.2.1) Global burden of malaria

Of all the mosquito-borne diseases, malaria is by far the most important in terms of morbidity and mortality. Malaria is a disease known since ancient times and Hippocrates, about 400 BC, described the three characteristic stages of an attack: chilly rigor, high fever and profuse sweating (Kettle, 1995). As recent as 1900, malaria was widely distributed and more than 77% of the world population in 140 countries was at risk, with more than 3.1 million deaths occurring among a total population of 1.6 billion (Carter and Mendis, 2002).

Currently, between 2000 and 2012, estimated malaria mortality rates fell by 45% in all age groups and by 51% in children under 5 years of age. With such progress, it is projected that by 2015 malaria mortality rates would decrease by 56% in all ages, and by 63% in children under 5 years of age. Modelling suggests that an estimated 3.3 million malaria deaths were averted between 2001 and 2012, and that 69% of these lives saved were in the 10 countries with the highest malaria burden in 2000. Malaria mortality rates among children in Africa have been reduced by an estimated 54% since 2000. It is estimated that approximately 3 million (90%) of the deaths averted between 2001 and 2012 are children under 5 years of age in sub-Saharan Africa (WHO, 2013).

The substantial reduction in malaria mortality rates have contributed significantly to progress towards achieving the target for Millennium Development Goal 4 (MDG 4), which is to reduce by two thirds the under-5 mortality rate between 1990 and 2015 (WHO, 2013). Notwithstanding the achievements made in malaria control, an

estimated 207 million cases and 627000 malaria deaths are estimated to have occurred in 2012. It is estimated that malaria would be the 10th leading cause of death in low income countries by 2030, representing 1.8% of total deaths and total disability-adjusted life year (DALY) lost of 2.5% (Mathers and Loncar, 2006).

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*. Five *Plasmodium* species are known to infect humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*). Tropical malaria is caused by *P. falciparum,* which is responsible for the most severe disease and the highest mortality in Africa. Tertian malaria is caused *P. vivax* and *P. ovale* and can form resting stages in the liver (hypnozoites). Once reactivated, it can cause a clinical relapse many months after the initial event (Greenwood *et al*., 2005). In recent years, some human cases of malaria have also occurred with *P. knowlesi* in Malaysian Borneo (Vythilingham *et al*., 2006; WHO, 2014), a species that causes malaria among monkeys and occurs in certain areas of South-East Asia. It is reported that about 58% of cases diagnosed as *P. malariae* in Malaysian Borneo are *P. knowlesi* (Sign *et al*., 2004).

2.2.2) Malaria control

Malaria is a preventable and treatable disease, provided that current recommended interventions are properly implemented. The WHO recommends a combination to control and eliminate malaria, which includes vector control interventions, preventive therapies, diagnostic testing, artemisinin-based combination therapies (ACTs), and

an intensive malaria surveillance (WHO, 2013). Many factors affect the choice of malaria control methods in a region, including endemicity, vector species and behaviour, seasonality, disease patterns, health service factors, and many more (WHO, 2014).

Vector control is the main way to reduce malaria transmission at the community level. It is the only intervention that can reduce malaria transmission from very high levels to close to zero (WHO, 2014). Malaria prevention through vector control seeks to reduce human-vector contact and by reducing the average lifespan of the local mosquito population at the community level. The major malaria prevention strategies recommended by WHO are the use of ITM and indoor residual spraying (IRS) which involves the application of residual insecticides to the inner surfaces of living places with the objective of targeting *Anopheles* mosquitoes that rest on walls after taking a blood meal (WHO, 2013).

ITM works both on the individual level (personal protection) and the community level. It is expected that if used on a large scale, ITM can have a mass killing effect on vector populations which can benefit the whole community, even those without nets. A number of studies have demonstrated that the use of ITMs is effective in reducing malaria-related morbidity and mortality (Binka *et al*., 1996; Hill *et al*., 2006; D'Alessandro *et al*., 1995). Individuals not sleeping under an ITM, but living within an area with high ITM coverage, have also been shown to be at a decreased risk of infection due to the resulting reduction in overall malaria transmission (Howard *et al*., 2000; Killeen *et al*., 2007).

Antimalarial medicines can also be used to prevent malaria, especially for travellers who go to malaria endemic areas. In addition, WHO recommends intermittent preventive treatment with sulfadoxine-pyrimethamine for pregnant women and infants living in high transmission areas (WHO, 2014).

2.3) *Culex species* and public health

2.3.1) Biology and behaviour of *Culex species*

The genus *Culex* is made up of about 768 species (Harbach, 2011). The adult females bite people and animals throughout the night, indoors and outdoors. During the day, they are inactive and are often found resting in dark corners of rooms, shelters and culverts. They also rest outdoors on vegetation and in holes in trees in forest areas (Rozendaal, 1997).

The sources of blood meals for *Culex* mosquitoes vary greatly among the various geographic populations. It may take blood meals from birds, livestock and humans but it is highly anthropophilic in some geographic areas. *Culex* mosquitoes are epidemiologically important vectors for a diverse array of pathogens, including many arboviruses, filarial worms, and protozoa, such as West Nile viruses (WNV), *Wuchereria bancrofti*, and *Plasmodium relictum*, an avian malaria parasite (Service, 2008).

Culex mosquitoes breed in a large variety of still or very slow moving waters, ranging from artificial containers and catchment basins of drainage systems to large permanent water bodies. Most importantly, *C. quinquefasciatus* has the ability to breed in organically polluted water bodies such as choked gutters and septic tanks. In many developing countries these are common breeding places in rapidly expanding urban areas where drainage and sanitation are inadequate. Under these conditions the mosquito population can increase rapidly (Rozendaal, 1997; Service 2004). Their ability of developing in organically highly polluted water bodies gives *C. quinquefasciatus* clear advantages over other species of mosquitoes, particularly in urban areas.

The combination of high anthropophilic females with high tolerance to insecticides and the ability of the larvae to develop in polluted waters make *C. quinquefasciatus* a very important mosquito in public health and difficult to control (Vinogradova, 1966).

2.3.2) Potential impact of *Culex* mosquitoes on malaria control

Urban areas in Ghana as well as many developing countries are characterized by extensive networks of open gutters, several wetlands and water bodies. As a result of inadequate urban infrastructure, partly due to rapid population growth, garbage and other household waste products are often deposited in open gutters and water bodies (Boadi and Kuituman, 2005), thus creating numerous polluted habitats that are suitable for the breeding of *Culex* mosquitoes, particularly *C. quinquefasciatus*.

This problem has not been adequately addressed, partly because *Culex* mosquitoes are currently not an important disease vector in Ghana and many West African countries. However, they cause considerable nuisance to people through their bites and this might very well be a serious yet under recognized factor jeopardizing the use of ITM.

ITM protection against a number of nuisance insects is cited as a reason for their popularity (Alaii *et al*., 2003). Most studies have suggested that if not for the protection given against *Culex* mosquitoes, the popularity and effectiveness of ITM would be hampered (Asidi *et al*., 2005). Therefore, it can be hypothesized that acceptability and utilization of ITM depends on the effectiveness of the net against nuisance mosquitoes, predominantly *C. quinquefasciatus* in urban areas. This suggestion is supported by the fact that people are more concerned about mosquitoes being a nuisance than a cause of malaria in many malaria endemic countries (Nganga *et al*., 2008).

Effectiveness of ITM can be compromised when the efficacy of the net decreases as well as when users do not use the net at all because of perceived problems or improper use of the net. A strong level of pyrethroid-resistance in mosquitoes can reduce the efficacy of the net and as such, discourage the sustained use of ITM because people may not perceive the personal protective effect of ITMs if the mosquitoes are not killed. Due to the importance attached to mosquito nuisance and due to the inability of local inhabitants to distinguish between the genus of a mosquito, it is possible that people living in places with high man biting rates from *Culex* mosquitoes before bedtime might prefer anti-mosquito strategies such as use of mosquito coils and aerosol spray, which are more effective in inhibiting mosquito

nuisance rather than being effective in preventing malaria (Lawrance and Croft, 2004).

In summary, the abundance and resistance status of *Culex* coupled with the value attributed to their nuisance by local people and their level of knowledge on the life history of mosquitoes can threaten the effectiveness of ITM and ultimately malaria control.

2.4) Insecticide use in public health and insecticide resistance

2.4.1) Insecticide use in vector control

Vector control with use of insecticide remains an important component of many vector-borne disease control programmes. Insecticidal properties of dichlorodiphenyltrichloroethane (DDT) were discovered around 1940 and its use in the global malaria eradication campaign led to the elimination of malaria in 37 of the 143 malaria endemic countries between 1950 and 1978 (WHO, 2012).

Presently, four classes of chemical insecticides consisting of the organochlorines (DDT exclusively), the organophosphates, the carbamates and the pyrethroids are the mainstay of vector control programmes. However, pyrethroids are the only recommended insecticide used in treating bed nets because of their relatively low toxicity to humans and rapid knock-down effect (Zaim *et al*., 2000). Other insecticide

groups, such as the benzyl phenyl urea as well as biological control agents such as *Bacillus thuringiensis* have been of limited use against mosquitoes.

Pyrethroids have multiple modes of action on the mosquito vector. They open sodium channels, which leads to continuous nerve excitation, paralysis and death of the vector. They also have an irritant effect, resulting in hyperactivity, rapid knockdown, feeding inhibition, shorter landing times and undirected flight, all of which reduce the biting ability of mosquitoes (WHO, 2009). Pyrethroids can be classified into two types according to the absence (type 1) or presence (type 2) of an alpha-cyano group in the alcohol moiety. Also, due to a complex chemical structure, the individual pyrethroid substances are often composed of different bonds, structures or isomers. The level of activity or toxicity is determined by the structure of the molecule (DeVries and Georghiou, 1980; Weerasinghe *et al*., 2001).

Organochlorines are used for IRS vector control in the form of DDT. The continued use of DDT for disease vector control is conditionally approved under the Stockholm Convention on Persistent Organic Pollutants in accordance with WHO recommendations and guidelines (WHO, 2006). DDT and pyrethroids have similar modes of action, and therefore cross resistance to these two classes of insecticide may occur (WHO, 2011).

Organophosphates and carbamates are also used for IRS. They are highly effective, but have relatively short residual activity compared to pyrethroids and DDT. Currently however, encapsulation (CS) technology is used to extend the residual performance of some organophosphate and carbamate insecticides (Oxborough *et al*., 2014). Organophosphates and carbamates inhibit cholinesterase, thereby preventing

neurotransmitter acetylcholine breakdown, resulting in neuromuscular overstimulation and subsequent death of the vector (WHO, 2009). This mode of action on mosquitoes differs from that of pyrethroids and organochlorines

2.4.2) Insecticide resistance in mosquitoes

A small percentage of the overall amount of insecticides is used in public health. However, there is wide spread of resistance among many vector species of public health importance. The first report on insecticide resistance was published about 100 years ago when Melander (1914) noticed that certain populations of scale insects were becoming less susceptible to sulphur-lime than they had been in the past, while the chemical was reported to be very effective at killing the insects in a previous experiment. The study found that 90% of the specimens that he had sprayed in Clarkston, USA, had survived. Even when he increased the amount of the active ingredient by ten times, 74% of them still survived (Melander, 1914).

Resistance to insecticides is defined as the development of the ability to survive doses of insecticides that previously were lethal to the majority of individuals in a population (IRAC, 2011). The level of resistance in insect vector populations is dependent both on the volume and frequency of applications of insecticides used against them and the inherent characteristics of the insect species involved (Hemingway and Ranson, 2000). Resistance became a major obstacle to the global malaria eradication programme of the 1950s and 1960s and rendered some

insecticides, particularly dieldrin, useless (Busvine, 1969). Presently, insecticide resistance is widespread and it is reported in nearly two thirds of malaria endemic countries. It affects all major vector species and all classes of insecticides (WHO, 2012). Insecticide resistance is expected to directly affect the re-emergence of vector borne diseases and threaten disease control in areas where vector borne diseases already exist (Brogdon and McAllister, 1998). There have been reported cases of failure of some malaria control strategies due to pyrethroid resistance (Hargreaves *et al*., 2000; N'Guessan *et al*., 2007). Therefore, it is very important to preserve useful insecticides by slowing and preventing the development of resistance in mosquitoes. To achieve this goal, it is necessary to understand and monitor the development of insecticide resistance and to find ways of preventing resistance development. In response to the spread of insecticide resistance in mosquito species, WHO has proposed various guidelines to encourage countries to plan and implement insecticide resistance management strategies (WHO, 2012).

2.4.3) Insecticide resistance mechanisms

Resistance mechanisms can be divided into major and minor groups. The major groups are metabolic resistance (alterations in the levels or activities of detoxification enzymes) and target-site resistance (i.e., insensitivity of the sodium channel, acetylcholinesterase and GABA receptor) whereas the minor group consists of reduced penetration and behavioural resistance (IRAC, 2011).

Metabolic resistance is the most common resistance mechanism that occurs in insects. It occurs due to changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than normal, preventing the insecticide from reaching the intended site of action. In mosquitoes, three enzyme systems are believed to be important: the esterases, the mono-oxygenases and the glutathione S-transferases. Glutathione S-transferase is often involved in DDT resistance. Several reports have shown esterases to be involved in organophosphate, carbamate, and to a lesser extent, pyrethroid resistance. Similarly, mono-oxygenases have been associated with pyrethroid resistance, the activation and/or detoxication of organophosphates and, to a lesser extent, carbamate resistance. Most insects possess these enzyme systems to help them detoxify naturally occurring foreign materials. However, they are often enhanced in resistant insect strains enabling them to metabolise or degrade insecticides before they are able to exert a toxic effect. Metabolic resistance mechanisms have been identified in vector populations for all major classes of insecticides currently used for vector control, including organophosphates, carbamates, pyrethroids and DDT (Hemmingway and Ranson, 2000; IRAC, 2011).

In mosquitoes, the esterase-based resistance mechanisms have been well studied in *Culex* mosquitoes. Broad-spectrum organophosphate resistance is conferred by elevated levels of esterases in *Culex*, which in most cases as a result of sequestration (Hemingway and Ranson, 2000). That is, the esterases act by rapidly binding and slowly turning over the insecticide. Two common esterase loci, estα and estβ, are involved alone or in combination in this type of resistance in *Culex* (Hemingway and Karunaratne, 1998; Hemingway and Ranson, 2000).

Several studies have shown that insecticide-resistant insects have elevated levels of glutathione S-transferase (GST) activity (Grant, 1991). GSTs are dimeric multifunctional enzymes that play a role in the detoxification of a large range of xenobiotics (Prapanthadara *et al*., 1996). The enzymes catalyse the nucleophilic attack of reduced glutathione (GSH) on the electrophilic centres of lipophilic compounds. Two families of insect GST are recognized and both appear to have a role in insecticide resistance, particularly DDT resistance (Hemmingway and Ranson, 2000).

The monooxygenases are a complex family of enzymes found in most organisms, including insects. These enzymes are involved in the metabolism of xenobiotics and have a role in endogenous metabolism. The P450 monooxygenases are generally the rate-limiting enzyme step in the chain. These enzymes are important in the adaptation of insects to toxic chemicals in their host plants. P450 monooxygenases are involved in the metabolism of most insecticides (Feyereisen, 1999), leading to an activation of the molecule in the case of organophosphates insecticides, or more generally to detoxification. P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen molecule into the substrate (IRAC, 2011).

Elevated monooxygenase activity is associated with pyrethroid resistance in several mosquito species. CYP6Z1, an adult-specific P450gene product, has been identified from *A. gambiae* associated with pyrethroid resistance (NiKou *et al*., 2003). An about 2.5-fold elevated level of CYP6F1 has been documented in *C. quinquefasciatus* associated with insecticide resistance (Kasai *et al*., 2000).

The second most common resistance mechanism encountered in insects is target-site resistance. Target-site resistance occurs due to the insensitivity of an active site to which an insecticide would normally bind and act. This insensitivity is due to any change in the protein structure of the target site leading to a lesser affinity or lesser action upon the molecule. This results in the insects being unaffected or less affected by the insecticide, compared to susceptible insects. For the main insecticides used in public health, this type of resistance may occur in either the voltage sensitive sodium channel, causing DDT and pyrethroid resistance, or the acetyl cholinesterase (AChE), causing organophosphate and carbamate resistance.

Knock down resistance (*kdr*) mutations, most commonly a substitution of leucine at codon 1014 in the S6 segment of domain II in the voltage gated sodium channels of nerve cell membranes (O'Reilly *et al*., 2006). Knockdown resistance was originally characterized in the house fly (*Musca domestica*) and it has been found that a leucine to phenylalanine substitution at amino acid residue 1014 in the S6 segment of domain II (IIS6) of the α-subunit is associated with moderate knockdown resistance of *kdr* strains. In super-*kdr* strains, a methionine to threonine replacement at residue 918 near the S4-S5linker in domain II is found along with a leucine to phenylalanine mutation, which results in much higher resistance. The third mutation at position 1014, leucine replaced by serine, has been reported in *C. pipiens* and *A. gambiae* (Martinez-Torres *et al*., 1999; Ranson *et al*., 2000).

AChE insensitivity is commonly caused by a glycine to serine point mutation at codon 119 (G119S) (Fournier and Mutero, 1994). The complete *ace*1 coding regions of susceptible and resistant *C. pipiens* strains were cloned and compared between insecticide resistant and susceptible mosquitoes. A single glycine to serine

substitution (G119S) was found (Weill *et al*., 2003). The same mutation has been identified in other AChE insensitive strains of *A. gambiae* and *C. quinquefasciatus* (Weill *et al*., 2003; 2004; Liu *et al*., 2005). As the G119S mutation creates an *Alu* 1 restriction site in the *ace*1 of resistant individuals, a PCR/RFLP test was successfully used to detect its presence in single mosquitoes of both *C. pipiens* and *A. gambiae* (Weill *et al*., 2004).

Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter in nervous systems of both vertebrates and invertebrates (ffrench-Constant *et al*., 1993). Binding of GABA to its receptor activates chloride ion selective channels (Hemingway *et al*., 2004). The GABA receptor is the target site of cyclodiene and phenylpyrazoles insecticides (ffrench-Constant *et al*., 2004). Resistance to dieldrin (*rdl*), a GABA receptor subunit gene, was cloned from a field-isolated Drosophila mutant, which was resistant to dieldrin, a cyclodiene insecticide (ffrench-Constant *et al*., 1993). Genetic mapping of dieldrin resistance in Drosophila melanogaster indicated that resistance was conferred by *rdl* on the left arm of chromosome III at map position 66F (ffrench-Constant *et al*., 2004). A mutation at a single codon in the *rdl* gene, are placement of alanine at position 302 with a serine or a glycine, conferred dieldrin resistance in Drosophila (ffrench-Constant *et al*., 1993). Alanine at position 302, which lies within the second transmembrane region of the *rdl* subunit, is crucial for insecticide binding and mutation of this amino acid can cause resistance due to a unique dual effect on insecticide binding (Zhang *et al*., 1994). The mutation of alanine to serine or glycine has been documented in resistant strains of several insects, including mosquitoes (Thompson *et al*., 1993).

Modifications in the insect cuticle or digestive tract linings that prevent or slow the absorption or penetration of insecticides can be found in some strains of resistant insects. This resistance mechanism can affect a broad range of insecticides (IRAC, 2011). Behavioural resistance describes any modification in insect behaviour that helps to avoid the lethal effects of insecticides. This normally occurs as a result of prolonged exposure of insects to an insecticide. Behavioural resistance does not have the same importance as physiological resistance but might be considered to be a contributing factor, leading to the avoidance of lethal doses of an insecticide (IRAC, 2011).

2.5) Factors that select for insecticide resistance

2.5.1) The role of agriculture

Agriculture is one of the most important economic sectors in Africa; however, exposure of mosquito vectors to agricultural insecticides has been implicated in the selection of resistance in mosquitoes (Diabate *et al*., 2002; Yadouleton *et al*., 2009, 2011). Most chemical classes of insecticides are either used in both agricultural and public health or have similar targets and modes of action (Khambay and Jewess, 2010). In West Africa, the increased rate of pyrethroid resistance and *kdr* mutation frequency were often attributed to the massive agricultural use of DDT and pyrethroids (Chandre *et al*., 1999; Diabate *et al*., 2002; Yadouleton *et al*., 2011). In Benin, it was reported that *A. gambiae* females frequently lay eggs in breeding sites

located around agricultural settings and that larvae from these sites underwent selection pressure from agricultural pesticides, favouring the emergence of resistance (Akogbeto *et al*., 2006).

In addition to pesticides and insecticides, chemicals commonly used in agriculture also include fertilizers, herbicides and fungicides. Although these compounds are usually non-toxic to insects, their presence in breeding sites has been reported to affect insecticide tolerance through the modulation of their detoxification system. For instance, *Aedes albopictus* larvae exposed to some fungicides (e.g. copper sulphate, triadimefon) often used in agriculture showed an increased tolerance to carbaryl and permethrin (Suwanchaichinda and Brattsten, 2001; Poupardin *et al*., 2008). Similarly, exposure of *Aedes aegypti* larvae to the herbicide glyphosate led to a significant increase of their tolerance of permethrin (Riaz *et al*., 2009).

2.5.2) The role of public health

For public health, insecticides are used in highly targeted strategies and persist longer, which allow significant build-up of selection pressure over many generations of vectors. The massive scaling-up of the use of ITM and IRS for malaria control has resulted in an increased selection of pyrethroid resistance in malaria-endemic countries (Trape *et al*., 2009; WHO, 2012). Also, massive use of domestic insecticides such as mosquito coils or aerosol sprays in many households have been reported to represent an additional selective pressure favouring pyrethroid resistance

in urban areas (Elissa *et al*., 1993; Diabate *et al*. 2002; Boakye *et al*., 2009; Kudom *et al*., 2013).

2.5.3) The role of urbanization

Rapid urbanization is mostly associated with increased pollution. Pollutants generated by traffic, industries, and domestic wastes, mostly end up in various water bodies. Urban pollutants are generally not toxic individually at environmental doses but are generally found in mixtures and several of these chemicals have been shown to affect mosquito tolerance to insecticides. This is supported by Suwanchaichinda and Brattsten (2002), who reported that exposure of *Aedes albopictus* larvae to benzothiazole, a major leachate compound of automobile tires, led to an increase in their tolerance to various insecticides. Similarly, exposure of *Aedes aegypti* larvae for 24 h to a sub-lethal dose of the fluoranthene increased their tolerance to permethrin and induced GST and P450 activities (Poupardin *et al*., 2008).

2.5.4) The role of microbial flora

Mosquitoes, like other insects, can harbour a wide range of microorganisms from pathogens to symbionts, which may have variable impacts on their life traits. Microbial flora that resides in the gut of mosquitoes is known to have significant

impacts on the biology and behaviour of the host. Nevertheless, there are not many studies on the relationship between the insecticide resistance status of mosquitoes and their microbial flora. Kikuchi *et al*. (2012) recently reported a novel insecticide resistance mechanism through the acquisition by the host of bacteria able to catabolise insecticides. They demonstrated that fenitrothion-degrading *Burkholderia* strains were able to establish a specific and beneficial symbiosis with *Riptortus pedestris* and confer resistance of the host insects against fenitrothion. Furthermore, experimental applications of fenitrothion to field soils massively enriched fenitrothion-degrading bacteria from undetectable levels to over 80% of the total culturable bacterial counts in the field soils, and more than 90% of *R. pedestris* reared with the enriched soil favouring symbiosis with the insecticide-degrading *Burkholderia* and subsequent insecticide tolerance to occur in the host insects (Kikuchi *et al*., 2012).

2.6) Insecticide resistance management

The simplest form of resistance management is mostly insecticide based, and this could take several forms, such as rotation, combinations, mixtures or mosaic. Successful resistance management depends upon reducing the selection pressure exerted by a particular insecticide or a particular mode of action (IRAC, 2011).

Combined pyrethroid and carbamate mixture or 'two-in-one' treated mosquito nets have been proposed as resistance management tools (Guillet *et al*., 2001). Synergists have also been reported to be capable of delaying control failure, due to

insecticide resistance, in an agricultural setting. Synergists can be defined as compounds that enhance the toxicity of some insecticides, although they usually have limited toxicity themselves. Synergists, including piperonyl butoxide (PBO), S,S,S-tributyl phosphoro-trithioate (DEF), and N-Octyl bicycloheptene dicarboximide (MGK-264), enhance the effect of several classes of insecticide, including the pyrethroids, organophosphates and carbamates. This is achieved by inhibiting the enzymes that metabolise insecticides, P450s and esterases, within the insect. Currently, some ITM (e.g. Permanet® 3.0) contain a mixture of a pyrethroid insecticide and a synergist, PBO to fight pyrethroid resistant-mosquitoes. Permanet 3.0 has been showed to be to more effective against malaria vectors with multiple resistance mechanisms that other ITM treated with single pyrethroid insecticide (Adeogun *et al*., 2012).

2.7) Mosquito borne diseases in Ghana and control

2.7.1) Burden of malaria and other mosquito-borne diseases in Ghana

The major mosquito borne diseases in Ghana are malaria, filariasis and yellow fever. Malaria has been a major cause of poverty and low productivity and the leading cause of workdays lost due to illnesses. In addition, malaria impacts adversely on productivity in all sectors of the economy (MOH, 2008).

Malaria is hyper-endemic in all parts of the country, with the entire population of about 24 million at risk. Transmission occurs all year round with slight seasonal

variations during the rainy season from April to July (MOH, 2008). The seasonal variation is marked in the northern parts of Ghana where there is a prolonged dry season from September to April. In 2006, outpatient malaria cases accounted for 37.5% of all outpatient illnesses, 36% of all admissions and 33.4% of all deaths in children under five years. In that same year, amongst pregnant women it accounted for 13.8% of all OPD attendances, 10.6% of admissions and 9.4% deaths (MOH, 2008). In 2012, over 11 million suspected cases of malaria were recorded and 2,855 people died as result of the disease (WHO, 2013).

The main parasite species causing malaria in Ghana are *P. falciparum* (80-90%), *P. malariae* (20-36%), and *P. ovale* (0.15%). For the past few years, however, malaria recorded was mainly caused by *P. falciparum* (WHO, 2011, 2013). The principal vectors are the *A. gambiae* complex and *A. funestus* (Afrane *et al*., 2004; Anto *et al*., 2009). The groups affected most by malaria are children under five years of age and pregnant women who constitute 20% and 4% respectively of the general population (MOH, 2008).

Yellow fever outbreaks normally recur every ten to twelve years in certain parts of Ghana. *Aedes aegypti, Aedes luteocephalus and Aedes africanus* have been implicated in the transmission of yellow fever in Ghana (Agdzi *et al.*, 1984, Appawu *et al*., 2006). Bancroftian filariasis is also present in most parts of Ghana (Gyapong *et al*., 1996) with about 12 million people requiring preventive chemotherapy. The disease is transmitted by *Mansonia* species (Ughasi *et al*., 2011) and *A. gambiae* and *A. funestus* (*Dunyo et al*., 1996) which are also vectors of malaria. Unlike East Africa and many Asian countries, *C. quinquefasciatus* has not been implicated in the transmission of filariasis in Ghana (Dunyo *et al*., 1996, Dzodzomenyo *et al*., 1999).

2.7.2) Malaria control in Ghana

Malaria control in Ghana dates back to the 1950s and the aim was to reduce malaria burden until it is no longer of public health significance. Interventions applied at the time included residual insecticide application against adult mosquitoes, mass chemoprophylaxis with pyrimethamine medicated salt and improvement of drainage systems (MOH, 2008).

Since 1998, Ghana has been implementing the Roll Back Malaria Strategy (RBM). In the year 2000, a 2000-2010 strategic plan was drawn which gave strategic direction to the Malaria Control Programme. Overall, the Ghana RBM emphasizes the strengthening of health services through multi and inter-sectorial partnerships and making treatment and prevention strategies more widely available. The goal was to reduce malaria-specific morbidity and mortality by 50% by the year 2010. Though Ghana made some progress, there were still gaps in achieving the targets in the previous plan. Lessons learnt from the implementation of the previous strategic plan helped in developing another strategic plan known as 2008-2015 strategic plan for malaria control in Ghana. The aim of this new approach is to give strategic directions in order to attain the goal of reducing the current malaria disease burden by 75% by the year 2015 which is in line with the objectives of the MDG. The plan covers the areas of improving multiple prevention, improving access to prompt and effective treatment, strengthening health systems at all levels, and creating and sustaining partnership. The specific objectives include 1) in 100% of households ownership of at least one ITM, 2) 80% of the general population sleeping under ITMs, 3) the increase of the number of children under five and pregnant women sleeping under treated nets from current levels to 85% (MOH, 2008).

In order to achieve these objectives, access to ITM has been scaled up and as a result, there has been a tremendous increase in the number of households protected by ITM over the past decade. A model predicts that about 40% of the population has access to ITM (WHO 2013). Percentage of households owning at least one ITM increased from 2% in 2000 to 47% in 2010. Besides ITM, IRS is also gradually scaled up in many areas in Ghana and the preliminary result of its impact on malaria control is encouraging. A private mining company (AngloGold Ashanti) initiated IRS activities within its catchment area, the Obuasi Municipal assembly, as part of its comprehensive integrated malaria control program. It is reported that over 74% of malaria cases have been reduced within a period of 2 years in the intervention area which comprises urban and rural communities. The U.S. President's Malaria Initiative (PMI) in Ghana has also implemented IRS operations in five districts in the Northern Region of the country (MOH, 2008).

Although much effort has been carried out to scale up ITM, acceptance and utilization of ITM use still remains a problem and if not properly addressed can jeopardize malaria control efforts in the country. Some reports have shown a significant gap between incidence of ITM use and ITM ownership (Eisele *et al*., 2009; Kudom and Mensah, 2010) and various reasons have been attributed to it. Kudom and Mensah (2010) observed that about 33% of students in the Cape Coast Metropolis that owned ITM did not use it. Few respondents knew or were able to describe different types of mosquitoes but to the majority, any mosquito bite can result in malaria. This implies a lack of understanding why sleeping under ITMs can prevent malaria. In addition, failure of ITM to protect users from nuisance from *Culex* mosquitoes due to a lack of insecticide efficacy against it has been cited as one of the major obstacles

to the sustained use of ITM (Samuelsen *et al.*, 2004). In this regard, for local communities to accept and maintain ITM use, it must be able to protect its users from mosquito bites from both vector and nuisance mosquitoes.

2.7.3) Insecticide resistance profile in Ghana

Insecticide resistance status among malaria vectors is well documented in different parts of Ghana. Varied resistance levels have been observed across different ecological zones, seasons and even different breeding habitats among *Anopheles* species (Yawson *et al.,* 2004; Anto *et al*., 2009; Kabula *et al*., 2011). More so, resistance has been spreading to areas that used to be susceptible. For instance, Kristan *et al.* (2003) found *A. gambiae* completely susceptible to deltamethrin in Tarkwa in 2000, however, by 2010 *A. gambiae* has developed resistance to the insecticide and mortality to deltamethrin was reduced from 100% to about 57% (Hunt *et al*. 2011). Resistance levels also varied between *A. gambiae* and *A. funestus* in places where they co-exist. *A. gambiae* appears to be more resistant to insecticides than *A. funestus*. For instance, in Obuasi mortality to DDT was 30.8 % in *A. gambiae* while *A. funestus* was 60.9%. A similar trend has been observed in different insecticides and different areas in Ghana (Hunt *et al*., 2011; Coetzee *et al*., 2006; Anto *et al.,* 2009).

The impact of ecology and seasons on insecticide resistance has also been assessed among *Anopheles* species. Anto *et al*. (2009) observed a significant

seasonal variation in susceptibility of *Anopheles* species to various insecticides. The study revealed a significant variation in susceptibility during rainy and dry season. For *A. funestus*, dry season susceptibility was 90.5% and that for the wet season was 87.3%. In the case of *A. gambiae*, susceptibility during the dry season was 89.8% and the wet season was 91.4% (Anto *et al*. 2009). Different susceptibility levels according to ecological zones have been reported by different studies in Ghana. Anto *et al*. (2009) concluded that mosquitoes from the Kassena-Nankana District in the Savannah region were susceptible to pyrethroid insecticides (Anto *et al*. 2009) while in the forest and coastal savannah zones resistance seems to be high (Hunt *et al*., 2011, Kudom *et al.,* 2012). Occurrence and distribution of *kdr* mutations, which is a major mechanism responsible for pyrethroid and DDT resistance in the country, appears to support this observation. Different studies have found very low frequencies of *kdr* mutations in the savannah zones in Ghana while high frequencies have been observed in Southern Ghana (Anto *et al*., 2009; Yawson *et al*., 2004). For example, Yawson *et al*. (2004) reported a *kdr* mutation frequency of 0.58% in Bonia and 0% in Korania in the savannah zone in the Northern Region of Ghana whereas a frequency of 100% was recorded in Kumasi in the forest zone.

Resistance levels of *Anopheles* species to different classes of insecticides vary. There is a widespread and relatively uniform distribution of DDT resistance among *Anopheles* species in Ghana. Resistance to pyrethroid, carbamate and organophosphate insecticides varies but *Anopheles* mosquitoes seem to be more susceptible to organophosphates (Hunt *et al.,* 2011; Kudom *et al.,* 2012). Furthermore, resistance to permethrin was more profound than to other pyrethroid insecticides (Anto *et al*., 2009; Adasi *et al.,* 2008).

Excessive use of agricultural pesticides and household use of insecticides have been implicated as the main modifiers on selection pressure in the development of resistance in *Anopheles* species (Boakye *et al*., 2009)

3) Rationale and Objectives

3.1) Rationale of the study

The rationale behind the investigations carried out in this study was the observation made by different studies on the potential impact of *Culex* mosquitoes on malaria control strategies. Strong level of pyrethroid-resistance in *Culex* is reported to represent an obstacle to malaria prevention, as people may not perceive the personal protective effect of ITM if nuisance due to *Culex* is still high (Chandre *et al.*, 1997). Several experimental hut trials have shown near to complete failure of ITM against pyrethroid-resistant *Culex* populations in many African countries (Ngufor *et al.*, 2014, Irish *et al*., 2008). However, there is little information on resistance status of *Culex* mosquitoes in Ghana. Few studies have reported varying resistance levels in *Culex* from Ghana's capital city of Accra and its surrounding towns (Wilding *et al*., 2012; Kudom *et al*., 2013) but the situation in the rest of the country remains largely unknown. *Culex* mosquitoes are the most widely distributed and abundant mosquitoes in Ghana and in order to improve and maintain the use of ITM, there is a need to attain a better understanding of their resistance profile and their impact on ITM usage.

3.2) Purpose of the study

The main objectives of the study were to determine the resistance status of *Culex* mosquitoes to the four classes of insecticide (pyrethroid, organochlorines, organophophate and carbamates) mostly used in public health and evaluate the personal protective efficacy of insecticide treated nets against the pyrethroid-resistant mosquitoes with the following specific objectives:

1. To determine spatial and temporal susceptibility status of *Culex* to the four classes of insecticides in urban towns in Ghana
2. To determine resistance mechanisms among resistant populations of *Culex*, if any.
3. To determine ecological and environmental determinants of resistance in *Culex*.
4. To evaluate the efficacy of different ITM against local pyrethroid-resistant *Culex*.

4) Materials and methods

4.1) Description of the study area

For the investigation presented in this study, mosquitoes were collected from all of the three major ecological zones in Ghana, namely coastal savannah, forest, and Guinea savannah, and each ecological zone (Figure 4.1.1), three large urban areas (based on the size of human population) were selected. In each urban area, mosquito larvae were sampled from three different land use settings: urban agricultural area, residential area and marshy or swampy area.

In an urban agricultural area, *Culex* larvae were sampled from water impoundments and other water bodies on vegetable farms, which are mostly used for irrigation. In the residential area category, *Culex* larvae were sampled exclusively from choked gutters, mostly found at the central part of the towns. The gutters were highly polluted with organic materials and surrounded by residential and commercial areas. Lastly, in the marshy or swampy areas, *Culex* larvae were sampled from low-lying waterlogged areas, often beside a water body that is poorly drained and liable to flood, or from slow-moving water bodies.

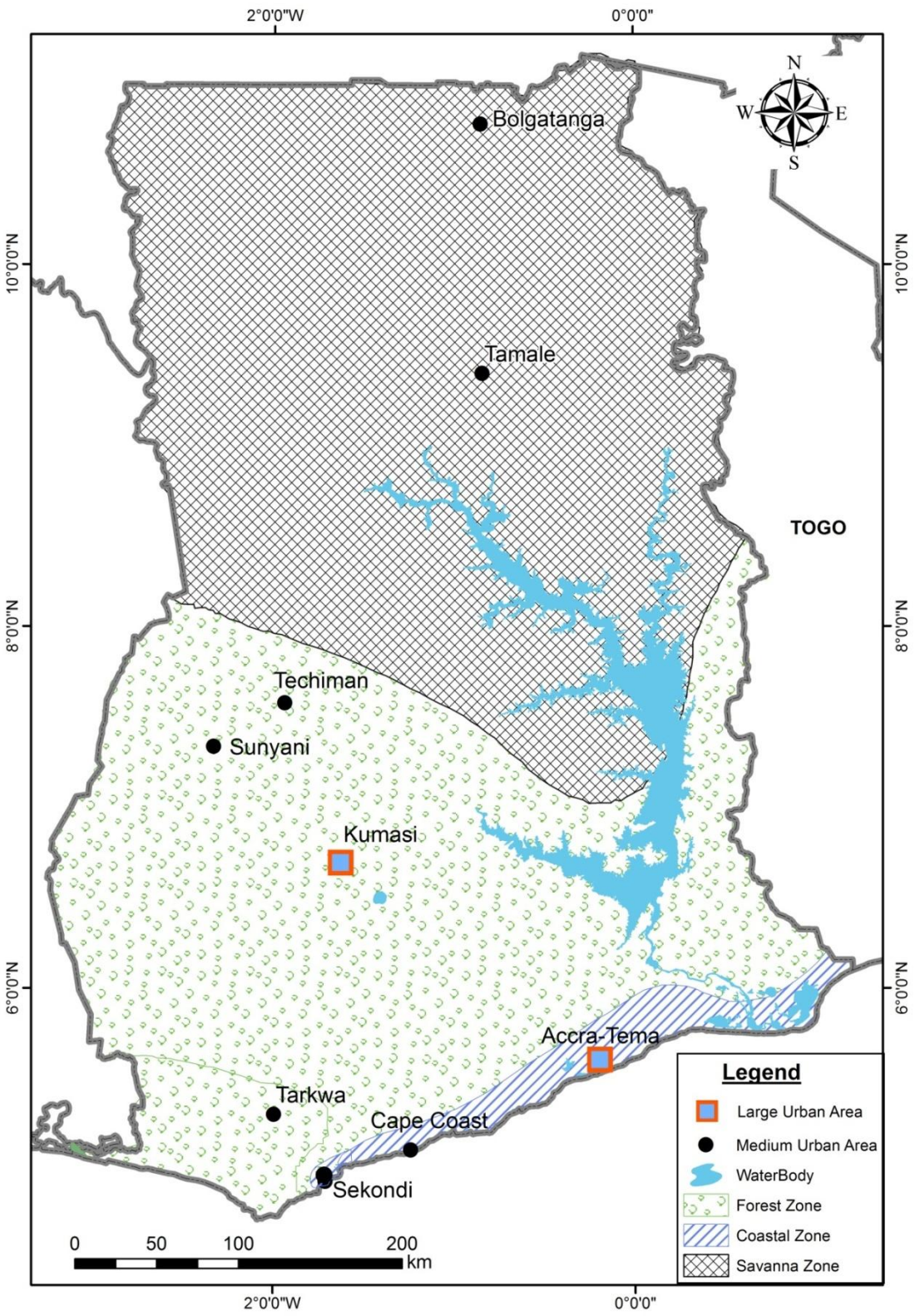


Figure 4.1.1: Map of Ghana showing the three ecological zones and urban towns where mosquitoes were collected.

4.2) Mosquito collection

List of materials used for mosquito collection

350 ml dipper	5000 ml plastic containers	Plastic trays (5 cm x 27 cm x 36 cm)
150 ml plastic disposable cups	25 cm^2 plastic mosquito cages	Pasteur pipettes

Larval collections were carried out from February 2012 to December 2012 in 9 urban areas of Ghana in both dry and rainy seasons. Larvae were sent to the laboratory for emergence. In each town (Table 4.2.1), larval survey was carried out on foot to locate mosquito breeding sites under the three categories of land use settings (Figure 4.2.1). *Culex* larvae were identified by their angular position on the surface of water (Figure 2.1.1.1). The larvae were collected with a 350 ml dipper and transferred into a 5000 ml plastic container and sent to the laboratory for emergence.

Once in the laboratory, the water with the mosquito juveniles was poured into plastic trays (5 cm x 27 cm x 36 cm) to a depth of 2 cm and each tray labelled according to the site and date of collection. The trays containing mosquito larvae were kept at 27-30 ºC and 76 ± 5% relative humidity. The larvae were fed with ground “Nutrafin” goldfish food (Rolf Hagen, USA). The development of the larvae were monitored regularly and all those that pupated were collected into a 150 ml disposable plastic cups with water using Pasteur pipette and then placed in a labelled cage for adult emergence. All emerged mosquitoes were fed on 10% sugar solution imbibed in cotton wool. Only female mosquitoes were used for the experiments. The female

mosquitoes that were later used for biochemical and PCR assay were stored in freezer (- 80 ºC).

In a single study site, about 50 female mosquitoes were used for the biochemical assay, 20 female mosquitoes for PCR assay and 150 female mosquitoes for susceptibility assay per insecticide per season (Figure 4.2.2).

A

B

C

Figure 4.2.1: Pictures showing different land use settings where mosquito larvae were collected (A- residential area; B- swampy area; C- urban agricultural area)

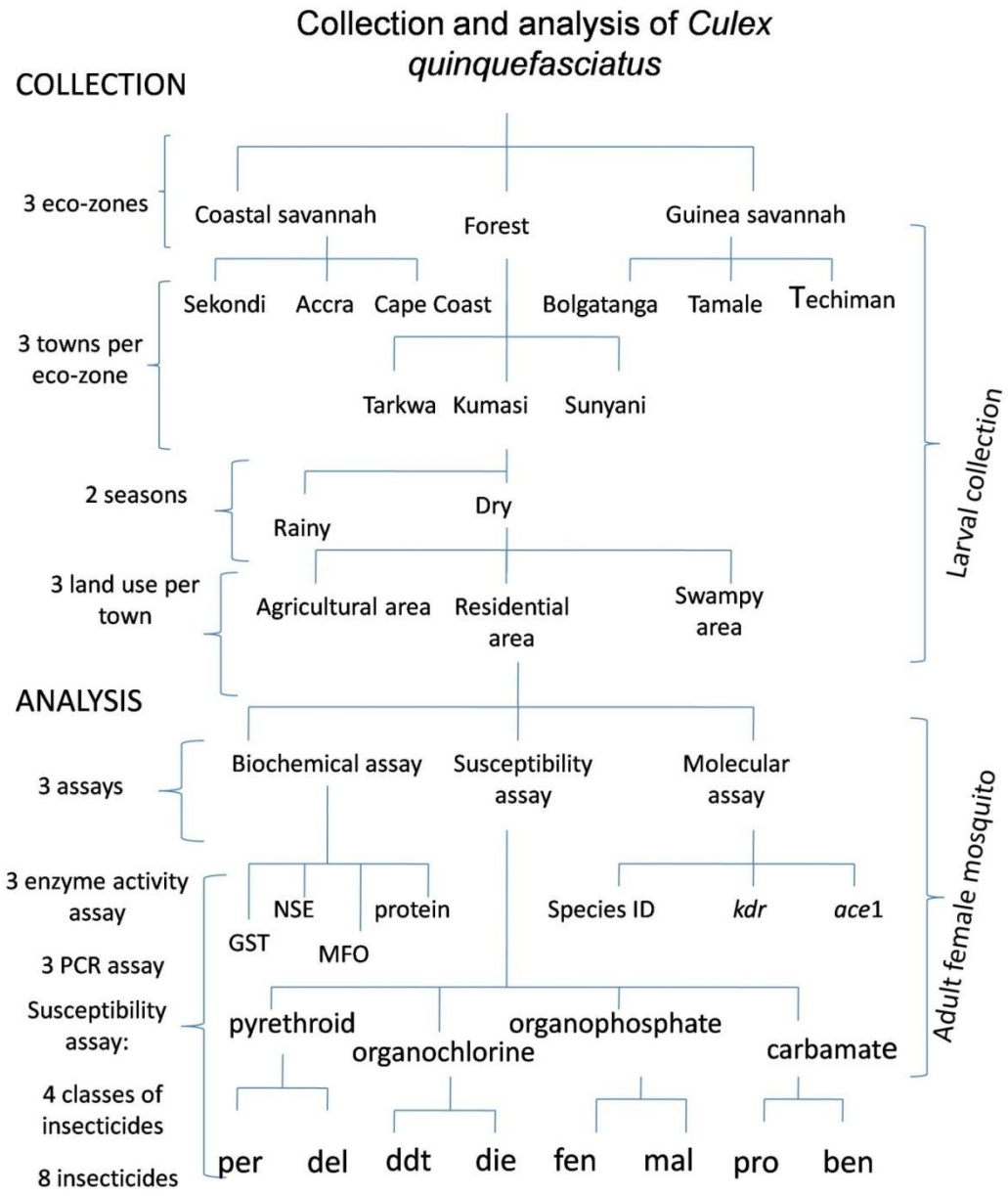


Figure 4.2.2: A flow chart showing the study design in the assessment of resistance status of *Culex* mosquitoes from Ghana. (GST- Glutathione-S-Transferase, NSE – non- specific esterase, MFO – mixed function oxidase; per – permethrin, del – deltamethrin, ddt – dichlorodiphenyltrichloroethane, die – dieldrin, mal – malathion, fen – fenitrothion, pro – propoxur, ben – bendiocarb).

Table 4.2.1: Ecological zone, urban areas, land use settings and GPS location or names of places where mosquito larvae were collected

Ecological zone	City	Residential area	Swampy area	Urban agricultural area
Coastal savannah	Accra	5.57473; -0.24994	-	Korle Bu
	Cape Coast	5.10234; -1.27883	5.10743; -1.29711	-
	Sekondi-Takoradi	4.93477; -1.72299	4.91309; -1.75322	4.904518; -1.771765
Forest zone	Kumasi	6.77819; -1.60643	6.71672; -1.65170	6.68254; -1.64022
	Sunyani	7.33680; -2.37097	7.33547; -2.37536	-
	Tarkwa	5.30246; -1.98793	5.30993; -1.98271	-
Savannah zone	Techiman	7.58541; -1.93786	-	-
	Tamale	9.40901; -0.83981	9.38657; -0.837096	-
	Bolgatanga	STC	10.76727; -0.85739	-

4.3) Susceptibility bioassay

List of materials used for susceptibility assay

WHO Insecticide impregnated papers	Permethrin (0.75 %)	Deltamethrin (0.05 %)	DDT (4.0 %)
	Dieldrin (4.0 %)	Bendiocarb (0.1 %)	Propoxur (0.1 %)
	Fenitrothion (1.0 %)	Malathion (5.0 %)	
WHO susceptibility test kit	aspirator	mosquito cages 25 cm x 25 cm x 25 cm	

The WHO insecticide susceptibility bioassay is a simple direct response-to-exposure test. Mosquitoes are exposed to known concentrations of an insecticide for a fixed period of time and at the end of which the number of fatalities is recorded. The test is designed to distinguish between baseline susceptibility and resistance to insecticides in adult mosquitoes.

In the present study, adult susceptibility assay was performed according to WHO guidelines (WHO, 2012). A clean sheet of white paper (12 cm x 15 cm), rolled into a cylinder shape, was inserted into a holding tube (Figure 4.3.1) and fastened into position with a steel spring-wire clip. The tube was attached to a slide. About 20 - 25 active female mosquitoes were aspirated in batches from the mosquito cage into the holding tube through the filling hole in the slide. Once the mosquitoes were transferred, the slide unit was closed and the holding tube set in an upright position for 30 minutes. At the end of this time, any damaged insects were removed. An exposure tube was prepared in much the same way. The exposure tube was lined with a sheet of insecticide-impregnated paper, which was fastened into position with

a copper spring-wire clip. The empty exposure tube was attached to the vacant position on the slide and with the slide unit mosquitoes were opened and blown gently into the exposure tube. Once all the mosquitoes were in the exposure tube, the slide unit was closed and the holding tube was detached and set to one side. Mosquitoes were kept in the exposure tube for a period of time recommended by WHO (Table 4.3.1). At the end of the recommended exposure period, the mosquitoes were transferred back to the holding tube. The exposure tube was detached from the slide unit. A pad of a cotton-wool soaked in sugar water (10 % sucrose) was placed on the mesh-screen end of the holding tube. Mosquitoes were maintained in the holding tube for 24 h (the recovery period). Temperature and humidity were recorded during the recovery period.

Exposure tubes for both permethrin and deltamethrin were held flat so that mosquitoes that were knocked down remained in contact with the paper during the entire period. New insecticide paper was used for new locations and each paper was not used more than six times. Each test of a batch of 20 – 25 mosquitoes was replicated four to five times and tests with untreated paper were run in parallel as a control. All tests were done with two to four day old, not blood-fed, female mosquitoes.

At the end of recovery period (i.e. 24 h post-exposure), the number of dead mosquitoes was counted and recorded. A mosquito was classified as dead if it was immobile or unable to stand or fly in a coordinated way. The mortality of test sample was calculated by summing the number of dead mosquitoes across all four exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes:

$$\text{Mortality} = \frac{\text{total number of dead mosquitoes}}{\text{Total sample size}} \times 100\ \%$$

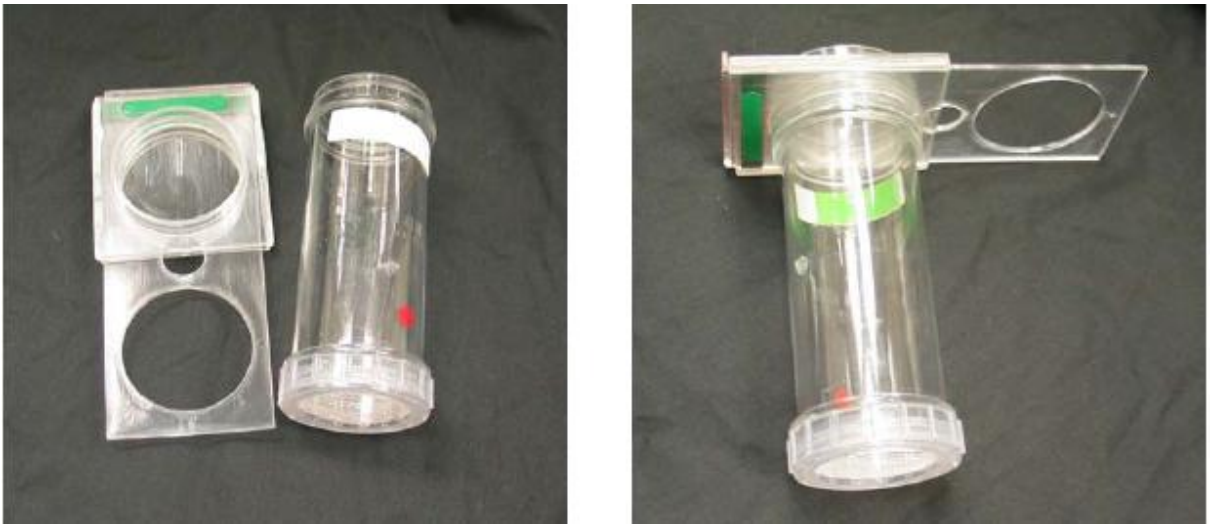


Figure 4.3.1: WHO susceptibility testing tube with a red dot for use as exposure tubes (source: MR4)

Table 4.3.1: Different classes of insecticides used in the study and WHO recommended time of exposure of the mosquitoes to the insecticides

Class of insecticide	Insecticide	Exposure time (h)
Pyrethroid	Permethrin	2
	deltamethrin	2
Organochlorines	DDT	4
	Dieldrin	1
Organophosphate	Malathion	1
	Fenitrothion	2
Carbamates	Bendiocarb	1
	Propoxur	2

4.4) Molecular analyses

4.4.1) DNA extraction

List of materials used for DNA extraction

DNeasy® extraction kit	vortex	centrifuge
pipettes and pipette tips	thermo mixer	pestle

Genomic DNA was extracted with DNeasy extraction kit (Qiagen®), according to the protocol provided by the manufacturer (DNeasy handbook). A whole mosquito was placed in a 1.5ml micro centrifuge tube and 180 µl Buffer ATL was added. The mosquito was homogenized with plastic pestle and 20 µl proteinase K was added. The sample was incubated at 56 °C in a thermo mixer until the mosquito was completely lysed. The sample was vortexed for 15 seconds and about 200 µl buffer

AL was added to the sample and mixed thoroughly by vortexing. Then, 200 µl ethanol (98 %) was added and thoroughly mixed again by vortexing. The mixture was transferred into the DNeasy Mini spin column placed in a 2 ml collection tube with a pipette. The mixture was then centrifuged at 8000 rpm for 1 min. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2 ml collection tube, 500 µl buffer AW1 was added, and centrifuged for 1 min at 8000 rpm. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in another new 2 ml collection tube, 500 µl buffer AW2 was added, and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed in a clean 2 ml micro centrifuge tube and 200 µl buffer AE pipetted directly onto the DNeasy membrane. It was then incubated at room temperature for 1 min, and then centrifuged for 1 min at 8000 rpm to elute. This procedure was repeated for about 20 mosquitoes each from the nine urban sites.

4.4.2) Species identification and detection of *kdr* and *ace*1 mutation

List of materials used for PCR assay

Thermo cycler (Takara®)
Primers (Table 4.3.2.1)
Mupid 2 plus® mini-gel system
0.2 ml micro tubes
Agarose powder
TAE buffer {Tris base, acetic acid in EDTA (Ethylenediaminetetraacetic acid)}
Double distilled water
Ethidium bromide
DNTP (deoxynucleoside triphosphate)
Taq polymerase (HotstarTaq: Qiagen®)

Molecular analysis was carried out in the laboratories of the Noguchi Memorial Institute for Medical Research (NMIMR). Mosquitoes for this study were mostly collected from polluted water bodies; hence *C. quinquefasciatus* was generally expected. Notwithstanding, there was a possibility of other *Culex* species existing alone or co-habiting with *C. quinquefasciatus* in the polluted breeding sites of which I was not able to distinguish morphologically. Therefore, PCR diagnostic assay were carried out targeting the two most populous *Culex* species in Ghana namely *C. quinquefasciatus* and *C. decens.*

Firstly, two primers ("ACEquin"and"B1246s") (Table 4.4.2.1) were used to amplify a 274 bp diagnostic fragment of the entire extracted DNAs and the procedure followed what was described by Smith & Fonseca (2004). This was done to identify *C. quinquefasciatus.* Another PCR assay was conducted with two primers (“F1457” and

"B1246") on the DNAs that fail to amplify in the previous assay. This was also conducted as described in Smith & Fonseca (2004). This was conducted to identify a possible sibling species of *C. quinquefasciatus* and other species that belong to *C. pipiens* complex.

Secondly, two primers (Cddir and Cdrev) (Table 4.4.2.1) were designed from *C. decens* cytochrome c oxidase subunit 1 (CO1). *C. decens* CO1 gene sequence was obtain from Genebank (Uniprot.org) with accession number Q5GCL4. The primer was design with Primer 3® software. PCR was conducted with Cddir and Cdrev on the DNAs that have failed to amplified in the previous two PCR assay.

Fragment size analysis was done by electrophoresis on a 2 % agarose gel and visualised by ethidium bromide staining under UV light. PCR was conducted in 20 µL volumes containing 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate (dNTP), 0.15 mg/mL of bovine serum albumin, 0.4 µM of each primers, one unit of Taq polymerase (HotstarTaq: Qiagen®), and 3 µl of genomic DNA. The amplification condition consisted of one cycle of denaturation at 95 °C for 15 minutes, followed by 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for one minute each, and one cycle of final extension at 72°C for five minutes.

Lastly, the universal DNA primers, LCO1490 and HCO2198 (Table 4.4.2.1) of Folmer *et al*. (1994) were used to amplified an 830 bp region of the mitochondrial cytochrome oxidase subunit I gene of four mosquitoes randomly selected from the mosquitoes that have not been amplified yet from the previous PCR assays. Each PCR contained 5 ml of 10 PCR buffer, 1.5 mM of $MgCl_2$, 35 ml of distilled water, 200

mM of each dNTP, 1 unit of Taq polymerase, 0.3 mM of each primer and 3 µl of DNA template. The PCR thermal conditon consisted of one cycle of 1 min at 94 °C; five cycles of 1 min at 94 °C, 1.5 min at 45 °C and 1.5 min at 72 °C; 35 cycles of 1 min at 94 °C, 1.5 min at 50 °C and 1 min at 72 °C and a final cycle of 5 min at 72 °C. To confirm a successful reaction, a 7 µl sample from each reaction was then run via electrophoresis through a 2 % agarose gel with ethidium bromide and visualized using ultraviolet (UV) light.

The four PCR products amplified by the universal DNA primers and two PCR products that were amplified by *C. decens* primers were purified using a QIAGEN QIAquick® PCR purification kit according to the manufacturers protocol and sequenced in both forward and reverse directions on an ABI 377 automated sequencer (Applied Biosystems) using the Big Dye v. 3 sequencing kit.

Detection of *kdr* mutations was performed as described by Martinez-Torres *et al.* (1999). Four primers, “Cdg1”, “Cdg2”, “Cgd3” and “Cdg4” were used for the PCR assay (Table 4.4.2.1) and two PCR reactions were run in parallel. In one reaction, the primers Cgd1, Cgd2 and Cgd3 were combined and, the other one, Cgd3 was replaced by Cgd4. The PCR reactions was conducted in a 20 µL volume containing 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 µM of each primer, one unit of Taq polymerase (HotstarTaq: Qiagen®), and 3 µl of genomic DNA. The amplification condition consisted of one cycle of denaturation at 95°C for 15 minutes, followed by 29 cycles at 94 °C for one minute, 49 °C for two minutes, 72 °C for two minutes each, and one cycle of final extension at 72 °C for 10 minutes according to Sarkar *et al*. (2009). Fragment size analysis was done by

electrophoresis on a 2 % agarose gel and visualised by ethidium bromide staining under UV light.

For detection of *ace*1 mutation in this study, 3 µl of genomic DNA was amplified with the primers “Moustidir1” and “Moustrev1” (Table 4.4.2.1). The primers generate a 194 bp fragment by PCR on genomic DNA, which is cut by them *Alu*1 restriction enzyme only in resistant mosquitoes (Weill *et al*., 2004)

PCR was conducted in 25µL volumes containing 1× PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 3.1 µL of each of the primers, one unit of Taq polymerase (HotstarTaq: Qiagen®) (Table 4.4.2.2). The PCR conditions included an initial denaturation step at 95 °C for 15 min followed by thirty five cycles of 94 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes.

Fifteen µL of the PCR product were digested with 5 units of *Alu*1 restriction enzyme in the final volume of 25 µL and incubated at 37 °C for 16 h. The restriction products were fractionated on a 2 % agarose gel and visualised by ethidium bromide staining under UV light.

Table 4.4.2.1: Oligonucleotides (primers) used in the molecular analysis

Type of PRC assay	Primers
Species identification	ACEquin 5'-CCTTCTTGAATGGCTGTGGCA-3'
	B1246s 5'-TGGAGCCTCCTCTTCACGG-3'
	F1457 5'-GAGGAGATGTGGAATCCCAA-3'
	B1246 5'-TGGAGCCTCCTCTTCACGGC-3'
	Cddir 5'-ACCTCGACGATACTCCGATTT-3'
	Cdrev 5'-TGTGTTCTGCAGGAGGAAGA-3'
	LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3'
	HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'
Detection of *kdr* mutation	Cdg1 5'-GTGGAACTTCACCGACTTC-3'
	Cdg2 5'-GCAAGGCTAAGAAAAGGTTAAG-3'
	Cgd3 5'-CCACCGTAGTGATAGGAAATTTA-3'
	Cdg4 5'-CCACCGTAGTGATAGGAAATTTT-3'
Detection of *ace*1 mutation	Moustdir1 5′-CCGGGNGCSACYATGTGGAA-3′
	Moustrev1 5′-ACGATMACGTTCTCYTCCGA-3′

Table 4.4.2.2: Preparation of the master mix for the different PCR assay

Reagents	Volume of reagents used for the PCR assay (µl)		
	Species identification	*kdr* detection	*ace*1 detection
Double distilled H_2O	13.2	12.8	12.9
10xPCR buffer	2	2	2.5
dNTP (10mM)	0.5	0.8	0.2
Primer (10mM)	0.4 each	0.4 each	3.1 each
Bovine serum albumin	0.3	-	-
Taq polymerase (5U/µL)	0.2	0.2	0.2
Genomic DNA	3	3	3
Total volume	20	20	25

4.5) Biochemical assays

List of materials used for biochemical assay

1.5 ml tubes	Micro plate-reading spectrophotometer
Plastic reusable pestles	Analytical balance
Pipettes and tips	Bottles of various sizes
Multichannel pipettes	Graduated cylinders
Forceps	pH meter
Timer	Laboratory stirrer
Micro plates	
Dibasic potassium phosphate	3,3',5,5'-Tetramethyl-Benzidine Dihydronchloride
Monobasic potassium phosphate	Methanol
Sodium acetate (NaOAc)	Cytochrome-C
α-naphthyl acetate	Reduced glutathione
ß-naphthyl acetate	1-chloro-2,4'-dinitrobenzene
Acetone	3% hydrogen peroxide
α-naphtol	Protein dye concentrate (Bio-rad)
ß-naphtol	Bovine serum albumin
Dianisidine	

The presence of enzyme activities relevant to insecticide-resistance is often performed using micro plate assays. This method is used to sample populations to determine if a specific mechanism is present and at what frequency it occurs. In the present study, Mixed Function Oxidase (MFO), Non-Specific Esterase (NSE), Glutathione-S-Transferase (GST) and protein were assayed in individuals 2–4 days old frozen adults (-80^{o}C) that had been reared from larvae and not previously

exposed to insecticides in the laboratory, according to the method described by Hemingway (1998). A total of about 450 mosquitoes, comprising 50 female individuals from each of the nine study sites were used for the biochemical assay.

***Reagent preparation*:**

0.25 M Potassium Phosphate Buffer [KPO4]

A glass beaker with 800 ml distilled water was placed on a laboratory stirrer and 6.6 g dibasic potassium phosphate was added.Then,1.7 g monobasic potassium phosphate was also added and the pH was adjusted to 7.2 with one of the potassium phosphates. The final volume was adjusted to 1000ml with addition of distilled water. The solution was stored at room temperature

Sodium Acetate Buffer [NaOAc]

A glass beaker with 900 ml of distilled water was placed on a laboratory stirrer and 83 ml 3M sodium acetate added. The pH was adjusted to 5 with glacial acetic acid. The final volume was also adjusted to 1000ml with addition of distilled water. The solution was stored at room temperature.

α- or ß-naphthyl acetate

- 56 mg α- or ß-naphthyl acetate was dissolved in 20 ml acetone
- 80 ml KPO_4 was then added to the solution

Dianisidine

- 100 mg 0-dianisidine tetrazotized was weighed and added to 100 ml distilled H_2O. The solution was immediately used

TMBZ solution

- Dissolve 20 mg 3, 3', 5, 5'-Tetramethyl-Benzidine Dihydronchloride was dissolved in 25 ml methanol.
- 75 ml 0.25 M Sodium Acetate, (pH 5.0) buffer.

GST solution

- 61 mg reduced glutathione was dissolved in 100 ml KPO4 buffer.
- 20 mg 1-chloro-2, 4'-dinitrobenzene (cDNB) was dissolved in 10 ml acetone.
- 90 ml 0.25 M KPO4 buffer was added to the solution

Protein dye reagent

- 20 ml protein dye concentrate (Bio Rad) was added to 80 ml dH_2O

***Mosquito preparation*:**

A single mosquito was placed in 1.5 ml tube and 100µl of KPO4 was added. The mosquito was homogenized with a plastic pestle. The final volume of the solution was adjusted to 1500 µl by the addition of KPO4. The solution was then centrifuged at 13,000 rpm (4 ^{o}C) for 30 seconds and place on ice for the tests (assays). This was repeated for all the individual mosquitoes used for the assay. The test was conducted at room temperature.

Each mosquito extract was loaded into micro plate wells in duplicate on the same plate for each enzyme assay. A new pipette tip was used for each sample. The micro plate consisted of 96 wells. Thus, 40 samples were loaded on each plate and the remaining 16 wells were loaded with both positive and negative controls. A plate-

reading spectrophotometer was used to collect data at the appropriate absorbing wavelength (nm).

4.5.1) Non-Specific Esterase microplate assay

Exactly, 100 µl mosquito homogenate was loaded in a micro plate wells and 100 µl α- or β-naphthyl acetate added to it. The mixture was incubated at room temperature for 10 minutes. Then, 100 µl dianisidine (dissolved in water) was added to the mixture and incubated for 2 minutes. The plate was then read using 620 nm filter for α-naphthyl and 540 nm filter for β-naphthyl.

4.5.2) Mixed Function Oxidase micro plate assay

In a micro plate well, 100 µl mosquito homogenate was loaded in duplicate and 200 µl of Tetramethyl-Benzidine (dissolved in sodium acetate buffer) added. A drop (approximately 25 µl) of 3% hydrogen peroxide was added and incubated for 5 minutes. The plate was then read using a 620 nm filter.

4.5.3) Glutathione S-Transferase microplate assay

Mosquito homogenate measuring, 100 µl mosquito homogenate was loaded in micro plate wells in duplicate and 100 µl of reduced glutathione added. Then, 100 µl 1-chloro-2, 4'-dinitrobenzenewas added. Plate was read immediately (T0) and again at 5 minutes (T5) using 340 nm filters. Final absorbance was determined by subtracting the T0 reading from the T5 reading. Glutathione S-Transferase in nmol of the mosquito samples was determined using Beer's law.

4.5.4) Protein assay

Mosquito homogenate measuring, 20 µl was loaded in micro plate wells in duplicate and 80µl of potassium phosphate buffer added. Then, 200 µl of protein dye reagent (bio rad) added and plate was read immediately using 620 nm filter.

4.6) Evaluation of insecticide treated nets against different pyrethroid-resistant mosquitoes

List of materials used for cone and tunnel bioassay

WHO cone	Glass tunnel	aspirator
Permanet 3.0®	LifeNet®	

Mosquito strains:

A. gambiae VKPER is a laboratory pyrethroid resistant strain, originally from the Kou Valley in Burkina Faso which has been selected for permethrin resistance and *kdr* mutation is exclusively responsible for the resistance.

C. quinquefasciatus were collected as larvae from polluted gutters in two suburban of Accra (Kaneshie and East Legon) and reared to adult stage in the laboratory. These mosquito populations are resistant to permethrin and deltamethrin.

C. decens were also collected as larvae from Cape Coast and reared to adult stage in the laboratory. This mosquito population is completely susceptible to permethrin but reduced susceptibility to deltamethrin

4.6.1) WHO cone test bioassay

Residual effect of an insecticide is an important characteristic to be considered when evaluating the efficacy of ITM. It is determined by placing a WHO plastic cone to an

insecticide-treated substrate (Figure 4.6.1.1), inserting mosquitoes through the hole at the top of the cone and closing the hole with a polyethylene plug (mosquitoes do not normally rest on the plastic cone or polyethylene plug and therefore mostly remain in contact with the treated substrate (WHO, 2006).

In this study, WHO cone bioassay test was carried out at NMIMR to evaluate efficacy of two new ITM, Permanet® 3.0, and LifeNet®. These two nets are among the popular brands in Ghana. Three field populations of *Culex* mosquitoes and a laboratory strain of *A. gambiae* VKPER were used for the experiment. Permanet 3.0 and LifeNet are deltamethrin-treated ITMs; however Permanet 3.0® had an additional synergist (PBO) on the roof of the net. A piece of netting measuring about 55 cm by 21 cm was cut from three parts of each of the nets; roof top, upper part of the side net and lower part of the side net. Four WHO cones were attached to each of the cut portions and about 5 mosquitoes exposed to the net for 3 minutes. Knockdown and final mortality was recorded at 1 h and 24 h respectively after exposure and the bioassay was repeated once.

Figure 4.6.1.1: WHO cone fixed on insecticide treated netting to determine the residual activity of the formulated insecticide on the net

4.6.2) Tunnel test bioassay

The tunnel test is a laboratory system designed to allow mosquitoes to freely express behavioural responses to insecticide that would occur when encountering ITMs under field conditions. Tunnel tests are used as a forerunner to experimental hut trials, and provide useful information on repellency, blood-feeding inhibition and mortality (WHO, 2006). The equipment consists of a 4-sided glass cylinder (25 cm high, 25 cm wide, 60 cm long) which is divided into two chambers by a netting insert which slots across the tunnel (Figure 4.6.2.1). In one of the chambers, an animal bait (usually Guinea pig or pigeon) is housed unconstrained in an open meshed cage and in the

other chamber about 100 unfed female mosquitoes aged 2-5 days are released at dusk and left overnight. The netting is deliberately holed with nine 1cm holes to give mosquitoes opportunity to penetrate into the baited chamber. The following morning, the number of mosquitoes alive or dead, fed or unfed in each chamber is scored.

Three indicators are used to assess the efficacy of a netting material in a tunnel test: passage, inhibition of blood-feeding and mortality. These indicators are calculated relative to the untreated netting (control) with respect to the three criteria; 1) passage (entry rate) - is the total number of female mosquitoes that are able to cross the netting material to the chamber that contains the animal bait. 2) The blood-feeding rate - is the proportion of blood fed female mosquitoes compared with the total number released into the tunnel. The reduction in the number of blood fed mosquitoes between a treatment tunnel (tunnel with treated netting) and a control tunnel (the tunnel with untreated netting) allows an assessment of the blood-feeding inhibition caused by the insecticide.3) The mortality rate - is the proportion of female mosquitoes found dead in the tunnel after and 24 h later. The difference in mortality between a control tunnel (natural mortality) and a treated tunnel allows assessment of the insecticide-induced mortality rate. If a treatment deters a significant number of mosquitoes from entering the baited chamber, the values given by proportions blood-feeding or killed in the treatment tunnel may underestimate the full personal protective effect. The personal protective effect of an ITM in a tunnel test study is determined by the reduction in the number of blood-fed mosquitoes in the treatment tunnel relative to the number blood fed in the control tunnel. It may be estimated using the following formula and expressed as a percentage: 100 x (Bc – Bt)/Bc, where Bc is the total number blood-fed in the control tunnel and Bt is the total number

blood-fed in the treatment tunnels. The overall insecticidal effect of an ITM in a tunnel test needs to take into account that significant numbers which were deterred and not killed by the treatment (WHO, 2006).

In this study tunnel test, a piece of netting measuring about 25cm^2 was cut from three parts of each of the nets; roof top, upper part of the side net and lower part of the side net. Nine holes of 1cm^2 each were deliberately made in the nettings to give mosquitoes opportunity to penetrate into the baited chamber. In one of the chambers, a Guinea pig was housed unconstrained in an open meshed cage and in the other chamber 100 unfed female mosquitoes aged 3-5 days were released at dusk and left overnight (6pm to 6am).The following morning, the number of mosquitoes alive or dead, fed or unfed in each chamber was scored. Live mosquitoes were given access to sugar solution and kept up to 24 h for final mortality.

Figure 4.6.2.1: Glass tunnel use in tunnel bioassay to evaluate insecticide treated net in the laboratory

4.7) Statistical analyses

Percentage mortality was calculated from the results of the susceptibility assays. Percentage mortalities from different land use and ecological zones and seasons were compared using a non-parametric test (Kruskal-Wallis and Mann Whitney test). Study sites were categorized into large urban area (metropolitan area with several sub-metro and human population over one million) and small urban area (a

metropolitan or municipal area with human population less than one million) as well as percentage mortality into resistant (mortality over 98 %) and susceptible (mortality less than 98 %) based on WHO criteria (WHO, 2012). Correlation was used to test the association between percentage mortality and results from biochemical assay or resistance status and urban size. Results from the esterase assay were compared between study sites with ANOVA and post hoc test using Fisher's Least Significant Difference. A binary logistic regression analysis was carried out to determine environmental factors that influence resistance status of *Culex* mosquitoes in urban areas of Ghana using the environmental factors ecology, seasons, urban size, land use and type of pyrethroid insecticide as predictors. Data from tunnel tests were compared between the two ITM, using Chi-square test. All the tests were done with SPSS® (version 20) and Stalcac, EpiInfo™.

5) Results

5.1) Insecticide susceptibility of *Culex* mosquitoes to different insecticides

A total of 21,396 mosquitoes from nine urban towns were tested for resistance against the eight insecticides (DDT, dieldrin, permethrin, deltamethrin, propoxur, bendiocarb, fenitrothion and malathion). Insecticide resistance status of *Culex* was successfully determined in 19 study sites comprising nine residential areas, seven swampy areas and three urban agricultural areas across the country.

Culex from two out of the three urban agricultural areas was completely susceptible to permethrin. Similarly, *Culex* populations that were collected in swampy areas were all completely susceptible to permethrin except swampy areas in Kumasi and Sunyani, which both recorded a mortality of 97 % (Table 5.1.1). With regards to the residential area category, permethrin-resistant populations were observed in five out of the nine urban towns where *Culex* were collected. Very low percentage mortality of the mosquito populations against permethrin were observed in residential area category in Kumasi (40 %), Accra (47 %), and Tamale (54 %) whereas complete mortality (100 %) was observed in the Cape Coast, Tarkwa and Bolgatanga.

Out of the 19 study sites, deltamethrin resistant populations were observed in 17 study sites. Mosquito population from swampy area in Sekondi was completely susceptible to deltamethrin and the population from residential area in Bolgatanga also recorded a mortality of 98 %.

In total, mortality caused by permethrin, 91.83 % ± 18.35 was significantly different from the mortality caused by deltamethrin, 68.76 % ± 28.01 S.D. (Mann Whitney U: $p < 0.0001$). Furthermore, percentage knockdown (90.7 %) and final mortality (91.4 %) were similar in mosquitoes exposed to permethrin while percentage knockdown (83.1 %) was significantly different from the final mortality (68.76 %) in mosquitoes exposed to deltamethrin (Figure 5.1.1)

Culex also displayed large variation in resistance to organophosphates and carbamates across the country and even in the same urban area; however the total mosquito populations that were exposed to carbamates had a mortality of 94.1 % ± 15.4 whereas mortality cause by organophosphates was 99.5 % ± 2.2 and the difference in the two mortalities was significant (Mann Whitney U: $p < 0.0001$). Besides Accra and Kumasi, which recorded a mortality of 99 % and 98 % respectively, the rest of the study sites were completely susceptible (100 % mortality) to malathion and fenitrothion. Unlike the pyrethroid insecticides, differential resistance was neither observed between propoxur and bendiocarb (carbamates) nor malathion and fenitrothion (organophosphates) (Figure 5.1.2).

Besides Tarkwa where complete mortality (100 %) to DDT was observed in the population from swampy area and 98 % in residential area, the rest of the study sites in the entire country recorded mortalities less than 98 % (Table 5.1.2). In addition, only Accra, Kumasi and Tamale recorded less than 98 % mortality against dieldrin, the rest of the study recorded 100 % mortality (Figure 5.1.2).

Table 5.1.1: Percentage mortality ± SD of *Culex* mosquitoes to permethrin (0.75 %) and deltamethrin (0.05 %) at different ecological and land use settings in urban areas in Ghana (exposure time: 2 h).

Ecological Zone	Urban town	% Mortality: Permethrin*			% Mortality: Deltamethrin*		
		Residential Area	Swampy Area	Urban farm	Residential Area	Swampy area	Urban farm
Coastal savannah	Accra	47 ± 5 (86)	-	100 (98)	56 ± 29 (90)	-	79 ± 13 (74)
	Cape Coast	100 (121)	100 (114)	-	67 ± 25 (110)	89 ± 5 (83)	-
	Sekondi	84 ± 3 (98)	100 (84)	100 (100)	60 ± 13 (108)	100 (78)	57 ± 23 (96)
Forest	Kumasi	40 ± 21 (97)	97 ± 3 (95)	89 ± 9 (94)	11 ± 19 (112)	37 ± 9 (85)	58 ± 17 (84)
	Sunyani	96 ± 6 (98)	97 ± 2 (97)	-	49 ± 26 (95)	44 ± 17 (92)	-
	Tarkwa	100 (64)	100 (86)	-	91 ± 4 (64)	95 ± 8 (71)	-
Guinea Savannah	Techiman	100 (100)	-	-	91 ± 8 (100)	-	-
	Tamale	54 ± 5 (70)	100 (100)	-	41 ± 4 (88)	84 ± 6 (96)	-
	Bolgatanga	100 (74)	100 (100)	-	98 ± 3 (63)	89 ± 5 (98)	-

*Values in bracket represent sample size.

Table 5.1.2: Percentage mortality of *Culex* mosquitoes to DDT (4 %) at different ecological and land use settings in urban areas in Ghana (exposure time: 4 h).

		% mortality after exposure to DDT*		
Ecological zone	Urban town	Residential area	Swampy area	Urban farm
Coastal savannah	Accra	6 (100)	-	73 (82)
	Cape Coast	91 (105)	95 (117)	-
	Sekondi	88 (79)	90 (64)	88 (95)
Forest	Kumasi	25 (90)	77 (86)	75 (104)
	Sunyani	81 (96)	75 (88)	-
	Tarkwa	98 (94)	100 (96)	-
Guinea savannah	Techiman	94 (100)	-	-
	Tamale	56 (86)	82 (100)	-
	Bolgatanga	87 (72)	84 (63)	-

*Values in bracket represent sample size.

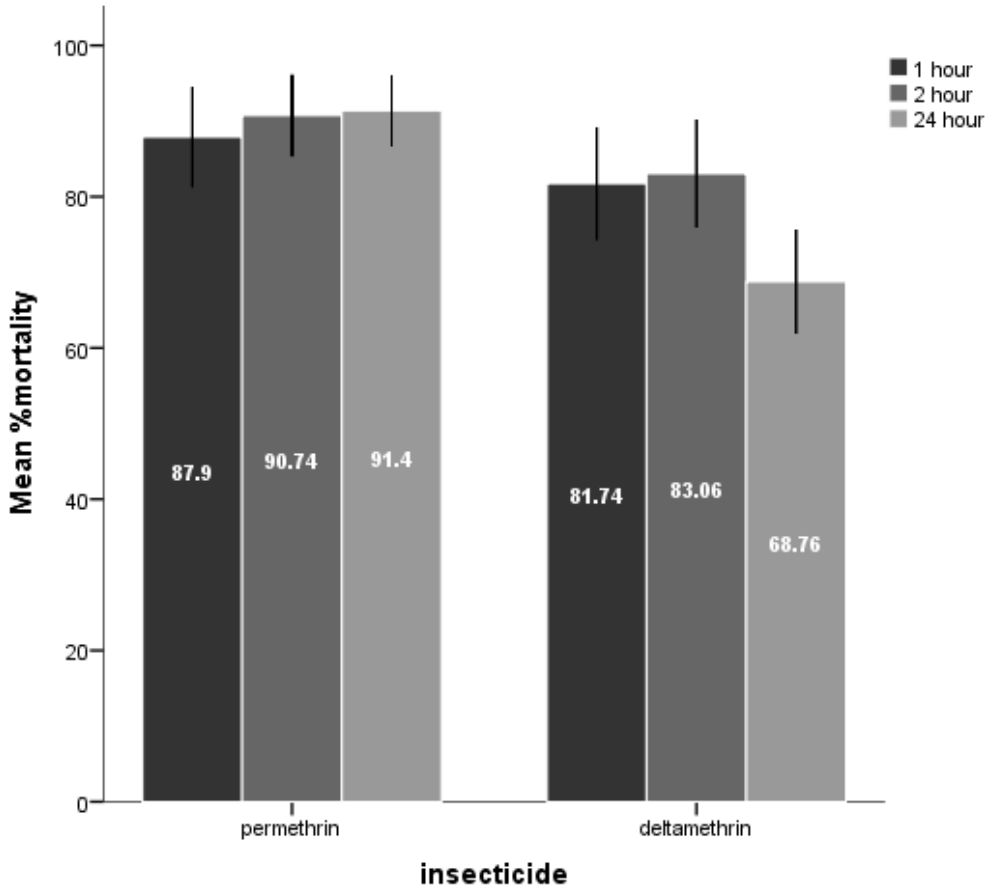
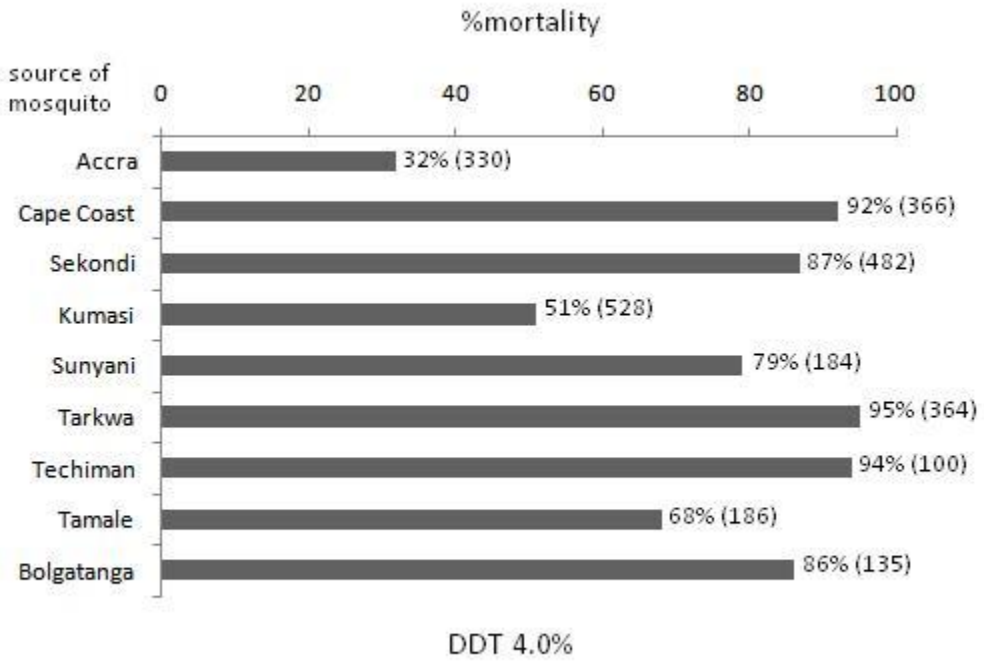


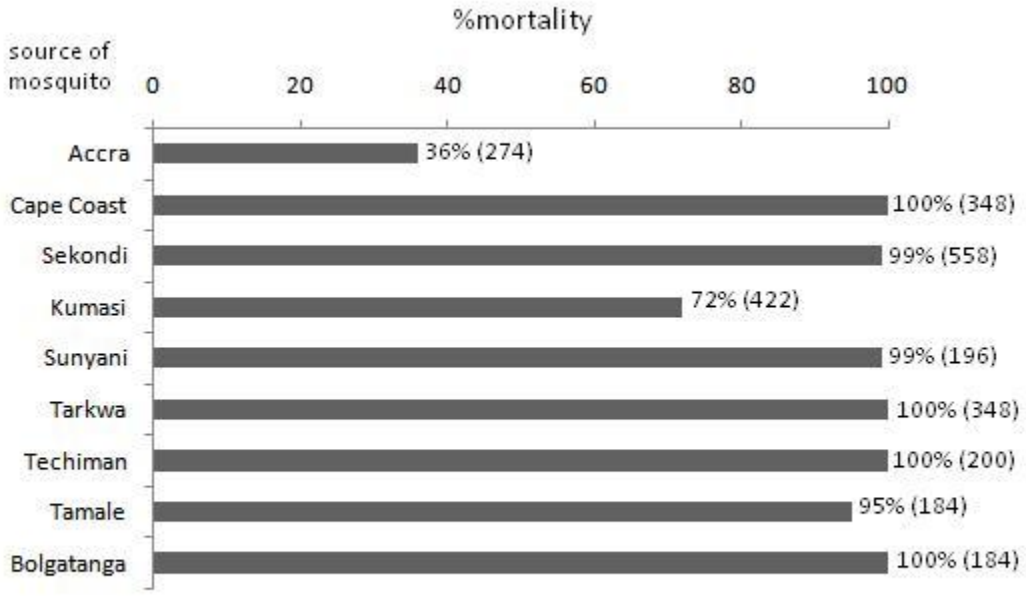
Figure 5.1.1: Percentage knock down at 1 h and 2 h and final mortality (24 h) of *Culex* mosquitoes in urban areas in Ghana after exposure to permethrin and deltamethrin insecticides (exposure time: 2 h) (Error bar: 95 % CI).

a.



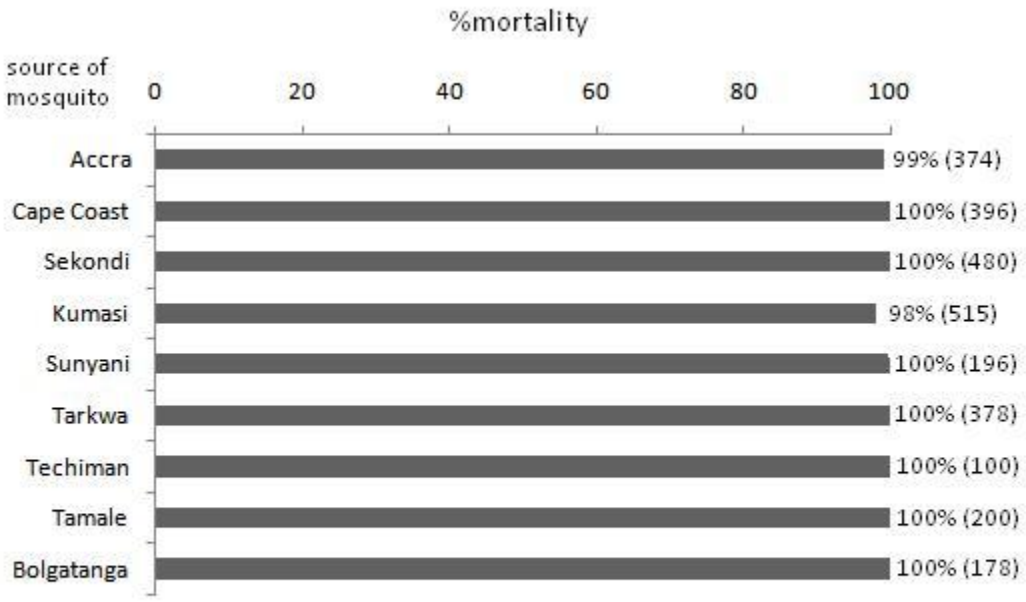
DDT 4.0%

b.



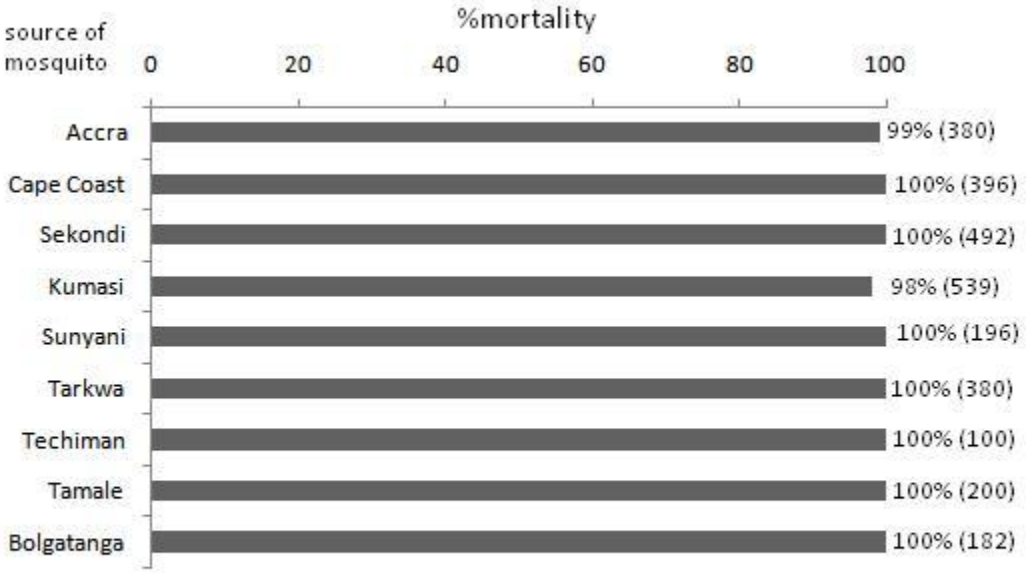
Dieldrin 4.0%

c.



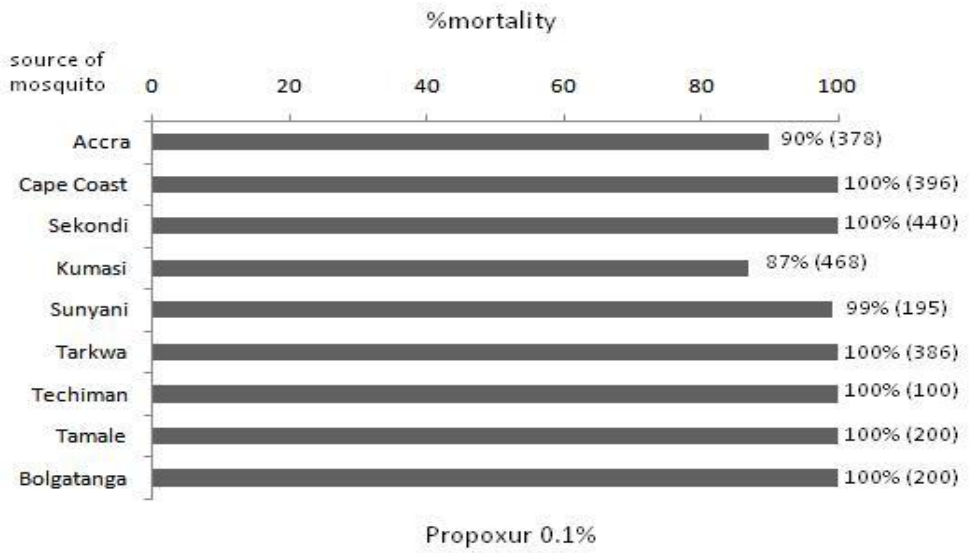
Malathion 5.0%

d.



Fenitrothion 1.0%

e.



f.

Figure 5.1.2: Resistance status of *Culex* mosquitoes to organochlorine, organophosphate and carbamate insecticides in urban areas in Ghana, a) DDT b) dieldrin c) malathion d) fenitrothion e) propoxur f) bendiocarb

5.2) Impact of environmental factors on insecticide resistance

5.2.1) Insecticide susceptibility status of *Culex* mosquitoes according to urbanization and land use settings

Different insecticide resistance status were observed in different urban areas (Kruskal-Wallis: $\chi 2 = 75.1$, df =8, $P < 0.0001$). However, percentage mortality to all the insecticides that were tested was similar in Accra and Kumasi (Mann Whitney U: $p = 0.49$). Mortality caused by DDT was similar in Accra (32 %) and Kumasi (51 %) (Mann Whitney U: $p < 0.355$) but significantly different from the rest of the study sites (Kruskal-Wallis: $\chi 2 = 29.5$; df = 8; $P < 0.0001$).

In addition, Pearson Chi-square test showed a strong relationship between resistance status and urban size (Pearson $\chi^2 = 48.2$; df = 1; $P < 0.0001$). This was more evident in the resistance status of the mosquitoes to carbamates and organophosphates, which was 61 times more likely to be found in large urban areas than in smaller urban areas (Pearson $\chi^2 = 77.8$, df = 1, $P < 0.0001$). In the case of pyrethroids, resistance to permethrin was 16 times more likely to be found in large urban areas than in small urban areas (Pearson $\chi^2 = 26.5$; df = 1; $P < 0.0001$) whereas resistance to deltamethrin appears to be uniformly distributed across the country. Thus, no significant correlation was found between deltamethrin resistance and urban size (Pearson $\chi^2 = 3.9$; df = 1; $P = 0.05$)

Furthermore, different levels of susceptibility were observed in different land use settings (Table 5.1.1, 5.2.1.1). The percentage mortality was lower in mosquitoes sampled from residential areas than from swampy or urban agricultural areas. This

was profound in pyrethroids and DDT insecticides. For instance, in Kumasi (forest zone) combined % mortality (data from pyrethroids, DDT and the two seasons) in residential area was 18.8 %, which was significantly lower than what was recorded in swampy area, 65.4 % or urban agricultural area, 79.9 % (Kruskal-Wallis: $\chi 2 = 39.2$; df = 2; $p < 0.0001$). A similar result was also observed in Sekondi-Takoradi (coastal savannah) where combined mean % mortality in residential area, 68.5 % was significant lower than mean % mortality in swampy area which is 91.8 % and urban agricultural area, 81.3 % (Kruskal-Wallis: $\chi 2 = 10.2$; df = 2; $p < 0.006$) (Figure 5.2.1.1). Nevertheless, there was no significant association between land use settings and resistance status to pyrethroids and DDT insecticides (Pearson $\chi 2 = 2.223$; df = 2; $P < 0.328$). Unexpectedly, mosquitoes from Korle Bu, a major urban agricultural area was susceptible to permethrin. A similar result was also observed in Sekondi (Table 5.1.1). For carbamate and organophosphate insecticides, ecology, seasons, and land use settings were marginally or non-significantly associated with resistance (Table 5.2.1.1).

Table 5.2.1.1: Percentage mortality (95 % CI) of *Culex* mosquitoes to organochlorines, organophosphate and carbamate insecticides, and different environmental factors associated with the distribution

Environmental factors		Mean mortality (%)					
		Organochlorines		Carbamate		Organophosphate	
		DDT	Die	Ben	Prop	Fen	Mal
Land use	Residential	64 % [a] (52-76)	75 % [a] (62-88)	87 % [a] (78-95)	93% [a] (86-99)	99 % [a] (98-100)	99 % [a] (98-100)
	Urban farm	80 % [a] (68-91)	88 % [a] (79-97)	98 % [a] (95-101)	99 % [a] (99-101)	99 % [a] (99-100)	100 % [b] (100-100)
	swampy	82 % [a] (75-89)	98 % [b] (95-101)	98 % [a] (94-101)	97 % [a] (94-100)	99 % [a] (99-100)	100 % [b] (100-100)
Season	rainy	64 % [a] (52-77)	80 % [a] (69-92)	89 % [a] (80-97)	96 % [a] (93-99)	99 % [a] (98-100)	99% [a] (98-100)
	dry	78 % [a] (71-86)	89 % [b] (82-97)	95.7 % [a] (92-99)	96 % [a] (90-101)	99 % [a] (99-100)	100 % [a] (99-100)
Ecology	Coastal savannah	77 % [a] (67-87)	83 % [a] (72-95)	94 % [a,b] (89-100)	97 % [a,b] (95-100)	99 % [a] (99-100)	100 % [a] (99-100)
	Forest	66 % [a] (54-78)	83 % [a] (73-94)	88 % [b] (80-97)	93 % [b] (86-99)	99 % [a] (98-100)	99 % [a] (98-100)
	Guinea savannah	80 % [a] (70-91)	98 % [a] (93-103)	100 % [a] (100-100)	100 % [a] (100-100)	100 % [a] (100-100)	100 % [a] (100-100)

*Values in columns for each environmental factor sharing same letter are not significantly different. (DDT – dichlorodiphenyltrichloroethane, ben – bendiocarb, pro – propoxur, fen – fenitrothion, mal – malathion)

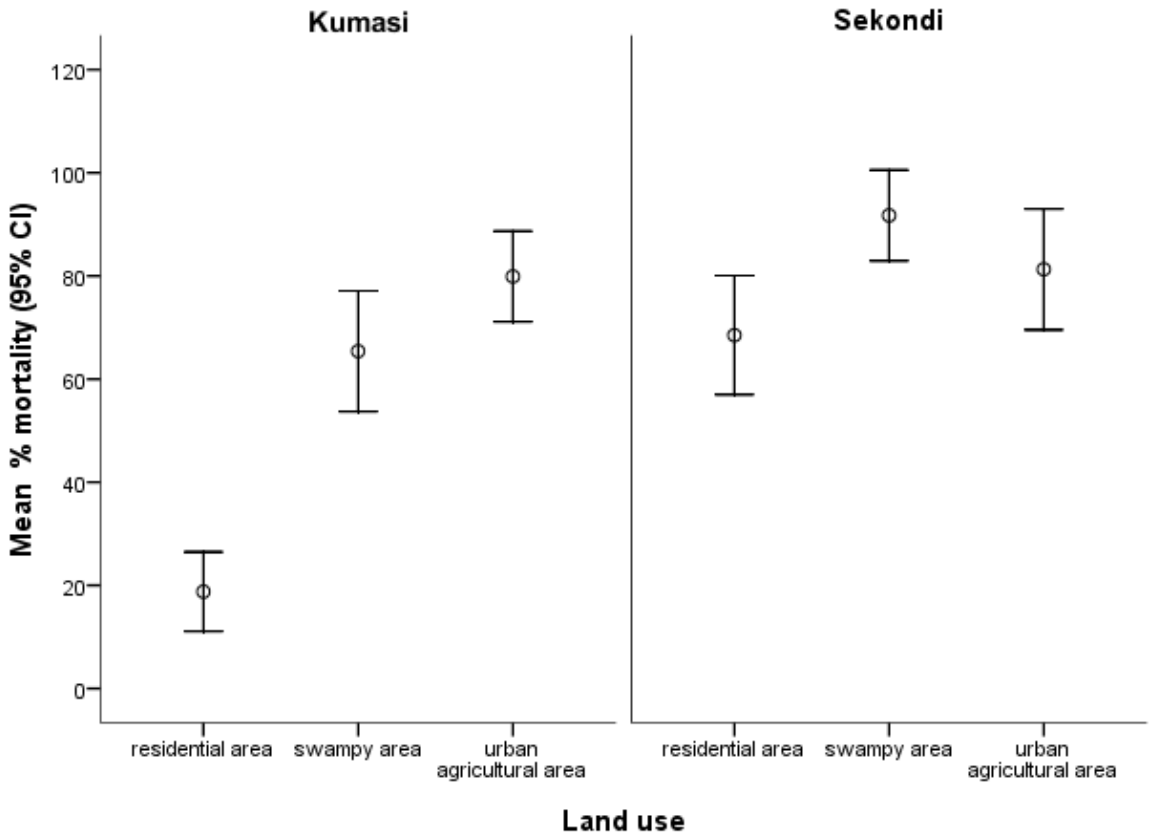


Figure 5.2.1.1: Means and 95 % confidence intervals of combined percentage mortality of *Culex* mosquitoes against pyrethroid and DDT insecticides at different land settings in Kumasi and Sekondi-Takoradi.

5.2.2) Insecticide susceptibility status of *Culex* mosquitoes according to ecology and seasons

Insecticide susceptibility was successfully determined in three ecological zones in Ghana. Different levels of susceptibility of *Culex* mosquitoes to all the insecticides that were tested were observed within the same ecological zone. Mosquitoes collected from study sites in Cape Coast were completely susceptible (100 % mortality) to permethrin while reduced mortality to permethrin was observed in Accra (47 %) and Sekondi (84 %), which are in the same ecological zone as Cape Coast. A similar trend was observed from study sites in the forest and Guinea savannah zones (Tables 5.1.1, 5.2.1.1).

There was a significant association between ecological zone and pyrethroid resistance status of *Culex* populations (Pearson χ^2 = 13.589; df = 2; P = 0.0001). However, it was not entirely consistent throughout the three ecological zones. Mosquitoes from the forest zone were nearly 2 times more likely to be resistant to pyrethroid insecticide than those in the coastal savannah zone, but the difference was not significant (p = 0.103). Significant difference was observed between forest and Guinea savannah zones (p < 0.0001).

In total, lower mortality (%) ± SD was recorded during the rainy season (77.8 ± 31.3) than during the dry season (85.1 ± 25.1) (Table 5.2.1.1; Figure 5.2.2.1). However, the difference was not significant (Mann Whitney U: 0.065).

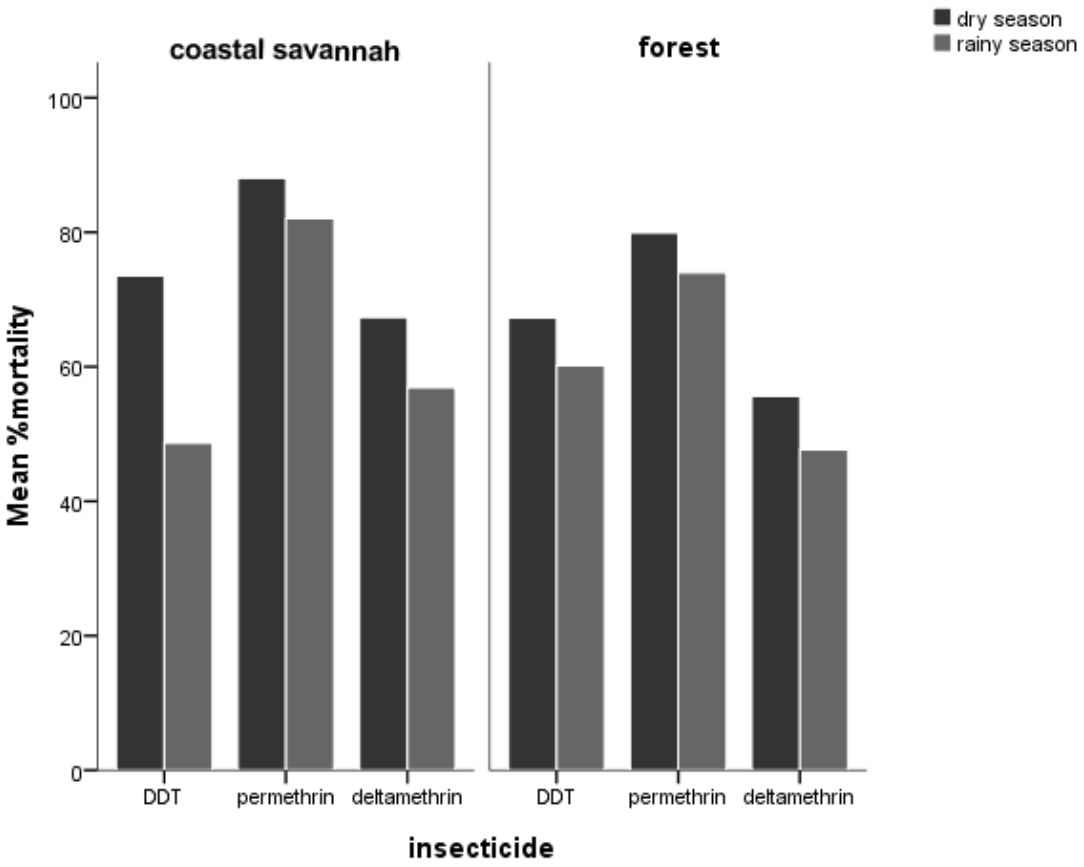


Figure 5.2.2.1: Percentage mortality of *Culex* mosquitoes to DDT, permethrin and deltamethrin in different seasons and ecological zones.

5.2.3) Modelling of environmental factors on pyrethroid resistance

In the binary logistic regression analysis, a test of full model against a constant only model was statistically significant (χ^2 = 121.78, df = 7, $p < 0.001$). Nagelkerke's R^2 was 0.64 and the model was able to correctly classify 90.5 % pyrethroid-resistant mosquitoes and 79.7 % not resistant mosquitoes (susceptible to pyrethroid).

The odds ratio for type of pyrethroid insecticide indicated that when holding all other variables constant, occurrence of deltamethrin resistance is about 55 times more likely than the occurrence of permethrin resistance in urban populations of *Culex* mosquitoes (Table 5.2.3.1). Inverting the odds ratio for size of urban area revealed that a pyrethroid-resistant population is 26 times more likely to be sampled from large urban areas than from small urban areas. Ecology and land use settings were dummy coded using forest and residential area as the reference group respectively. Inverted odds ratios for these dummy variables indicated that pyrethroid-resistant *Culex* mosquitoes were 7.4 times more likely to be sampled from residential areas than from urban agricultural areas whilst the same resistant mosquito was 3.3 times more likely to be sampled from the forest zone than from the coastal savannah zone or 7.6 times more likely to be sampled from the forest zone than from the Guinea savannah zone.

Table 5.2.3.1: Logistic regression predicting resistance status from environmental factors – ecology, season, land use settings and type of pyrethroid in *Culex* mosquitoes in urban areas in Ghana.

Predictor*	B	Wald	P-value	Odds Ratio
Season	-0.739	2.434	0.119	0.478
Insecticide (deltamethrin)	4.016	50.939	< 0.001	55.487
Urban size (small)	-2.432	12.851	< 0.001	0.088
Ecology				
Coastal savannah	-1.158	4.296	0.038	0.314
Guinea savannah	-2.023	7.786	0.005	0.132
Land use				
Swampy area	1.038	3.870	0.049	0.354
Agricultural area	1.998	7.743	0.005	0.136

*rainy season, permethrin, large urban area, forest, residential area were used as reference for season, type of insecticide, urban size, ecology and land use settings respectively for the regression analysis.

5.3) Enzyme activity in *Culex* mosquitoes

Among the four enzymes that were assayed, β-esterase recorded the highest absorbance whereas oxidase recorded the least absorbance (Figure 5.3.1, 5.3.2). A significant relationship was only found between β- esterase and the susceptibility level of *Culex* mosquitoes to all the insecticides tested (Pearson r = - 0.885; P = 0.002). Linear regression showed a stronger relationship between β- esterase and carbamates and organophosphate insecticides (regression $r^2 = 0.71$; p = 0.004) than pyrethroid insecticides (regression $r^2 = 0.49$; p = 0.037). Strong negative correlation was also observed between DDT induced mortality and the mean absorbance of GST (r = -0.856, P = 0.003).High absorbance from all the enzymes assayed were observed in Accra and Kumasi, which was significantly different from the rest of the study sites (Table 5.3.1).

Table 5.3.1: Mean absorbance (95 % CI) of α- esterase and β- esterase of *Culex* mosquitoes from different urban areas in Ghana

Urban size	Study sites	Mean absorbance (95 % CI)	
		α- esterase*	β- esterase*
Large urban areas (population > one million)	Accra	0.545[a]	1.028[a]
		(0.493-0.597)	(0.932-1.125)
	Kumasi	0.528[a,b]	0.912[b]
		(0.483-0.573)	(0.835-0.988)
Small urban areas (population < one million)	Cape Coast	0.328[c]	0.700[c]
		(0.304-0.351)	(0.607-0.792)
	Sekondi	0.395[c,d]	0.712[c]
		(0.373-0.417)	(0.679-0.744)
	Sunyani	0.474[b,e]	0.811[b,c]
		(0.408-0.573)	(0.693-0.930)
	Tarkwa	0.409[d,f]	0.735[c]
		(0.371-0.447)	(0.675-0.794)
	Techiman	0.398[c,d]	0.733[c]
		(0.374-0.422)	(0.689-0.777)
	Tamale	0.452[e,f]	0.808[c]
		(0.416-0.487)	(0.721-0.894)
	Bolgatanga	0.448[e,d]	0.784[c]
		(0.404-0.492)	(0.712-0.857)

*Values in columns sharing the same letter (a, b or c) are not significantly different.

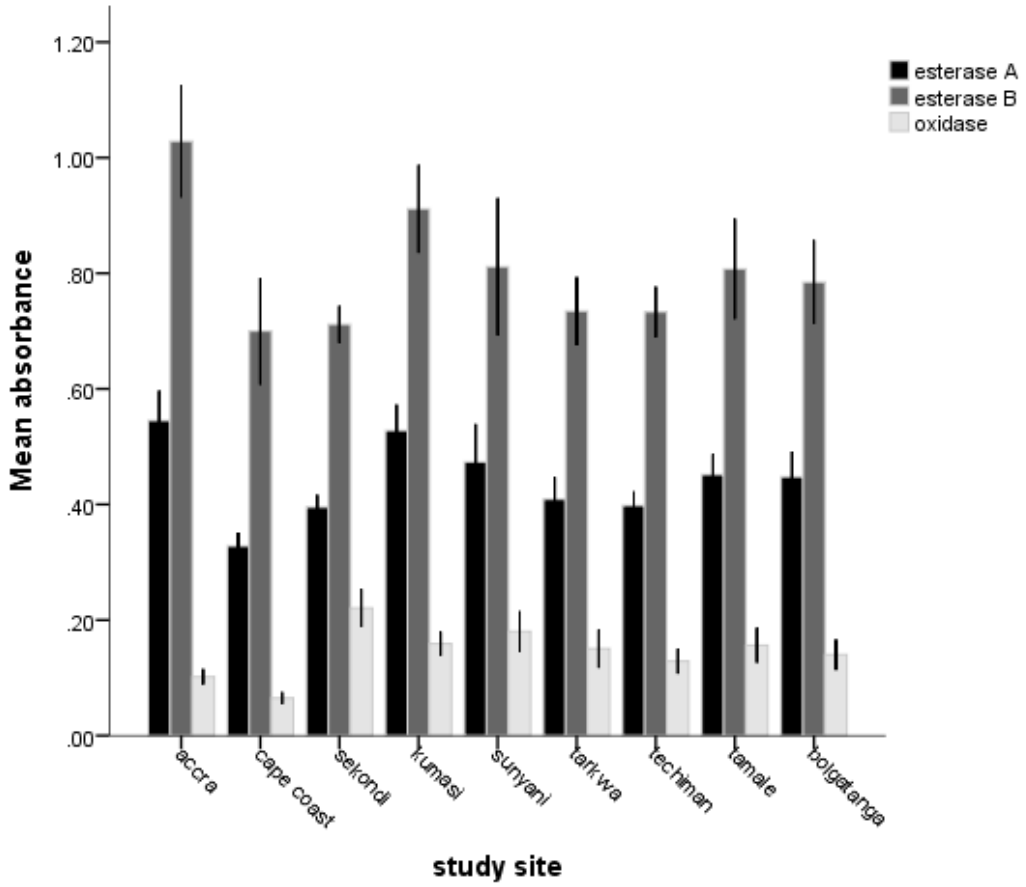


Figure 5.3.1: The mean absorbance of α- esterase (esterase A), β- esterase (esterase B) and oxidase in *Culex* mosquitoes from different urban areas in Ghana. (Error bar: 95 % CI).

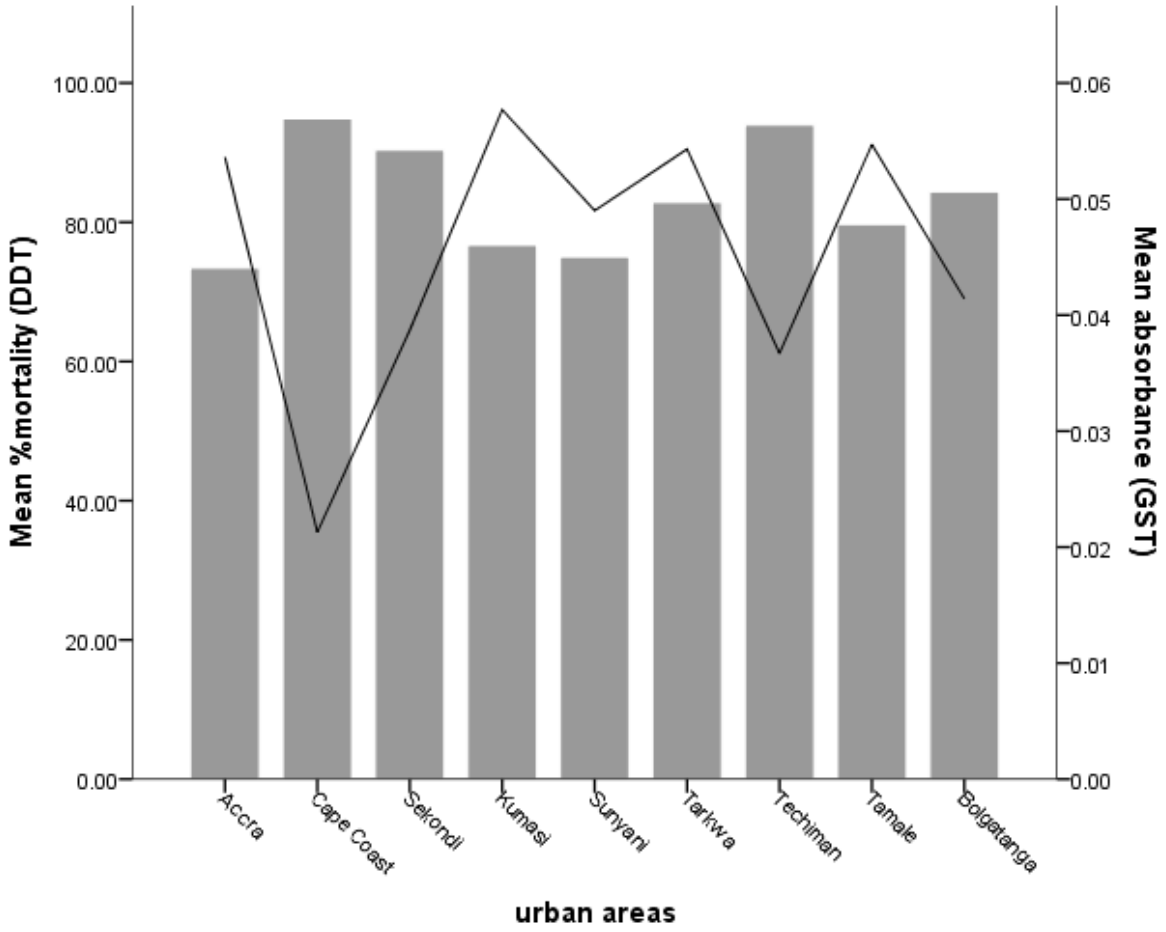


Figure 5.3.2: A combined line (mean absorbance of GST) and bar chart (mean % mortality) showing the relationship between GST and susceptibility level of *Culex* mosquitoes to DDT.

5.4) Species identification and distribution of *kdr* and *ace*1 mutation

Culex species from Accra, and Kumasi were all identified as *C. quinquefasciatus* whereas species *C. decens* were found in the rest of the study sites. The sequence blast search of the DNAs that were amplified by the universal primers did not match any distinct species. They were 93 % similar to *C. fuscocephala*, a *Culex* species found in Asia.

In the case of *kdr* mutation, out of 68 mosquitoes, 71 % did not have *kdr* mutation. The mutation was present in two (Accra and Kumasi) out of the five urban towns in which their mosquitoes were analysed. In Accra, 50 % (12/24) of the mosquitoes had *kdr* mutation whereas in Kumasi about 64 % (7/11) had the mutation (Table 5.4.1). Yet still, in Accra, 100 % of the *kdr* mutation was heterozygotes whereas in Kumasi 70 % of the *kdr* mutation was heterozygotes and 30 % being homozygotes.

Concerning *ace*1 mutation, out of 76 mosquitoes, about 70 % did not have the mutation. However, *ace*1 mutation was found in 4 out of 5 towns in which their mosquitoes were analysed (Table 5.4.2).

Table 5.4.1: Frequencies of susceptible and resistant genotypes of *Culex* mosquitoes from Ghana (N = sample size).

Study sites*	N	*kdr* negative	*kdr* positive	
			Heterozygote	Homozygote
Accra	24	50 % (12/24)	50 % (12/24)	-
Cape Coast	26	100 % (26/26)	-	-
Sekondi-Takoradi	4	75 % (3/4)	25 % (1/4)	-
Kumasi	11	36.4 % (4/11)	45.5 % (5/11)	18.2 % (2/11)
Techiman	3	100 % (3/3)	-	-
Total	68	70.6 % (48/68)	26.5 % (18/68)	2.9 % (2/68)

*Mosquitoes from residential area category.

Table 5.4.2: Distribution of *ace*1 mutations (G119S) in *Culex* mosquitoes from different urban areas in Ghana (N = sample size)

Study sites*	N	*ace*1 negative	*ace*1 positive	
			heterozygote	Homozygote
Accra	25	48% (12)	40% (10)	12%(3)
Cape Coast	20	100% (20)	0%	0%
Kumasi	16	81.3% (13)	12.5(2)	6.2%(1)
Sunyani	9	36.4% (4)	45.5% (5)	18.2% (2)
Techiman	5	80% (4)	20%(1)	0%
Total	76	69.7% (53)	23.6% (18)	7.8% (6)

*Mosquitoes from residential area category

5.5) Efficacy of long lasting insecticide treated net against pyrethroid-resistant *Culex* mosquitoes

A complete (100 %) mortality (258/258) was observed in pyrethroid-resistant *Anopheles gambiae* VKPER exposed to the nets in the cone bioassay while low mortality was observed in pyrethroid-resistant *Culex* mosquitoes (Figure 5.5.1). The roof of Permanet 3.0, which had additional synergist, PBO, recorded the highest mortality in all the three *Culex* mosquito populations: Kaneshie, 58.5 % (24/41), East Legon, 95.2 % (40/42) and Cape Coast, 92.9 % (26/28). Excluding the roofs of both nets, Permanet 3.0 and LifeNet caused similar mortality (Permanet 3.0, 12.1 % (29/240), LifeNet, 20.8 % (47/225), Mann Whitney U: $p = 0.43$). In total, the mortality caused by the two ITMs was highest in *C. decens* from Cape Coast 56.3 % (90/160), followed by *C. quinquefasciatus* from East Legon 19.2 % (52/271) and *C. quinquefasciatus* from Kaneshie recorded the least mortality 10.9 % (32/293) (Kruskal-Wallis: $\chi^2 = 24.35$, $df = 2$, $p < 0.001$). Mosquitoes exposed to untreated net showed 0 % mortality.

In the tunnel test bioassay, the roof of the Permanet 3.0 caused high mortality in both East Legon, 90.9 % (90/99) and Kaneshie 80 % (76/95) populations. Excluding the roof of both nets, LifeNet caused higher mortality than Permanet 3.0 to the East Legon population (LifeNet, 77.6 % (156/201); Permanet 3.0, 66.8 % (145/217); $\chi^2 = 6.03$, $p = 0.014$) but both ITM caused similar mortality to the Kaneshie population {LifeNet, 11.3 % (16/141); Permanet 3.0, 12.4 % (25/201); $\chi^2 = 0.09$, $p = 0.76$}. Percentage blood feeding and passage were lower in both treated nets than the untreated control (Table 5.5.1).

Table 5.5.1: Percentage mortality, blood feeding and passage on netting materials cut from Permanet 3.0 and LifeNet in a tunnel test bioassay against *Culex* mosquitoes sampled from East Legon and Kaneshie, Accra.

parameter	Source of mosquito*					
	East Legon			Kaneshie		
	Control	Permanet 3.0	LifeNet	Control	Permanet	LifeNet
% Mortality	10[a] (9/90)	74.4[b] (235/316)	76[b] (237/312)	5.1[a] (6/118)	34.1[b] (101/296)	12.1[c] (29/240)
% Blood feeding	6.7[a] (6/90)	1.3[b] (4/316)	0[b] (0/312)	37.3[a] (44/118)	3.4[b] (10/296)	5[b] (12/240)
% Passage	20[a] (18/90)	10.4[b] (46/312)	14.7[a,b] (46/312)	41.5[a] (49/118)	26.7[b] (79/296)	22.1[b] (53/240)

*Values with same letter in a row are not significantly different for a population.

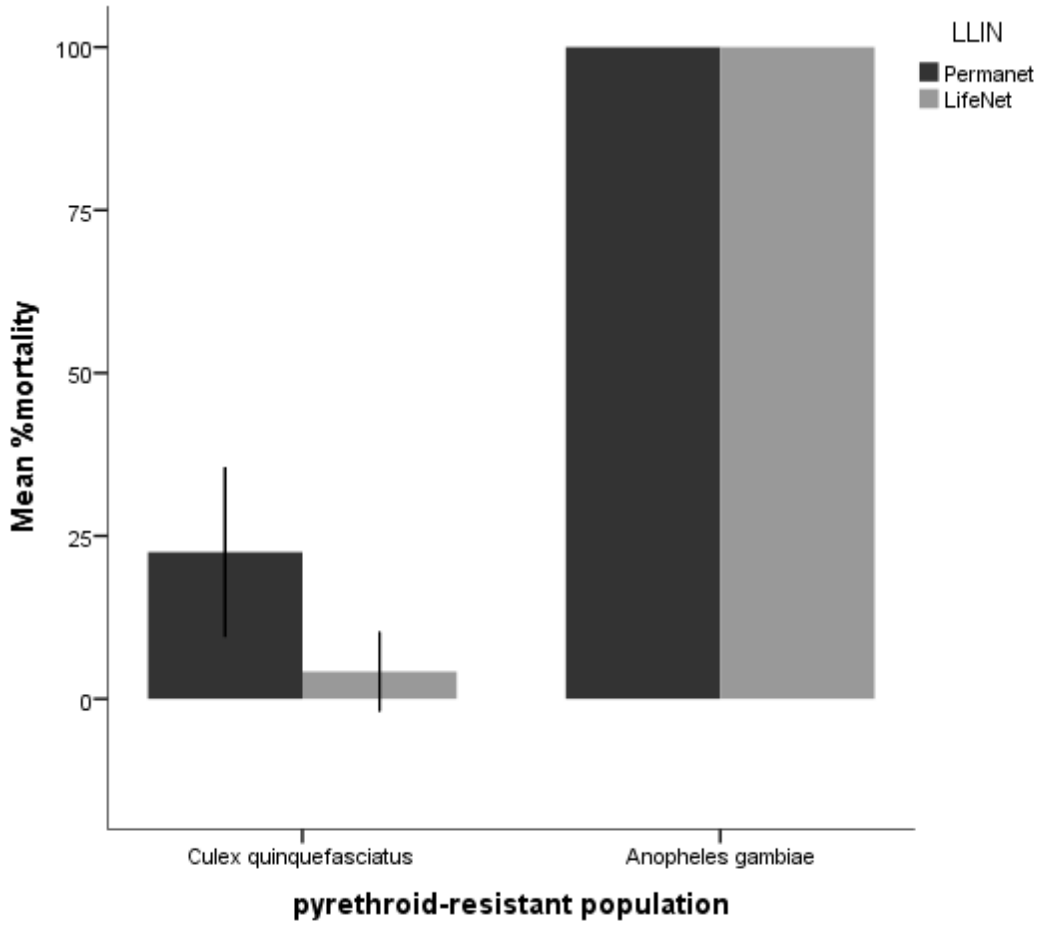


Figure 5.5.1: Efficacy of Permanet 3.0 and LifeNet against pyrethroid-resistant *Culex* mosquitoes (field strain) and *Anopheles gambiae* (laboratory strain: VKPER) assessed by WHO cone bioassay (Error bar: 95 % CI)

6) Discussion

6.1) Distribution of pyrethroid-resistant *Culex* mosquitoes in Ghana

Toxicological results from this study showed that urban populations of *Culex* mosquito in Ghana have varied susceptibilities to pyrethroid insecticides. Resistance to deltamethrin was evident across the country; however the mosquitoes were relatively susceptible to permethrin. There may be three or more *Culex* species involved in this study, but, interestingly resistance pattern to pyrethroid insecticides were similar. Mosquitoes were more resistant to deltamethrin than permethrin across the different species. Notwithstanding, higher resistance level was observed in *C. quinquefasciatus* than in *C. decens* or the unknown *Culex* species.

As expected, high frequency of *kdr* mutation was observed in the *C. quinquefasciatus* populations. This was understandable since *kdr* mutation is a major mechanism responsible for pyrethroid resistance. Similar finding has been reported by Wilding *et al*. (2012) among *C*. *quinquefasciatus* mosquitoes from Accra.

The significant difference in susceptibility levels between permethrin and deltamethrin found in the study was unexpected; however, it may indicate the existence of one or more additional resistance mechanisms. To my knowledge, there were no previous data on differential pyrethroid resistance in *Culex* species from Ghana. On the contrary, existing data on pyrethroid susceptibility pattern of *Anopheles* species in Ghana shows that they are relatively more resistant to permethrin than deltamethrin (Okoye *et al*., 2008; Boakye *et al*., 2009; Hunt *et al*., 2011; Kabula *et al*., 2011)

In principle, resistance to pyrethroids can be due to detoxification of the insecticide by metabolic enzymes (metabolic resistance mechanism) or decreased sensitivity of the target site of the insecticide (target site mutation). Pyrethroids are classified into two groups depending on the absence (type 1, e.g. permethrin) or presence (type 2, e.g. deltamethrin) of an α-cyano group in the alcohol moiety (Gammon *et al.,* 1981; Weerasinghe *et al*., 2001). The high resistance levels observed for deltamethrin in this study may therefore be the result of an additionally enhanced activity of metabolic enzymes which have high substrate specificity to the α-cyno group of pyrethroid insecticides. This suggestion is supported by several studies that have observed similar differential resistance between the two groups of pyrethroids in *Culex* mosquitoes and other insects groups (Weerasinghe *et al*., 2001; Hama, 1987; DeVries and Georghiou, 1980).

Although the results from the biochemical assays show evidence of esterase, monoxygenase and GST elevation in highly pyrethroid-resistant areas, there was no clear association between enzyme levels and pyrethroid resistance phenotypes across study sites from which can be concluded that biochemical mechanisms were in involved. However, Permanet® 3.0 had an additional synergist (PBO) on the roof top of the net and the high mortality of the mosquitoes after exposure to the roof presents further evidence for the existence of a biochemical resistance mechanism.

While resistance has been associated with both agricultural and domestic use of insecticide (Diabate *et al*., 2002; Boakye *et al*., 2009), I suspect the latter to be the major cause of pyrethroid resistance in the study population investigated here. This is supported by the low resistance level observed in the mosquito populations sampled from urban agricultural farms. Increased use of aerosol sprays and mosquito coils in

urban households have been reported by different studies in Ghana (Afrane *et al*., 2004; Boakye *et al*., 2009; Kudom *et al*., 2013) and it is suspected to be the major selection pressure responsible for the pyrethroid resistance of *Culex* species in this study.

6.2) Multiple insecticide resistance mechanisms are responsible for carbamates and organophosphate resistance in *Culex* mosquitoes from Ghana

Culex mosquitoes from the study population displayed large variation in resistance to organophosphates (malathion, fenitrothion) and carbamates (propoxur, bendiocarb) across the country and even in the same urban area. The study populations were more resistant to carbamates than organophosphates. Similar variation of resistance to these insecticides has also been observed in *Culex* mosquitoes from Ghana's neighbouring countries (Chandre *et al.*, 1997) and in *Anopheles* species from Ghana (Achondoh *et al*. 2008; Anto *et al*. 2009; Kudom *et al*., 2012). The resistance pattern in the two major mosquito species in Ghana to carbamate insecticides may offer some challenges to insecticide resistance management strategies in the country that include employment of carbamate insecticides.

The PCR diagnostic assay that was performed in the present study detected *ace*1 mutations (G119S) in some of the mosquito populations. Nevertheless, very low frequencies of homozygote resistance were found. Expectedly, high frequencies of *ace*1 mutations were found in mosquitoes in areas where the bioassay test showed the mosquitoes to be resistant to organophosphate and carbamate insecticides, suggesting the involvement of the mutation in the resistance of the mosquito population to the insecticides. Acetyl cholinesterase, the target site for organophosphates and carbamates, is a synaptic enzyme that hydrolyzes the neurotransmitter acetylcholine to terminate nerve impulses, thereby blocking nervous transmission and leading to the death of the insect. Selection of a modified acetyl cholinesterase less sensitive to these insecticides has been shown to be a common resistance mechanism in mosquitoes (Alout *et al*., 2008). The low frequency of homozygote resistance can be explained by the high fitness cost that is associated with *ace*1 mutation, such as long development time and decreased male reproductive success (Raymond *et al*., 2001). Despite *ace*1 mutations being reported to provide cross resistance to organophosphates and carbamates (Alout *et al*., 2008), the resistance level greatly varied between the two classes of insecticides. However, some studies have suggested that *ace*1 mutations have a greater impact on carbamate than organophosphate resistance (Djobenou *et al*., 2007).The reason for this is unclear at this time.

Results from the biochemical analysis in the present study showed a strong negative correlation between percentage mortality from the bioassay and mean absorbance of β-esterase. However, such significant association was not observed in oxidase. This may suggest the involvement of esterase activity in the resistance of the mosquitoes

to organophosphate and carbamate insecticides. Enhanced levels or modified activities of esterases and other detoxifying enzymes have been reported to prevent some insecticides from reaching their site of action (Hemmingway and Ranson, 2000). Meanwhile, several studies have shown close association between organophosphate and carbamate resistance and high levels of esterases (Georghiou and Pasteur, 1980; Alout *et al*., 2011).

There are strong arguments in favour of agricultural and domestic use of insecticides as the major cause of resistance in urban areas (Diabate *et al*., 2002) but these factors cannot fully explain the cause of resistance in the present study. Despite the use of organophosphates in urban farms, mosquitoes collected from those areas were susceptible to the insecticides. Moreover, the history of the domestic use of carbamates and organophosphates in urban areas is not well known for Ghana. In a related study, *Anopheles* species that were sampled from an urban vegetable farm in Accra were susceptible to organophosphate, though several organophosphate insecticides were detected in the water in which the mosquito was breeding (Achondoh *et al*., 2008). Chandre and colleagues (1997) were also not able to relate carbamate and organophosphate resistance in *C. quinquefasciatus* in Ivory Coast and Burkina Faso to the use of agricultural pesticides but rather implicated domestic use of insecticides to be associated with corresponding resistances. Other environmental variables such as ecology, seasons and land use settings were marginally or non-significantly associated with carbamate or organophosphate resistance in the present study, suggesting that these environmental factors do not have a clear impact on the resistance.

6.3) Insecticide resistance status of *Culex* mosquitoes from Ghana in relation to DDT and dieldrin insecticide use

Resistance to DDT was evident across the country and it appears to be uniformly distributed among the *Culex* species involved in this study. Similar levels of resistances were observed in different ecological zones, seasons and various land use settings. However, resistance to dieldrin was most profound in *C. quinquefasciatus*. The presence of *kdr* mutation leads to a reduction in the sensitivity of both DDT and pyrethroid insecticides. However the presence of *kdr* mutations alone in some of the mosquito population in this study cannot fully explain the cause of DDT resistance in the study area. That is, the pattern of DDT resistance in the country suggests the presence of an additional resistance mechanism in the present study.

Metabolic resistance is the most common resistance mechanism that occurs in insects. It occurs due to changes in a mosquito's enzyme systems that result in a more rapid detoxification of the insecticide than what is normal, preventing the insecticide from reaching the intended site of action (Hemmingway and Ranson, 2000). Several studies have shown that insecticide-resistant insects have elevated levels of Glutathione S-Transferase (GST) activity in crude homogenates, which suggests a role for GSTs in resistance (Grant, 1991), particularly to DDT (Hemmingway and Ranson, 2000). Strong negative correlation was observed between percentage mortality caused by DDT and mean absorbance of GST in this study. This presents evidence of the involvement of GST activity in DDT resistance in the *Culex* mosquitoes from Ghana. However, elevated GST activities have also been

detected in some insects species resistant to organophosphates (Fournier *et al*., 1992), other organochlorines (Grant *et al*., 1992) and implicated in resistance to pyrethroid insecticides (Kostaropoulos *et al*., 2001).

Resistance to dieldrin has been associated with mutations occurring in amino butyric acid (GABA), namely an alanine-296 substitution to glycine. Another mutation of the same codon conferring the substitution alanine to serine has also been shown to be associated with dieldrin resistance (ffrench-Constant *et al*., 2000; Du *et al*., 2005). However the present study failed to determine such mutations in the mosquito population studied here. The results from biochemical assays revealed high absorbance of the three detoxifying enzymes in the *C. quinquefasciatus* populations from Accra and Kumasi where mosquitoes were highly resistant to dieldrin. It is possible that metabolic resistance mechanisms are involved in the dieldrin resistance observed in the present study. This observation was made based on a related study that found no association between dieldrin resistance phenotype and the GABA mutation (alanine-296 to glycine substitution) in *A. gambiae* from Obuasi, Ghana (Brooke *et al*., 2006). The study, however, implicated metabolic resistance mechanisms as an additional cause of dieldrin resistance in the *A. gambiae* population based on the results of biochemical and synergist assays (Brooke *et al*., 2006).

Although no formal survey of general use of organochlorine insecticides was undertaken during larval collections, it is likely that dieldrin and DDT are still used in urban agricultural activities. Several studies have found traces of organochlorines in water, sediment, vegetables, fruits, meat, human blood, and even mothers' breast milk in different urban areas in Ghana (Ntow, 2001; Darko and Aquaah, 2007;

Bempah and Donkor, 2011). In an experiment to determine organochlorine residues in vegetables found in Accra, Bempah *et al*. (2012) reported that the most frequently found and abundant pesticide was DDT and traces of dieldrin were also found particularly on tomatoes. Therefore, the current use of organochlorine in agriculture coupled with the past use in vector control may be the main cause of DDT and dieldrin resistance observed in this study.

The use of DDT in public health still remains controversial (Walker *et al*., 2003). However, it is currently used in a number of countries for vector control and is still one of the insecticides recommended by WHO for indoor residual spraying (Sadasivaiah *et al*., 2007). Widespread distribution of DDT resistance as shown in the present study and other works among major mosquitoes in Ghana (Kudom *et al*., 2012; Hunt *et al*., 2011; Okoye *et al*., 2008; Achonduh *et al*., 2008) suggest, however, that the use of DDT for vector control in the country is most likely to be counterproductive.

6.4) Impact of urbanization on insecticide resistance status of *Culex* mosquitoes

The present study revealed a strong relationship between resistance status and the degree of urbanization. Resistance to carbamates and organophosphates was 61 times more likely to be found in large urban areas than in smaller urban areas. Likewise, resistance to permethrin was 16 times more likely to be found in large urban areas than in small urban areas. Incidentally, *Culex* mosquitoes in large urban areas were identified as *C. quinquefasciatus* whereas the rest of the study sites were made up of *C. decens* and other unknown *Culex* species. Therefore, the different distribution of *Culex* species across the study sites may have accounted for the differential insecticide resistance status between the different urban areas.

Although the level of pollution was not quantified in the breeding sites in the study areas, it was nevertheless observed that Kumasi and Accra had more extensive networks of open gutters and the choked gutters where mosquitoes were collected from were also more polluted than those in other urban areas. It is therefore not surprising that the *Culex* species found in these areas were *C. quinquefasciatus.*

Thus far, little attention has been paid to numerous polluted breeding sites scattered in urban areas in Ghana. However, urban pollutants have been shown to increase mosquito tolerance to insecticides (Poupardin *et al*., 2008; Riaz *et al*., 2009). In the present study, polluted breeding habitats are suspected to have played a role in insecticide resistance in the mosquito species. This suspicion stems from the significant difference in resistance level observed within the pyrethroid insecticides. Similar result was observed in *A. gambiae* and it was attributed to pollution in the

breeding habitats of the mosquitoes (Kabula *et al.,* 2011). The conclusion was made based on ammonium level (an indicator of how polluted a breeding site is) in the breeding sites which was positively associated with occurrence of deltamethrin resistance and negatively associated with permethrin resistance in *A. gambiae* (Kabula *et al.,* 2011). However, there is not much evidence from the present and the previous study (Kabula *et al.,* 2011) to explain the cause of high levels of deltamethrin resistance in mosquitoes that breed in polluted water.

Although, there is not much evidence from this study in support of the role of pollution on insecticide resistance, there are conclusive evidences from different studies in support of this ascension (Poupardin *et al*., 2008; Nkya *et al*., 2013). The presence of *C. decens* in polluted breeding sites, which is not known to breed in such habitats couple with various reports that have observed *A. gambae*, a major malaria vector also breeding in polluted habitats (Keating *et al*., 2003; Kudom *et al*., 2012) reinforce the need to take a critical look at the numerous polluted breeding sites scattered in the country. Both solid and liquid waste are poorly managed in urban areas in Ghana and what enters into gutters or water bodies from commercial and domestic activities is not much regulated. This presents a situation where apart from pesticides officially sanctioned for public use, mosquitoes could also be exposed to unknown chemicals or insecticides in polluted breeding habitats, which can select for resistance mechanisms that can confer high level of resistance to new insecticides that officially have not been recognized to be used in the country before.

6.5) Efficacy of long lasting insecticide treated nets against pyrethroid-resistant *Culex* mosquitoes from Ghana

Complete mortality was observed in pyrethroid-resistant *A. gambiae* VKPER exposed to the nets in the cone bioassay while low mortality was observed in pyrethroid-resistant *Culex* mosquitoes. This result indicates that ITM can be effective against pyrethroid-resistant *A. gambiae* but not pyrethroid resistant *Culex* species. This finding is in consistent with several experimental hut trials, which have shown reduced efficacy of ITM against pyrethroid resistant *Culex* species in many African countries (Ngufor *et al.*, 2014, Irish *et al*., 2008). The roof of Permanet 3.0®, which had additional synergist, PBO recorded the highest mortality in all the three *Culex* populations. The result may suggest that ITM with synergist can be an effective resistant management tool against pyrethroid-resistant *Culex* mosquitoes.

Reduced efficacy of ITM against pyrethroid-resistant mosquitoes and its implication for malaria control are well known (Strode *et al.,* 2014). However, the impact of reduced efficacy of ITM on attitude of local people towards the use, acceptability or sustained use of ITM has not received much attention. Most studies have suggested that if not for the protection given against *Culex* mosquitoes, the popularity and effectiveness of ITM would be hampered (Asidi *et al*., 2005). Hence, the question as to what happens to the popularity and effectiveness of ITM if the net fails to protect users from *Culex* mosquitoes is critical and needs to be explored.

Furthermore, partly due to the importance of mosquito nuisance and, to a lesser degree, due to the inability of local inhabitants to distinguish between the genera of mosquitoes, it is possible that reduced protective efficacy of ITM against pyrethroid-

resistant *Culex* mosquitoes can encourage people to use other anti mosquito strategies (e.g. mosquito coil, aerosol spray), that may prevent nuisance but not necessarily effective against malaria. This may lead to an increase in malaria cases. Also, it may lead to excessive use of domestic insecticide as reported in many urban areas in Ghana (Afrane *et al*., 2004; Coetzee *et al*., 2006; Boakye *et al*., 2009; Kudom *et al*., 2013), which may in turn cause resistance in both *Anopheles* and *Culex* species (Elisa *et al*., 1996; Diabate *et al*., 2002; Boakye *et al*., 2009; Kudom *et al*., 2013).

The 2008-2015 strategic plan for malaria control in Ghana involves 100% coverage of household's ownership of at least one ITM and 80% incidence of ITM use. With the high level of resistance in *Culex* mosquitoes observed in the present study, it would be difficult to achieve this target without supplementing the ITM component of malaria control with an effective management of anthropogenic habitats and educating the general public about the biology and behaviour of mosquitoes. Informal interaction during larval collection among inhabitants from various parts of Ghana showed that they were oblivious about the life stages of mosquitoes and surprisingly, most people could not identify mosquito larvae. Thus, the abundance and resistance status of *Culex* mosquitoes coupled with the value attached to their nuisance by local people and their level of knowledge on the life history of mosquitoes can threaten the effectiveness of ITM and ultimately malaria control (Figure 6.5.1).

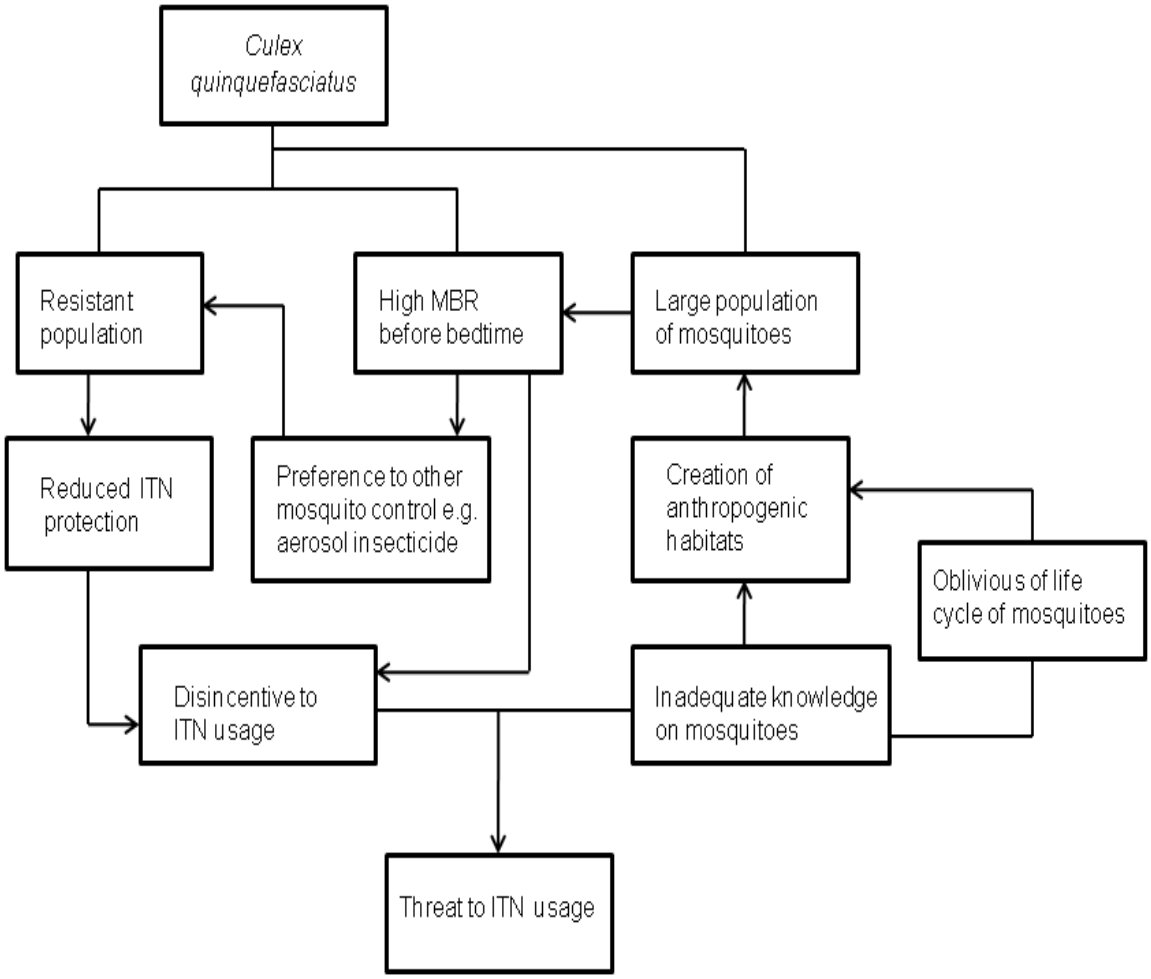


Figure: 6.5.1: A diagram showing a relationship between *Culex* mosquitoes and malaria control in urban areas in Ghana.

7) Conclusion

In this study, insecticide resistance status of *Culex* species in urban areas of Ghana was determined. *C. quinquefasciatus, C. decens* and other unknown *Culex* species (unidentified) were found inhabiting in organic polluted habitats in urban areas of the country. The pattern of resistance to insecticides within a class of insecticide was similar across different species, ecological zones, seasons and land use settings. This was more evident in pyrethroid insecticides where mosquitoes were found to be more resistant to deltamethrin than permethrin irrespective of the species or environmental factors. Notwithstanding, resistance level was higher in *C. quinquefasciatus* than in *C. decens* or the unknown *Culex* species

Environmental factors such as ecology and land use were observed to be influential on level of insecticide resistance of *Culex* species. However, the role of urbanization and its associated problems such as pollution were more profound and it appears to have contributed to pattern of distribution of *C. quinquefasciatus and C. decens* observed in the study.

Results from the biochemical assay showed an association between enzyme levels and the degree of insecticide resistance among the *Culex* mosquitoes. This may suggest the involvement of metabolic resistance mechanism in the study populations. In addition, target site mutations (*kdr* and *ace*1) were also observed particularly in *C. quinquefasciatus* from the study populations. This is the first description showing evidence of the existence of multiple insecticide resistance mechanisms in *C. quinquefasciatus*. The pattern of resistance and the mechanisms involved can be expected to have a number of implications on resistance management strategies.

Due to low levels of resistance to organophosphates, resistance management strategies comprising the use of organophosphates may be successful against pyrethroid-resistant *Culex* mosquitoes. However, such strategies must be carried out with caution since populations with *ace*1 mutations and high levels of esterases, which can confer high resistance to organophosphate, already exist.

Various insecticide selection pressures particularly from agriculture and domestic use of insecticides were suspected to be the cause of resistance to the insecticides. It also appears that the presence of urban pollutants in mosquito breeding sites probably has a direct or indirect impact on mosquito resistance. For this reason, proper management of waste, particularly in urban areas, and effective regulation of use of pesticides appear to be critical in resistance management programs.

The results also show that ITM has lost its efficacy against pyrethroid-resistant *Culex* mosquitoes and this has the potential to affect acceptance and utilization of the use of ITM among the local people. ITM with synergist was seen as a possible tool that could be used to manage pyrethroid-resistant *Culex* populations. In order not to jeopardize the efficacy of ITM through insecticide resistance, there is also the need to reduce insecticide use, especially at the household level. One way is to reduce the mosquito population, especially in urban areas where most of the important larval habitats have been shown to be anthropogenic (Keating *et al*., 2003, Klinkenberg *et al*. 2008; Kudom *et al*., 2012). Such habitats can easily be managed through proper waste management, proper construction of drains and the change of the inhabitants' behaviour through proper education.

The study came with some limitations. It was difficult to distinguish morphologically the *Culex* species that were involved in this study. The safest way to collect a single species particularly *C. quinqefasciatus* was to have sampled resting adults from inside homes. However, factors such as age of the mosquitoes, previous exposure to insecticide may not be known when adults are collected from houses and this can affect the quality of results from the susceptibility assay. Detections of target site mutations were mostly successful in *C. quinqefasciatus* but not other *Culex* species found in the study area and this was understandable because primers available for the PCR assay were purposely designed and optimised for *C. quinqefasciatus.* Further research is needed particularly in the area of urbanization and mosquito control, taxonomy of *Culex* species inhabiting in urban areas in Ghana and development of molecular tools for easy identification of *Culex* species.

Acknowledgement

I am grateful to my supervisors, Dr. G. Froeschl, PD. Dr. Dr. H. Rinder and Prof. D. Boakye for their direction and up building comments, which contributed in making this project successful.

I am also grateful to Kwadwo Frimpong, Selasie P. Bansah, Osei K. Akuoko, K. Yirenkyi Sekye and all the staff of Noguchi Memorial Institute who helped me during the molecular, biochemical and tunnel test assays. My appreciation goes to the following Institutions; University for Development Studies, Tamale, University of Mines and Technology, Tarkwa, Takoradi Polytechnic, Takoradi, Opoku Ware School, Kumasi, Sunyani Senior High School, Sunyani, Techiman Senior High School, Techiman for providing me with laboratory space for mosquito rearing and insecticide susceptibility assay. I also appreciate the contribution of Parasitology Unit of Noguchi Memorial Institute and Department of Entomology and Wildlife, University of Cape Coast to the project. Special thanks to Dr. B. A Mensah, Head of Department, Entomology and Wildlife, University of Cape Coast.

I acknowledge the Federal Ministry for Economic Cooperation and Development (BMZ), the German Academic Exchange Services (DAAD) together with its Exceed-Programme for offering me scholarship for this PhD program.

Special thanks also go to my wife Florence A. Kudom, my daughter Fosua A. Kudom, family members, and friends and to everyone who contributed in making this project successful.

References

Achonduh, O.A., Gbewonyo, W.S.K., Boakye, D.A., Wilson, M.D. (2008).Susceptibility Status of Anopheles gambiae s.l. (Diptera: Culicidae) from Cabbage Growing Areas Associated with Pyrethroid and Organophosphate Use in Accra, Ghana**.** *West African Journal of Applied Ecology*, 12:1

Adasi, K., Hemingway, J. (2008). Susceptibility to three pyrethroids and detection of knockdown resistance mutation in Ghanaian *Anopheles gambiae* sensu stricto. *J.vec. Ecol*, 3:2

Adeogun, A.O., Olojede, J.B., Oduola, A.O., Awolola, T.S. (2012). Village-Scale Evaluation of PermaNet 3.0: an Enhanced Efficacy Combination Long-Lasting Insecticidal Net against Resistant Populations of Anopheles gambiae s.s. *Malaria Chemotherapy, Control & Elimination*, 1:9

Afrane, Y.A., Klinkenberg, E., Drechsel, P., Owusu-Daaku, K., Garms, R., Kruppa, T. (2004). Does irrigated urban agriculture influence the transmission of malaria in the city of Kumasi, Ghana? *Acta Tropica*, 89:125–134

Agadzi, V. K., Boatin, B. A., Appawu, M. A., Mingle, J. A. A., Addy, P. A. (1984). Yellow Fever in Ghana. Bulletin of the World Health Organization 62(4): 577-583.

Akogbeto, M.C., Djouaka, R.F., Kinde-Gazard, D.A. (2006). Screening of pesticide residues in soil and water samples from agricultural settings. *Malar.J*, 5: 22.

Alaii, J.A., Borne, W.V.D., Kachur, S.P., Shelley, K., Mwenesi, H., Vulule, J.M., Hawley, W.A., Nahlen, B.L., Phillips-Howard, P.A. (2003). Community reactions

to the introduction of permethrin-treated bed nets for malaria control during a randomized controlled trial in Western Kenya. *Am J Trop Med Hyg,* 68 (Suppl 4):128-136

Alout, H., Labbe, P., Pasteur, N., Weill, M. (2011). High incidence of ace-1 duplicated haplotypes in resistant *Culex pipiens* mosquitoes from Algeria. *Insect Biochem. Mol. Biol*, 41:29-35

Alout, H., Djogbénou, L., Berticat, C., Chandre, F., Weill, M. (2008).Comparison of *Anopheles gambiae* and *Culex pipiens* acetylcholinesterase 1 biochemical properties. *Comp Bio chem. Physiol B*, 150:271–277

Anto, F., Asoala, V., Anyorigiya, T., Oduro, A., Adjuik, M., Owusu-Agyei, S., Dery, D., Appawu, M., Dadzie, S., Bimi, L., Hodgson, A. (2009). Insecticide resistance profiles for malaria vectors in the Kassena-Nankana district of Ghana. *Malaria Journal*, 8: 81.

Appawu, M. Dadzie, S. Abdul, H. Asmah, H. Boakye, D. Wilson, M. Ofori-Adjei, D. (2006). Surveillance of viral haemorrhagic fevers in Ghana: entomological assessment of the risk of transmission in the northern regions. *Ghana Medical Journal*, 40(4): 137-141.

Asidi, A.N., N'Guessan, R. Koffi, A.A., Curtis, C.F., Hougard, J.M., Chandre, F., Corbel, V., Darriet, F., Zaim, M., Rowland, M.W. (2005). Experimental hut evaluation of bed nets treated with an organophosphate (chlorpyrifos methyl) or a pyrethroid (lambdacyhalothrin) alone and in combination against insecticide-resistant *Anopheles gambiae* and *Culex quinquefasciatus* mosquitoes. *Malar. J*, 4: 25

Bempah, C. K., Buah-Kwofie, A., Enimil, E., Blewu, B., Agyei-Martey, G. (2012). Residues of organochlorine pesticides in vegetables marketed in Greater Accra Region of Ghana. *Food Control*, 25 (2): 537-542.

Bempah, C.K., Donkor, A.K. (2011). Pesticide residues in fruits at the market level in Accra Metropolis, Ghana, a preliminary study. *Environmental Monitoring and Assessment,* 175: 551-561

Binka, F. N., Kubaje, A., Adjuik, M., Williams, L. A, Lengeler, C., Maude, G. H., Armah, G. E., Kajihara, B., Adiamah, J. H., Smith, P. G. (1996). Impact of permethrin treated bed nets on child mortality in Kassena-Nankana district, Ghana: a randomized controlled trial. *Trop Med Int Health*, 1:147-154.

Boadi, K.O., Kuitunen, M. (2005). Environment, wealth, inequality and the burden of disease in the Accra metropolitan area, Ghana. *International Journal of Environmental Health Research,* 15: 193-206

Boakye, D.A., Adasi, K., Appawu, M., Brown, C.A., Wilson, M.D. (2009). Patterns of Household Insecticide Use and Pyrethroid Resistance in Anopheles gambiae Sensu Stricto (Diptera: Culicidae) within the Accra Metropolis of Ghana. African Entomology, 17: 125-130

Brogdon, W. G., McAllister, J. C. (1998). Insecticide resistance and vector control. *Emerg Infect Dis*, 4: 605-13

Brooke, B.D., Hunt, R.H., Matambo, T.S., Koekemoer, L.L., Van Wyk, P., Coetzee, M. (2006). Dieldrin resistance in the malaria vector *Anopheles gambiae* in Ghana. *Med Vet Entomol,* 20: 294-299.

Busvine, J.R. (1969). The impact on malaria of insecticide resistance in anopheline mosquitoes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 63(Suppl 1):S19–S30.

Carter, R., Mendis, K.N. (2002). Evolutionary and historical aspects of the burden of malaria. *Clin. Microbiol. Rev*, 15(4):564

Chandre, F., Darriet, F., Manga, L., Akogbeto, M., Faye, O., Mouchet, J., Guillet, P. (1999).Status of pyrethroid resistance in *Anopheles gambiae* s.l. *Butt. World Health Organ*, 77: 230-234.

Chandre F., Darriet F., Darder M., Cuany, A., Doannio, J.M.C.,Pasteur, N., Guillet, P. (1998) Pyrethroid resistance in *Culex quinquefasciatus* from West Africa. *Medical and Veterinary Entomology*, 12: 359–366.

Chandre, F., Darriet, F., Doannio, J.M.C.,Riviere, F.,Nicole, Pasteur, N, Guillet, P. (1997). Distribution of Organophosphate and Carbamate Resistance in *Culex pipiens quinquefasciatus* (Diptera: Culicidae) in West Africa. *J. Med. Entomol,* 34: 664-671

Coetzee, M., Van Wyk, P., Booman, M., Koekemoer, L.L., Hunt, R.H. (2006). Insecticide resistance in malaria vector mosquitoes in a gold mining town in Ghana and implications for malaria control. *Bull Soc Path Exot*, 99:400-3.

Corbel, V., N'Guessan, R., Brengues, C., Chandre, F., Djogbenou, L., Martin, T., Akogbeto, M., Hougard, J.M., Rowland, M. (2007).Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Trop*, 101: 207-216

D'Alessandro, U., Olaleye, B.O., McGuire, W., Langerock, P., Bennett, S., Aikins, M.K., Thomson, M.C., Cham, M.K., Cham, B.A., Greenwood, B.M. (1995). Mortality and morbidity from malaria in Gambian children after introduction of a treated bed nets programme. *The Lancet*, 345:479-483.

Darko, G., Acquaah, S.O. (2007). Levels of organochlorine pesticides residues in meat. *International Journal of Environmental Science & Technology*, 4: 521-524

DeVries, D.H., Georghiou, G.P. (1980). A wide spectrum of resistance to pyrethroid insecticides in Musca domestica. *Experientia*, 36:226-227

Diabate , A., Baldet, T., Chandre, F., Akogbéto, M., Guiguemde, R.T., Darriet, F., Brengues, C., Guillet, P., Hemingway, J., Graham, J. S., Hougard, J.M.(2002). The role of agricultural use of insecticides in resistance to pyrethroids in *Anopheles gambiae* s.l. in Burkina Faso. *Am J Trop Med Hyg*, 67: 617–622

Dickinson, K., Paskewitz, S. (2012). Willingness to pay for mosquito control: how important is West Nile virus risk compared to the nuisance of mosquitoes? *Vector Borne Zoonotic Dis*, 12: 886–892.

Djogbénou, L., Weill, M., Hougard, J.M., Raymond, M., Akogbeto, M., Chandre, F. (2007). Characterization of insensitive acetylcholinesterase (ace-1R) in

Anopheles gambiae (Diptera: Culicidae): resistance levels and dominance. *J Med Entomol,* 44:805–810.

Du, W., T.S. Awolola, Howell, P., Koekemoer, L.L., Brooke, B.D., Benedict, M.Q. Coetzee, M. Zheng, L. (2005). "Independent mutations in the Rdl locus confer dieldrin resistance to *Anopheles gambiae* and *An. arabiensis*." *Insect Molecular Biology,* 14: 179-183.

Dunyo, S.K., Appawu, M., Nkrumah, F.K., Baffoe-Wilmot. A., Pedersen, E.M., Simonsen P.E. (1996). Lymphatic filariasis on the coast of Ghana. *Trans R Soc Trop Med Hyg,* 90:634-8.

Dzodzomenyo, M.D., Dunyo, S.K., Ahorlu, S.K., Coker, W.Z., Appawu, M.A., Pedersen, E.M., Simonsen P.E. (1999). Bancroftian filariasis in an irrigation project community in southern Ghana. *Trop Med Int Hlth,* 4:13-18.

Eisele, T.P., Keating, J., Littrell, M., Larsen, D., Macintyre, K. (2009). Assessment of insecticide-treated bed net use among children and pregnant women across 15 countries using standardized national surveys. *Am J Trop Med Hyg*, 80:209-214

Elissa, N., Mouchet, J., Riviere, F., Meunier, J.Y Yao, K. (1993). Resistance of Anopheles gambiae s.s. to pyrethroids in Cote d'Ivoire. *Ann. Soc. Belge. M´ed. Trop*, 73: 291–294.

Fang, J. (2010). A world without mosquitoes. *Nature*, 466: 432-434

Feyereisen, R. (1999). Insect P450 enzymes. *Annu Rev Entomol,* 44: 507-33

ffrench-Constant, R.H., Daborn, P.J., Le Goff, G.(2004). The genetics and genomics of insecticide resistance. *TRENDS in Genetics*, 20: 163-170.

ffrench-Constant, R.H., Anthony, N., Aronstein, K., Rocheleau, T., Stilwell, G. (2000) Cyclodiene insecticide resistance: from molecular to population genetics. Annu. Rev. Entomol.45:449– 466.

ffrench-Constant, R. H., Rochelaeau, T. A., Steichen, J.C., Chalmers, A. E. (1993). A point mutation in a *Drosophila* GABA receptor confers insecticide resistance. *Nature*, 363: 449-451.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol,* 3**:** 294–299.

Fournier, D., Mutero, A. (1994). Modification of acetylcholinesterase as a mechanism of resistance to insecticides. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology,* 108: 19-31.

Fournier, D., Bride, J.M., Hoffman, F., Karch, F. (1992) Acetylcholinesterase: Two types of modifications confer resistance to insecticides. *J Biol Chem*, 267: 14270–14274.

Gammon, D.W., Brown, M.A., Casida, J.E. (1981). Two classes of pyrethroid action in the cockroach. *Pesticide Biochemistry and physiology*, 15 (2):181-191

Georghiou, G.P.,Pasteur, N. (1980). Organophosphate resistance and esterase pattern in a natural population of the southern house mosquito from California. *J. Econ. Entomol*, 73: 489-492

Gyapong, J.O., Adjei, S. & Sackey, S.O. (1996). Descriptive epidemiology of lymphatic filariasis in Ghana. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 90: 26-30

Guillet, P., N'Guessan, R., Darriet, F., Traore-Lamizana, M., Chandre, E., Carnevale, P. (2001). Combined pyrethroid and carbamate "two in one' treated mosquito nets: field efficacy against pyrethroid-resistant Anopheles gambiae and Culex quinquefasciatus. *Med Vet Entomol*, 15: 105-112.

Grant, D.F., Hammock, B.D. (1992). Genetic and molecular evidence for a trans-acting regulatory locus controlling glutathione S-transferase-2 expression in *Aedes aegypti. Mol. Gen. Genet*, 234: 169-176.

Grant, D.F. (1991). Evolution of glutathione S-transferase subunits in Culicidae and related Nematocera: Electrophoretic and immunological evidence for conserved enzyme structure and expression. *Insect Biochem*, 21:435-45

Greenwood, B.M., Kalifa Bojang, K., Whitty, C.J.M., Targett, G.A.T. (2005): Malaria. *Lancet*, 365: 1487–98

Halasa, Y.A., Shepard, D.S., Fonseca, D.M., Farajollahi, A., Healy, S. Gaugler, R., Bartlett-Healy, K., Strickman, D. A., Clark, G.G. (2014). Quantifying the Impact of Mosquitoes on Quality of Life and Enjoyment of Yard and Porch Activities in New Jersey. *PLoS ONE*, 9(3): e89221.

Hama, H. (1987). Development of pyrethroid resistance in the Diamondback Moth, Plutella xylostella Linne (Lepidoptera: Yponomeutidae). *Appl. Ent. Zool,* 22(2):166-175

Harbach, R.E. (2011). Classification within the cosmopolitan genus Culex (Diptera: Culicidae): the foundation for molecular systematics and phylogenetic research. *Acta Trop*, 120:1-14

Hargreaves, K., Koerkemoer, L.L., Brooke, B., Hunt, R.H., Mthembu, J., Coetzee, M. (2000). Anopheles funestus resistant to pyrethroid insecticides in South Africa. *Med. Vet. Entomol*, 14: 181–189.

Hemingway J., Hawkes N.J., McCarroll, L., Ranson, H. (2004). The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem Mol Biol*, 34: 653–665.

Hemingway, J., Ranson, H. (2000). Insecticide Resistance in Insect Vectors of Human Disease. *Annual Review of Entomology*, 45: 371-391.

Hemingway, J., Karunaratne, S.H.P.P. (1998). Mosquito carboxylesterases: a review of the molecular biology and biochemistry of a major insecticide resistance mechanism. *Med. Vet. Entomol*, 12: 1-12.

Hemingway, J. (1998). *Techniques to Detect Insecticide Resistance Mechanisms (Field and Laboratory Manual).* Document WHO/CDS/CPC/MAL/98.6.World Health Organization, Geneva, Switzerland.

Hill, J., Lines J., Rowland, M. (2006). Insecticide-treated nets. *Advances in parasitology*, 61:78-128

Howard, S.C., Omumbo, J., Nevill, C., Some, E.S., Donnelly, C.A., Snow, R.W. (2000). Evidence for a mass community effect of insecticide-treated bed nets on the incidence of malaria on the Kenyan coast. *Trans R Soc Trop Med Hyg*, 94:357-360.

Hunt, R.H., Fuseini, G., Knowles, S., Stiles-Ocran, J., Verster, R., Kaiser, M.L., Choi, K.S., Koekemoer, L.L., Coetzee, M. (2011) Insecticide resistance in malaria vector mosquitoes at four localities in Ghana, West Africa. *Parasites and Vectors*, 4:107.

IRAC. (2011). *Prevention and Management of Insecticide Resistance in Vectors of Public Health Importance*. (2nd Ed). A manual produced by: Insecticide Resistance Action Committee

Irish, S.R., N'Guessan R., Boko P.M., Metonnou, C., Odjo, A., Akogbeto, M. & Rowland, M. (2008). Loss of protection with insecticide-treated nets against pyrethroid-resistant *Culex quinquefasciatus* mosquitoes once nets become holed: an experimental hut study. *Parasites & Vectors,* 1:17 doi:10.1186/1756-3305-1-17

Kabula, B.I., Attah, P.K., Wilson, M.D., Boakye, D. (2011). Characterization of Anopheles gambiae s.l and insecticide resistance profile relative to physicochemical properties of breeding habitats within Accra Metropolis, Ghana. *Tanzania Journal of Health Research*, 13:3

Khambay, B.P.S., Jewess, P.J. (2010). *Pyrethroids*. In: Gilbert, L.I., Gill, S.S. (Eds.), Insect Control: Biological and Synthetic Agents. Elsevier Academic Press, pp. 1-29.

Kikuchi, Y., Hayatsu, M., Hosokawa, T., Nagayama, A., Tago, K., Fukatsu, T. (2012).Symbiont-mediated insecticide resistance. *Proc. Nat. Acad. Sci*. 109: 8618-8622.

Kasai, S., Weerashinghe, I.S., Shono, T., Yamakawa, M., 2000. Molecular cloning, nucleotide sequence and gene expression of a cytochrome P450 (CYP6F1) from the pyrethroid-resistant mosquito, *Culex quinquefasciatus* Say. *Insect Biochem. Mol. Biol,* 30: 163-171.

Keating, J., Macintyre, K., Mbogo, C., Githeko, A., Regens, J.L., Swalm, C., Ndenga, B., Steinberg, L.J., Kibe, L., Githure, J.I., Beier, J. C. (2003). A geographic sampling strategy for studying relationships between human activity and malaria vectors in urban Africa. *Am J Trop Med Hyg,* 68: 357-65.

Kettle, D.S. (1995). *Medical and veterinary entomology*. (2nd ed). CABI publishing, London, UK

Kristan, M.., H. Fleischmann, A., Della Torre, A. Stich, Curtis,C.F. (2003). Pyrethroid resistance/susceptibility and differential urban/rural distribution of *Anopheles arabiensis* and *An. gambiae*s.s. malaria vectors in Nigeria and Ghana. *Med. Vet. Entomol*, 17: 326-332.

Killeen, G.F., Smith, T.A., Ferguson, H.M., Mshinda, H., Abdulla, S., Lengeler, C., and Kachur, S. P. (2007). Preventing childhood malaria in Africa by protecting adults from mosquitoes with insecticide-treated nets. *PLoS Med.* 4, e229. doi: 10.1371/journal. pmed.0040229

Killeen, G.F., Fillinger, U., Kiche, I, Gouagna, L.C., Knols, B.G. (2002). Eradication of Anopheles gambiae from Brazil: lessons for malaria control in Africa? *Lancet Infect. Dis*, 2: 618–627.

Klinkenberg E, McCall, P.J., Wilson, M.D., Amerasinghe, F.P., Donnelly, M.J. (2008). Impact of urban agriculture on malaria vectors in Accra, Ghana. *Malaria Journal*, 7**:** 151.

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E., Papadopoulou-Mourkidou, E.. (2001). Glutathione S-transferase in the defense against pyrethroids in insects. *Insect Biochemistry and Molecular Biology*, 31: 313-319.

Kudom, A.A., Mensah, B.A., Nunoo J. (2013). Assessment of anti-mosquito measures in households and resistance status of Culex species in urban areas in southern Ghana: implications for the sustainability of ITN use. *Asian Pacific Journal of Tropical Medicine*, 6 (11):859-864.

Kudom, A.A., Mensah, B.A., Agyeman, T.K. (2012). Characterization of mosquito larval habitats and assessment of insecticide-resistance status of Anopheles gambiae senso lato in urban areas in south western Ghana. *J vector ecol*, 37(1): 77-82.

Kudom, A.A., Mensah, B.A. (2010). The potential role of the educational system in addressing the effect of inadequate knowledge of mosquitoes on use of insecticide-treated nets in Ghana. *Malaria Journal*, 9:256.

Lawrance, C.E., Croft, A.M. (2004). Do mosquito coils prevent malaria? A systematic review of trials. *J Travel Med*, 11(2):92-6.

Liu, H., Xu, Q., Zhang, L., Liu, N. (2005). Chlorpyrifos resistance in mosquito *Culexquinquefasciatus.J. Med. Entomol*, 42: 815-820

Mathers, C.D., Loncar, D. (2006). Projections of global mortality and burden of disease from 2002 to 2030. *PloS Med*, 3(11): e442

Martinez-Torres, D., Chevillon, C., Brun-Barale, A., Berge J.B., Pasteur, N., Pauron, D. (1999). Voltage-dependent Na+ channels in pyrethroid-resistant *Culex pipiens* L. mosquitoes. *Pesticide Science*, 55: 1012–1020.

Melander, A.L. (1914). Can insects become resistant to sprays? *Journal of Economic Entomology*, 7:167:173

MOH. (2008).Strategic plan malaria control Ghana for in 2008-2015. Ministry of Health, Accra, Ghana.

Nikou, D., Ranson, H., Hemingway, J. (2003). An adult-specific CYP6 P450 gene is overexpressed in a pyrethroid-resistant strain of the malaria vector, *Anopheles gambiae*. *Gene*, 318: 91-102

Nganga, P. N., Shililu, J., Jayasinghe, G., Kimani, V., Kabutha, C., Kabuage, L., Kabiru, E., Githure, J., Mutero, C. (2008). Malaria vector control practices in an irrigated rice agro-ecosystem in central Kenya and implications for malaria control. *Malar J*, 7: 146

N'Guessan, R., Corbel, V., Akogbeto, M., Rowland, M. (2007). Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerg Infect Dis*, 13:199-206

Ngufor, C., N'Guessan, R., Fagbohoun, J., Odjo, A., Malone, D., et al. (2014). Olyset Duo H (a Pyriproxyfen and Permethrin Mixture Net): An Experimental Hut Trial

against Pyrethroid Resistant Anopheles gambiae and Culex quinquefasciatus in Southern Benin. *PLoS ONE*, 9(4): e93603. doi:10.1371/journal.pone.0093603

Nkya, T.E., Akhouayri, I., Kisinza,W., David, J-P. (2013). Impact of environment on mosquito response to pyrethroid insecticides: Facts, evidences and prospects. *Insect Biochem. Mol. Biol*, 43:407-416

Ntow, W.J. (2001). Organochlorine Pesticides in Water, Sediment, Crops, and Human Fluids in a Farming Community in Ghana. *Archives of Environmental Contamination and Toxicology*, 40: 557-563.

Ogoma, S.B., Kannady, K**.,** Sikulu,M., Chaki, P.P., Govella, N.J., Mukaban**,** W.R**.,** Killeen**,** G.F. (2009). Window screening, ceilings and closed eaves as sustainable ways to control malaria in Dares Salaam, Tanzania. *Malar J*, 8:221

Okoye, P.N., Brooke, B.D., Koekemoer, L.L., Hunt, R.H., Coetzee, M. (2008). Characterisation of DDT, pyrethroid and carbamate resistance in Anopheles funestus from Obuasi, Ghana. *Trans R Soc Trop Med Hyg*, 102: 591-598

O'Reilly, A.O., Khambay, B.P., Williamson, M.S., Field, L.M., Wallace, B.A., Davies, T.G. (2006). Modelling insecticide-binding sites in the voltage-gated sodium channel. *Biochem J,* 396: 255-263.

Oxborough, R.M.,Kitau, J., Jones, R., Feston, E., Matowo, J.,Mosha, F.W., Rowland, M.W. (2014). Long-lasting control of Anopheles arabiensis by a single spray application of micro-encapsulated pirimiphos-methyl (Actellic® 300 CS). *Malaria J*, 13:37.

Pates, H., Curtis, C. (2005). Mosquito behavior and vector control. *Annual Review of Entomology*, 50, 53-70.

Poupardin, R., Reynaud, S., Strode, C., Ranson, H., Vontas, J., David, J.P. (2008).Cross induction of detoxification genes by environmental xenobiotics and insecticides in the mosquito Aedes aegypti: impact on larval tolerance to chemical insecticides. *Insect Biochem. Mol. Biol*, 38: 540-551.

Poulin, B., Lefebvre, G., Paz, l. J. (2010). Red flag for green spray: adverse trophic effects of Bti on breeding birds. *Appl. Ecol*, 47: 884–889

Prapanthadara, L., Koottathep, S., Promtet, N., Hemingway, J., Ketterman, A.J. (1996).Purification and characterization of a major glutathione S-transferase from the mosquito *Anopheles dirus* (Species B).*Insect Biochem. Mol. Biol*, 26: 277-285

Ranson, H., Jensen, B., Vilule, J.M., Wang, X., Hemingway, J., Collins, F.H. (2000).Identification of a point mutation in the voltage-gated sodium channel gene of Kenyan *Anopheles gambiae* associated with resistance to DDT and pyrethroids. *Insect Mol. Biol*, 9: 491-497

Raymond, M., Berticat, C., Weill, M., Pasteur, N., Chevillon, C. (2011).Insecticide resistance in the mosquito Culex pipiens: what have we learned about adaptation? Genetica, 113:287–296

Riaz, M.A., Poupardin, R., Reynaud, S., Strode, C., Ranson, H., David, J.P. (2009). Impact of glyphosate and benzo[a]pyrene on the tolerance of mosquito larvae to chemical insecticides. Role of detoxification genes in response to xenobiotics**.** *Aquat. Toxicol*, 93: 61-69.

Rozendaal, J. A. (1997). *Vector control: Methods for use by individuals and communities*. World Health Organization, Geneva, Switzerland.

Sadasivaiah, S. Tozan, Y. Breman, J.G. (2007). Dichlorodiphenyltrichloroethane (DDT) for Indoor Residual Spraying in Africa: How can it be used for Malaria control? *Am. J. Trop. Med. Hyg*, 77(6):249-263.

Samuelsen, H., Toe, L.P., Baldet, T., Skovemand, O. (2004). Prevention of mosquito nuisance among urban population in Burkina Faso. *Social Science and Medicine,* 59: 2361–2371

Sakar, M., Borkotoki, A., Baruah, I., Bhattacharyya, I.K., Srivastava, R.B. (2009). Molecular analysis of knock down resistance (kdr) mutation and distribution of kdr genotypes in a wild population of *Culex quinquefasciatus* from India. *Tropical Medicine and International Health*, 14: 1097-1104.

Service, M. (2008). *Medical entomology for students*. (4th ed). Cambridge University Press. UK

Singh, B., Sung, L.K., Matusop, A., Radhakrishnan, A., Sunita S G Shamsul, S.S.G., Cox-Singh, J., Thomas, A. (2004). A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. Lancet, 363:1017–24

Smith J.L., Fonseca, D.M. (2004). Rapid assays for identification of members of the *Culex* (Culex) *pipiens* complex, their hybrids, and other sibling species (Diptera: culicidae). *American Journal of Tropical Medicine and Hygiene*, 70: 339–345.

Strode, C., Donegan, S., Garner, P., Enayati, A.A., Hemingway, J. (2014). The Impact of Pyrethroid Resistance on the Efficacy of Insecticide-Treated Bed Nets

against African Anopheline Mosquitoes: Systematic Review and Meta-Analysis. *PLoS Med* 11(3): e1001619. doi:10.1371/journal.pmed.1001619

Suwanchaichinda, C., Brattsten, L.B. (2002). Induction of microsomal cytochrome P450s by tire-leachate compounds, habitat components of Aedes albopictusmosquito larvae. *Arch. Insect Biochem. Physiol*, 49: 71-79

Suwanchaichinda, C., Brattsten, L.B. (2001). Effects of exposure to pesticides on carbaryl toxicity and cytochrome P450 in Aedes albopictus larvae (Diptera: Culicidae). *Pestic.Biochem. Physiol,* 70: 63-73.

Tempelis, C.H. (1975). Host feeding patterns of mosquito, with a review of advances in analysis of blood meals by serology. *Journal of Medical Entomology*, 11:635-653.

Thompson, M., Steichen, J.C., ffrench-Constant, R.H. (1993). Conservation of cyclodiene insecticide resistance associated mutations in insects. *Insect Mol. Biol*. 2:149-154.

Trape, J.F., Tall, A., Diagne, N., *et al*. (2011). Malaria morbidity and pyrethroid resistance after the introduction of insecticide-treated bed nets and artemisinin-based combination therapies: a longitudinal study. *Lancet infect dis*, 11:925-32

Ughasi, J., Bekhard, H.E., Coulibaly, M., Adabie-Gomez, D., Gyapong, J., Appawu, M., Wilson, M.D., Boakye, D.A. (2012). Mansonia Africana and Mansonia uniformis are vectors in the transmission of *wuchereria bancrofti* lymphatic filariasis in Ghana. *Parasites and Vectors,* 5:89.

USDA. (2014). *Aedes communis: The Pollinating Mosquito* www.fs.fed.us/wildflowers/pollinators/pollinator-of-the_month/aedes_communis.shtml. Accessed: January, 2014

Utzinger, J., Tozan, Y., Singer, B.H. (2001). Efficacy and cost-effectiveness of environmental management for malaria control. *Trop. Med. Int. Hlth*, 6: 677–687

Vinigradova, E.B. (1966). Blood-sucking mosquitoes of the Culex pipiens L. complex (Diptera, Culicidae), their practical importance, taxonomy and biology. *Ent. Obozr*, 45(2):241-257 (In English)

Vythilingama, I.,Tana, C.H., Asmadb, M., Chana, S.T., Leec, K.S., Singh, B. (2006). Natural transmission of *Plasmodium knowlesi* to humans by *Anopheles latens* in Sarawak, Malaysia. *Trans R Soc Trop Med Hyg,* 100 (11): 1087-1088.

Walker, R.K., Ricciardone, M.D., Jensen, J. (2003). Developing an international consensus on DDT: a balance of environmental protection and disease control. *Int. J. Hyg. Environ. Health*, 206:423-435

Webb, C.E., Russell, R.C. (2007). *Living with Mosquitoes on the Central Coast region of NSW*. University of Sydney, Sidney, Australia.

Weerasinghe, I.S., Kasai, S., Shono, T. (2001). Correlation of pyrethroid structure and resistance level in *Culex quinquefasciatus* say from Saudi Arabia. *J. Pestic. Sci*, 26: 158-161.

Weill, M., Malcolm, C., Chandre, F., Mogensen, K., Berthomieu, A., Marquine, M., Raymond, M. (2004). The unique mutation in *ace-1* giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol Biol*, 13: 1-7.

Weill, M., Lutfalla, G., Mogensen, K., Chandre, F., Berthomieu, A., Berticat, C., Pasteur,N., Philips, A., Fort, P., Raymond, M.(2003). Insecticide resistance in mosquito vectors. *Nature*, 423, 136-137

WHO. (2014). *Malaria*. Fact sheet number 94. World Health Organization, Geneva, Switzerland

WHO. (2013). *World Malaria Report*. 2013. World Health Organization, Geneva, Switzerland. (http://www.who.int/malaria/publications). Accessed: January, 2014

WHO. (2012). *Global action plan for insecticide resistance management in malaria vector (GPIRM)*. World Health Organization, Geneva, Switzerland.

WHO. (2011). *World Malaria Report. 2011*. World Health Organization, Geneva, Switzerland. (http://www.who.int/malaria/publications). Accessed: January, 2014

WHO. (2009). *Global insecticide use for vector-borne disease control*. (4th Ed).World Health Organization, Geneva, Switzerland

WHO. (2006). *Guidelines for testing mosquito adulticides for indoor residual spraying and treatment of mosquito nets*. WHO/CDS/NTD/WHOPES/GCDPP/2006.3.

Wilding, C.S., Smith, I., Lynd, A., Yawson, A.E., Weetman, D., Paine, M.J.I., Donnelly,M.J. (2012). A cis-regulatory sequence driving metabolic insecticide resistance in mosquitoes: Functional characterization and signatures of selection. *Insecticide biochemistry and molecular biology,* 42:699-707

Yadouleton, A., Martin, T., Padonou, G., Chandre, F., Asidi, A., Djogbenou, L.,Dabire, R., Aikpon, R., Boko, M., Glitho, I., Akogbeto, M. (2011). Cotton pest

management practices and the selection of pyrethroid resistance in *Anopheles gambiae* population in northern Benin. *Parasit, Vectors,* 4: 60.

Yadouleton, A.W., Asidi, A., Djouaka, R.F., Braima, J., Agossou, C.D., Akogbeto, M.C., (2009). Development of vegetable farming: a cause of the emergence of insecticide resistance in populations of Anopheles gambiae in urban areas of Benin. *Malar. J*. 8:103

Yawson, A.E., McCall, P.J., Wilson, M.D., Donnelly, M.J. (2004). Species abundance and insecticide resistance of Anopheles gambiae in selected areas of Ghana and Burkina Faso. *Med. Vet. Entomol*, 18: 372-377

Zaim, M., Aitio, A., Nakashima, N. (2000). Safety of pyrethroid-treated nets. *Med. Vet. Entomol*, 14:1-5.

Zhang, M., Scott, J.G. (1994). Cytochrome b5 involvements in cytochrome P450 monooxygenase activities in house fly microsomes. *Arch. Insect Biochem. Physiol,* 27: 205-216.

Appendices

Appendix 1: Pictures of different mosquito breeding habitats where larvae were collected. a)Accra residential area category (Kaneshie), b) Kumasi swampy area category (Bohyen) c) Sunyani residential area category (Penkwasi)

a

b

c

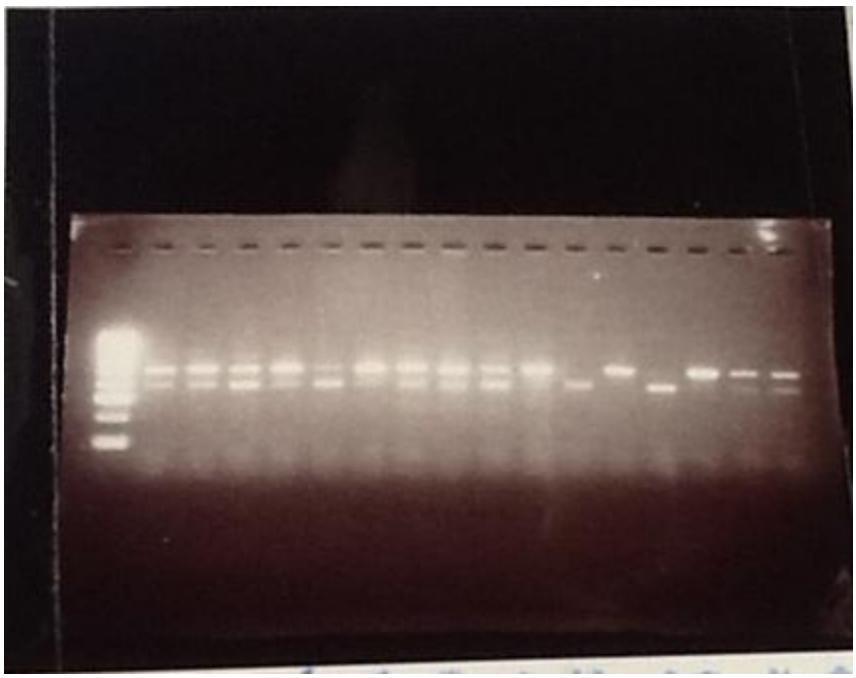
Appendix 2: The output of Primer 3 software showing the sequence of Cytochrome c oxidase of *Culex decens* and the position where primer was design from.

```
OLIGO             startlen     tmgc%  any  3'seq
LEFT PRIMER        684   21   59.15   50.00  5.00  1.00
ACCTCGACGATACTCCGATTT
RIGHT PRIMER       886   20   59.55   50.00  6.00  3.00
TGTGTTCTGCAGGAGGAAGA
SEQUENCE SIZE: 887

PRODUCT SIZE: 203, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00

    1 TTATTAACTGATCGAAATTTAAATACTTCATTTTTTGACCCAATTGGAGGAGGAGATCCT

   61 ATTTTATATCAACATTTATTTTGATTTTTTGGTCACCCAGAAGTATACATTTTAATTTTA

  121 CCAGGATTTGGAATAATTTCTCACATTATTTCTCAAGAAAGAGGTAAAAAGGAAACATTT

  181 GGAACATTAGGAATAATTTATGCAATATTAGCAATTGGTTTATTAGGATTTATTGTATGA

  241 GCTCATCATATATTTACAGTTGGTATAGATGTTGATACTCGAGCTTATTTTACTTCAGCT

  301 ACAATAATTATTGCCGTTCCTACAGGAATTAAAATTTTTAGTTGATTAGCTACTCTTCAT

  361 GGAACACAATTAAATTATACTCCAGCATTATTATGATCACTAGGATTTGTATTTTTATTT

  421 ACAGTAGGAGGATTAACTGGAGTWGTATTAGCTAATTCATCTATTGATATTGTTCTTCAT

  481 GATACTTACTATGTAGTTGCTCATTTTCATTATGTATTATCAATAGGGGCTGTATTTGCT

  541 ATTATAGCAGGATTTGTTCATTGATATCCTTTATTAACAGGATTAGTAATAAATCCAACA

  601 TGATTAAAAATTCAATTTACTATTATATTTATTGGAGTAAATTTAACATTCTTTCCTCAA

  661 CATTTCTTAGGATTAGCAGGAATACCTCGACGATACTCCGATTTTCCAGATAGTTACCTA
                              >>>>>>>>>>>>>>>>>>>>

  721 ACATGAAATATTGTATCATCATTAGGAAGTACAATTTCAWTATTTGCTATTATTTTCTTT

  781 TTATTTATTATTTGAGAAAGTATAATTTCTCAACGAACACCTTCATTCCCTATACAATTA

  841 TCTTCATCAATTGAAYGATATCATACTCTTCCTCCTGCAGAACACAA
                                <<<<<<<<<<<<<<<<<<<
```

Appendix 3: *S*pecies identification (A) and detection of *kdr* assays (B). Lane 1 1kb ladder, (A) lanes 2-6 *Culex quinquefasciatus* species, (B) lanes 2-10 heterozygote resistance, lane 11-12 susceptible

A

B



Appendix 4: Multiple comparisons of the mean absorbance from α-esterase, β-esterase and oxidase using ANOVA

(I) enzyme	(J) enzyme	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
α-esterase	esterase B	-0.3594458*	0.0132870	0.000	-0.385528	-0.333363
	oxidase	0.2962227*	0.0131703	0.000	0.270370	0.322076
β-esterase	esterase A	0.3594458*	0.0132870	0.000	0.333363	0.385528
	oxidase	0.6556685*	0.0133481	0.000	0.629466	0.681871
oxidase	esterase A	-0.2962227*	0.0131703	0.000	-0.322076	-0.270370
	esterase B	-0.6556685*	0.0133481	0.000	-0.681871	-0.629466

*. The mean difference is significant at the 0.05 level.

Curriculum vitae

Personal profile

Name: Andreas Adutwum Kudom

Address: Department of Entomology and Wildlife, University of Cape Coast, Cape Coast, Ghana

Nationality: Ghanaian

Sex: Male

Profile

I am a medical entomologist with interest in bio-ecology of insects in urban areas. My research interest includes insecticide resistance in mosquitoes, habitat ecology of mosquitoes in urban areas and effect of human behaviour on mosquito and malaria control.

Education

Ludwig - Maximilian University, Munich, Germany - PhD in Medical Research (International Health), 2014

University of Montpellier 2– France and University Of Abomey Calavi - Benin - International Master in Medical and Veterinary Entomology (MSc.), 2009

University of Cape Coast – Cape Coast, Ghana - BSc. Entomology and Wildlife, 2006

Working experience

Principal research Assistant, University of Cape Coast, 2009 up to date

Senior research assistant, University of Cape Coast, 2007 to 2009

Project works

- Insecticide resistance status of *Culex quinquefasciatus* in urban towns in Ghana and efficacy of long lasting nets against them (PhD Thesis)
- Exploration of the residuality of a suspension concentrates formulation of chlorfenapyr alone or combination with alphacypermethrin for protection against resistance anophelines and culicine mosquitoes and resistant management (MSc. Thesis)
- A survey of pollinators on pineapple in central region of Ghana. (BSc. Thesis)

Grants and Scholarships

BES: Ecologist in Africa-research grant (4767/5805), 2013 - 2015

DAAD: PhD scholarship, 2011 - 2014

Erasmus Mundus, Travel grant 9-15th July 2012

IRD/LSTMH: MSc scholarship, 2008 - 2009

Membership

Member of Ghana science association

Member of British ecological association

List of Publications

1. N'Guessan R., Ngufo C., **Kudom A.A.,** Boko P., Odjo A., Malone D., Rowland M. (2014). Mosquito Nets Treated with a Mixture of Chlorfenapyr and Alphacypermethrin Control Pyrethroid Resistant *Anopheles gambiae* and *Culex quinquefasciatus* Mosquitoes in West Africa. *PLoS ONE* 9(2): e87710

2. **Kudom, A.A**., Mensah, B.A. and Nunoo, J. (2013). Assessment of anti-mosquito measures in households and resistance status of Culex species in urban areas in southern Ghana: implications for the sustainability of ITM use. *Asian Pacific Journal of Tropical Medicine* 6(11):859-864.

3. Sims, C.A., **Kudom A.A.,** Mensah, B.A., Wubah D.A., Wubah, J.A. (2012): Permethrin and Dichlorodiphenyltrichloroethane (DDT) resistant Anopheles gambiae and *Culex quinquefasciatus* in Southern Ghana. *Journal of Young Investigators,* 24 (6): 77-80

4. **Kudom, A.A**., Mensah, B.A. and Agyemang, K.T. (2012).Characterization of mosquito larval habitats and assessment of insecticide resistance status of Anopheles gambiae senso lato in urban areas in South-Western Ghana. *Journal of Vector Ecology,*37: 77 – 82.

5. **Kudom, A.A.**, Mensah, B.A. and Botchey, M. (2011).Aqueous neem extract versus neem powder on *Culex quinquefasciatus*: Implications for control in anthropomorphic habitats**.** *Journal of Insect Science*, 11:142

6. Mensah, B.A. and **Kudom, A.A**. (2011).Nutritional ecology of the mimetic butterfly, *Hypolimnas missipus* L. (Lepidoptera: Nymphalidae) in Ghana. *Journal of Lepidoptera Research* 44:29-33

7. Mensah, B.A. and **Kudom, A.A.** (2011). Foraging dynamics and poITMation efficiency of *Apis mellifera* and *Xylocopa olivacea* on *Luffa aegyptiaca* Mill (Cucurbitales: Cucurbitaceae). *Journal of PoITMation Ecology* 4(5): 34-38

8. **Kudom, A.A.** and Mensah, B.A. (2010). The potential role of the educational system in addressing the effect of inadequate knowledge of mosquitoes on use of insecticide-treated nets in Ghana. *Malaria Journal*, 9:256

9. **Kudom, A. A.** and Kwapong, P.K. (2010).Floral visitors of *Ananas comosus* in Ghana: A prelimnary assessment. *Journal of poITMation ecology* 2(5):27-32.

10. Deikumah, J.P. and **Kudom, A.A**. (2010). Biodiversity status of urban remnant forests in Cape Coast Metropolis, Ghana. *Journal of Science and Technology* 30:3

11. Mensah, B.A. and **Kudom, A.A.** (2010). Relating bee activity to poITMation of *Luffa aegyptiaca* Mill. in Ghana. *Journal of Apicultural Research and Bee World* 42(2):192-196.